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Novel alternatives to toxic antifouling agents:
towards effectiveness and environmental
compatibility

Sandra Cristina Lopes Pereira

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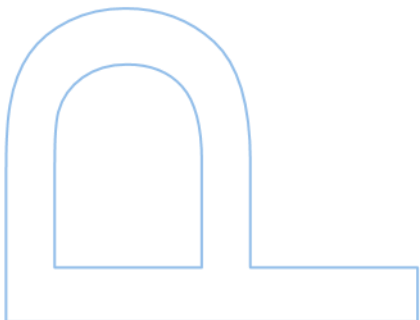
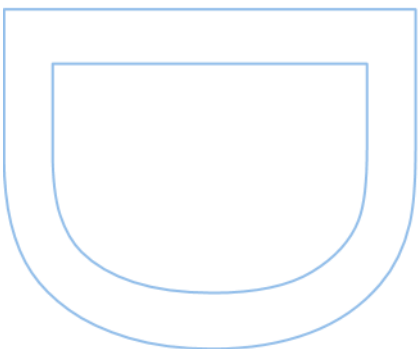
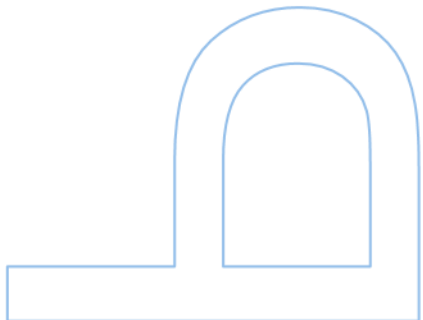
Universidade do Minho

Novel alternatives to toxic antifouling agents: towards effectiveness and environmental compatibility

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Faculty of Sciences of the University of Porto
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Novel alternatives to toxic antifouling agents: towards effectiveness and environmental compatibility

Sandra Cristina Lopes Pereira

Thesis carried out as part of the Marine Biotechnology and
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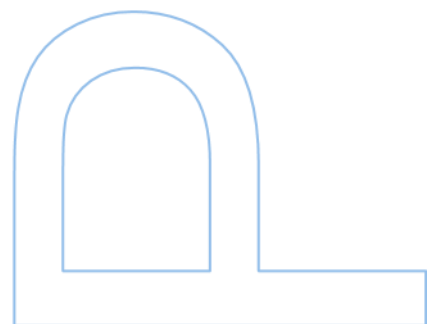
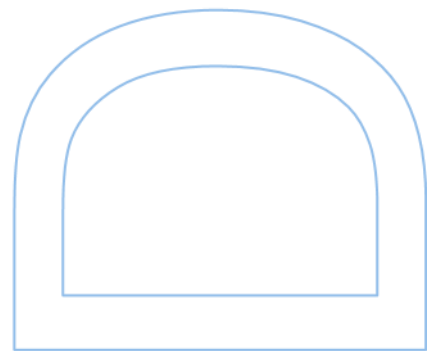


All the corrections determined by
the jury, and only those, were
made.

The Supervisor,

Joana Reis de Almeida

Matosinhos, 19 / 05 / 2026



Dedicado à minha mãe e ao meu irmão.

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Resumo

A bioincrustação marinha representa um grande desafio para as indústrias marítimas, comprometendo o desempenho e a integridade de estruturas e embarcações submersas. Tradicionalmente controlada através da utilização de biocidas em revestimentos marinhos, esta abordagem tem suscitado preocupações ambientais significativas, levando à implementação de regulamentações mais rigorosas que exigem novos agentes anti-incrustantes (AI) eficazes, ambientalmente responsáveis e com mecanismos de ação bem caracterizados. Os estudos desenvolvidos no âmbito desta tese tiveram como foco a exploração da diversidade química de cianobactérias da Coleção de Culturas Biotecnologia Azul e Ecotoxicologia (LEGE-CC) para aplicações AI. O trabalho envolveu a avaliação da eficácia AI, dos alvos moleculares, da toxicidade ambiental e do desempenho em revestimentos de metabolitos de cianobactérias selecionados. Após bioprospecção, cinco compostos foram investigados em detalhe: dois nucleósidos de ocorrência natural, adenosina e 2'-desoxiadenosina, isolados de forma bio-guiada a partir de *Leptothoe* sp. LEGE 181152; dois análogos, arabinosídeo de hipoxantina e 2'-desoxiinosina; a oxadiazina nocuolina A, previamente isolada de *Nodularia* sp. LEGE 06071; e os péptidos cíclicos portoamidas, isolados de *Phormidium* sp. LEGE 05292, com atividade AF conhecida contra plantígrados de mexilhão ($EC_{50} = 3.16 \mu M$) e bactérias formadoras de biofilme marinho.

A eficácia AI foi avaliada contra organismos representativos das comunidades micro e macro incrustantes, incluindo bactérias marinhas formadoras de biofilme, a diatomácea *Navicula* sp. e plantígrados de *Mytilus galloprovincialis*. Não foi observada inibição do crescimento bacteriano pelos quatro nucleósidos nem pela nocuolina A. Em contraste, os cinco compostos inibiram o crescimento de *Navicula* sp., com a nocuolina A a apresentar a maior inibição ($EC_{50} = 1.56 \mu M$; $LC_{50} = 3.1 \mu M$). Todos os compostos inibiram eficazmente a fixação de mexilhões (adenosina $EC_{50} = 6.63 \mu M$; 2'-desoxiadenosina $EC_{50} = 8.74 \mu M$; arabinosídeo de hipoxantina $EC_{50} = 5.50 \mu M$; 2'-desoxiinosina $EC_{50} = 8.54 \mu M$; nocuolina A $EC_{50} = 3.90 \mu M$), sem apresentarem letalidade ($LC_{50} > 100 \mu M$).

Os plantígrados de mexilhão foram utilizados como modelo biológico para o isolamento bio-guiado dos compostos e para a investigação dos respetivos modos de ação. Para esse efeito, recorreu-se a uma abordagem integrada que incluiu ensaios enzimáticos, estudos de docking molecular e proteómica não direcionada. Os quatro nucleósidos influenciaram a via da acetilcolinesterase (AChE), com os estudos de *docking* a

apoiarem a afinidade de ligação. Adicionalmente, o arabinosídeo de hipoxantina e a 2'-desoxiinosina modularam a atividade da tirosinase (TYR). A nocuolina A não parece atuar através destas vias enzimáticas. A análise proteómica diferencial entre mexilhões expostos à 2'-desoxiadenosina, ao arabinosídeo de hipoxantina e à 2'-desoxiinosina revelou alterações na expressão do fator de von Willebrand (vWF) e de proteínas com domínios semelhantes ao fator de crescimento epidérmico (EGF), associados às proteínas de adesão *proximal thread matrix protein-1* (PTMP1) e *mussel foot protein-2* (Mfp-2), respetivamente. Foram também alteradas proteínas relacionadas com o metabolismo do ATP, bem como proteínas associadas ao movimento ciliar (2'-desoxiadenosina e arabinosídeo de hipoxantina). A exposição à nocuolina A provocou alterações em proteínas associadas ao stress oxidativo, regulação muscular e metabolismo do ATP.

A compatibilidade ambiental foi avaliada através de ensaios ecotoxicológicos com *Artemia salina*, *Amphibalanus amphitrite* e embriões de *Danio rerio*, assim como por estudos de previsão de risco *in silico* (EPI Suite™, ECOSAR, MAMPEC). Não foi observada toxicidade para os quatro nucleósidos nem para as portoamidas. No entanto, a nocuolina A revelou-se tóxica para *A. salina* (LC₅₀ = 2.48 µM), *A. amphitrite* (LC₅₀ = 0.02 µM) e embriões de *D. rerio* (LC₅₀ = 0.06 µM), apresentando potencial risco ambiental.

Dado o comprovado potencial, as portoamidas foram ainda alvo de incorporação em protótipos de revestimentos AI para prova de conceito em laboratório e em ambiente marinho relevante (TRL 6) no Porto de Leixões (Portugal). Ensaios de validação em laboratório demonstraram que os protótipos contendo 0,7% (p/p) de portoamidas reduziram eficazmente a formação de biofilme e a fixação de mexilhões. A prova de conceito em mar confirmou o desempenho superior dos protótipos com revestimento de portoamidas face a um biocida AI comercial (ECONEA®), atrasando a macro-incrustação inicial e melhorando a eficácia AI global.

Em suma, este trabalho destaca o potencial AI dos nucleosídeos, uma classe química pouco explorada, como fonte de soluções sustentáveis e ecológicas para revestimentos marinhos. Apesar da potente bioatividade da nocuolina A, a sua toxicidade ambiental limita a sua aplicabilidade. Em contraste, as portoamidas demonstraram cumprir os requisitos de segurança e eficácia, mantendo a atividade AI após incorporação bem-sucedida em revestimentos AI e sob condições reais de exposição. Em conjunto, estes resultados contribuem para o desenvolvimento de sistemas AI inovadores baseados em

produtos naturais de cianobactérias, alinhados com as exigências regulamentares e os objetivos de inovação sustentável.

Palavras-chave: Bioincrustação Marinha, Tecnologias Anti-Incrustantes de Base Biológica, Cianobactérias, Produtos Naturais, Alvos Moleculares, Toxicidade Marinha

Abstract

Marine biofouling remains a major challenge for maritime industries, affecting the performance and integrity of submerged structures and vessels. Traditionally managed through the use of booster biocides in marine coatings, these compounds have raised significant environmental concerns, prompting stricter regulations that demand new antifouling (AF) agents to be effective, environmentally responsible, and mechanistically well-characterized. This PhD research focused on exploring the chemical diversity of cyanobacteria from the LEGE Culture Collection (LEGE-CC) for AF applications. The work encompassed the evaluation the AF efficacy, molecular targets, environmental toxicity, and coating performance of selected cyanobacterial metabolites. Five compounds were investigated: two naturally occurring nucleosides – adenosine and 2'-deoxyadenosine – bio-guided isolated from *Leptothoe* sp. LEGE 181152; two analogues – hypoxanthine arabinoside and 2'-deoxyinosine; the oxadiazine nocuolin A, previously isolated from *Nodularia* sp. LEGE 06071; and the cyclic peptides portoamides, previously isolated from *Phormidium* sp. LEGE 05292, with known AF activity against mussel plantigrades ($EC_{50} = 3.16 \mu M$) and marine biofilm-forming bacteria.

AF efficacy was assessed against representative micro- and macrofouling organisms, including marine biofilm-forming bacteria, the diatom *Navicula* sp., and *Mytilus galloprovincialis* plantigrades. No bacterial growth inhibition was observed for the four nucleosides and nocuolin A. In contrast, all five compounds inhibited the growth of *Navicula* sp., with nocuolin A being the most potent ($EC_{50} = 1.56 \mu M$; $LC_{50} = 3.1 \mu M$). All compounds effectively inhibited mussel settlement (adenosine $EC_{50} = 6.63 \mu M$; 2'-deoxyadenosine $EC_{50} = 8.74 \mu M$; hypoxanthine arabinoside $EC_{50} = 5.50 \mu M$; 2'-deoxyinosine $EC_{50} = 8.54 \mu M$; nocuolin A $EC_{50} = 3.90 \mu M$), without displaying lethality ($LC_{50} > 100 \mu M$).

Mussel plantigrades were used as a model to guide the isolation and to study the mode of action of the compounds. Enzymatic assays, molecular docking, and untargeted proteomics were employed. All four nucleosides influenced the acetylcholinesterase (AChE) pathway, with docking studies supporting their binding affinity. Additionally, hypoxanthine arabinoside and 2'-deoxyinosine also modulated tyrosinase (TYR) activity. Nocuolin A did not appear to act via these enzymatic pathways. Proteomic analysis of mussels exposed to 2'-deoxyadenosine, hypoxanthine arabinoside, and 2'-deoxyinosine revealed altered expression of von Willebrand factor (vWF) and epidermal growth factor-like (EGF) proteins, domains associated with adhesion proteins proximal thread matrix

protein-1 (PTMP1) and mussel foot protein-2 (Mfp-2), respectively. Proteins related to ATP metabolism were also affected, along with cilia-associated proteins (2'-deoxyadenosine and hypoxanthine arabinoside). Nocuolin A exposure led to changes in proteins linked to oxidative stress, muscle regulation, and ATP metabolism.

Environmental safety was evaluated through ecotoxicity assays using *Artemia salina*, *Amphibalanus amphitrite*, and *Danio rerio* embryos, along with *in silico* predictions (EPI Suite™, ECOSAR, MAMPEC). No toxicity was observed for the four nucleosides or for portoamides. However, nocuolin A was toxic to *A. salina* ($LC_{50} = 2.48 \mu M$), *A. amphitrite* ($LC_{50} = 0.02 \mu M$), and *D. rerio* embryos ($LC_{50} = 0.06 \mu M$), and was predicted to pose environmental risks.

Portoamides were tested in a proof-of-concept coating formulations and produced prototypes were validated under real marine conditions in Leixões Port (Portugal). Lab-scale tests demonstrated that coatings containing 0.7 wt% portoamides effectively reduced biofilm formation and mussel settlement. Field trials confirmed the superior performance of portoamides-based coatings compared to a commercial AF biocide (ECONEA®), delaying early-stage macrofouling and enhancing overall AF effectiveness.

In summary, this work highlights the AF potential of nucleosides, a still underexplored chemical class, as a sustainable, eco-friendly solution for marine coatings. Despite the potent bioactivity of nocuolin A, its environmental toxicity limits practical applicability. In contrast, portoamides fulfilled key safety and efficacy requirements, maintaining AF activity after successful incorporation into coatings, and under real exposure conditions. Overall, these findings contribute to the advancement of engineered AF systems based on cyanobacterial natural products, aligning with regulatory demands and sustainable innovation goals.

Keywords: Marine Biofouling, Bio-Based Antifouling Technologies, Cyanobacteria, Natural Products, Molecular Targets, Marine Toxicity

Scientific Production

Patent

Reis, M., Almeida, J.R., Vasconcelos, V., Morais, J., Ferreira, L., **Pereira, S.**, Gonçalves, C., Neves, J. 2022. Bioactive compounds obtained from cyanobacteria *Leptothoe* sp. LEGE 181152. PT117824 (20/02/2023). EPO 4233857 (30/08/2023).

Participation in courses and seminars

(Speaker) Sandra Pereira – “Cyanobacteria-derived Nucleosides as Antifouling Agents: From Efficacy to Environmental Safety”. CIIMAR’s Neptune Seminar, Matosinhos, Portugal, November 26, 2025.

(Speaker) Sandra Pereira – “Uncovering molecular targets of promising antifouling compounds – an untargeted proteomic approach”, Principles of High-throughput Proteomics Research Course, School of Health of Polytechnic of Porto (E2S | P.Porto), Porto, Portugal, December 3-6, 2024.

(Speaker) Sandra Pereira – “Bio-Descoberta de Nucleosídeos com Propriedades Anti-Incrustantes por Isolamento Bio-guiado”, UC: Bioassays in drug discovery, MSc in Pharmaceutical Chemistry, Faculty of Pharmacy of University of Porto (FFUP), Porto, Portugal, March 22, 2022.

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Communications (*presenting author)

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List of Abbreviations

13R-hpa	¹³ C-R-hydroxypheophytin A
13S-hpa	¹³ C-S-hydroxypheophytin A
3-APS	3-Alkylpyridinium
AChE	Acetylcholinesterase
ADP/ATP	Adenosine Diphosphate/Adenosine Triphosphate
AF	Antifouling
AM1	Austin Model 1
ANOVA	Analysis of variance
AP-2	Adaptor protein complex 2
ASV	Amplicon Sequence Variant
ATP	Adenosine Triphosphate
BCF	Bioconcentration factor
BF	Biodegrades Fast
BPR	Biocidal Products Regulation
CADD	Computer-aided drug design
CECT	Spanish Type Culture Collection
CERENA	Centro de Recursos Naturais e Ambiente
Cfu	Colony-Forming Unit
CI	Confidence Intervals
CID	Collision-Induced Fragmentation
CLSM	Confocal Laser Scanning Microscopy
COPI	Coatomer protein complex I
COSY	Homonuclear Correlation Spectroscopy
CuSO ₄	Copper Sulphate (II)
DEP	Differentially Expressed Proteins
DMSO	Dimethyl Sulfoxide
DNBF	Does Not Biodegrade Fast
DTNB	Dithiobisnitrobenzoate
DTT	Dithiothreitol
EC ₅₀	Half-maximal effective concentration
ECOSAR	Ecological Structure Activity Relationships
EGF	Epidermal Growth Factor-like
EPI	Estimation Programs Interface
EPS	Extracellular Polymeric Substances

ER	Endoplasmic Reticulum
EU	European Union
FASP	Filter-Aided Sample Preparation
FC	Fold change
FNSW	Filtered Natural Seawater
FRS	Fouling Release Systems
GO	Gene Ontology
GTP	Guanosine triphosphate
hCMEC/D3	Non-cancerous cell line
HCT 116	2D and 3D models of human carcinoma
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High-Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence
HTS	High-Throughput Sequencing
IAS	Invasive Aquatic Species
IMO	International Maritime Organization
K ₂ Cr ₂ O ₇	Potassium dichromate
LC ₅₀	Median Lethal Dose
LC ₅₀ /EC ₅₀	Therapeutic Ratio
LC-HR-ESI-MS/MS	Liquid Chromatography-High Resolution Electrospray Ionization Tandem Mass Spectrometry
LCL	Lower Confidence Limit
LC-MS/MS	Liquid Chromatography with Tandem Mass Spectrometry
L-DOPA	3,4-dihydroxyphenyl-L-alanine
LEGE-CC	Blue Biotechnology and Ecotoxicology Culture Collection
LEGE-NPL	Cyanobacterial Natural Products Library
LOEC	Lowest Observed Effect Concentration
Log K _{oc}	Organic Carbon-Water Partition Coefficient
Log K _{ow}	Octanol-Water Partition Coefficient
MAMPEC	Marine Antifoulant Model to Predict Environmental Concentrations
MeCN	Acetonitrile
MED	Minimum effective dose
MeOH	Methanol
Mfp	Mussel foot protein
MIC	Minimum Inhibitory Concentration
mRNA	Ribonucleic Acid messenger

MS/MS	Tandem Mass Spectrometry
NMP	N-methyl-2-pyrrolidone
NMR	Nuclear Magnetic Resonance
NOEC	No Observed Effect Concentration
NRB	Not Readily Biodegradable
NSAF	Normalized Spectral Abundance Factors
OCT	Optical Coherence Tomography
OECD	Organisation for Economic Co-operation and Development
Pam	Portoamides A and B
PAS	Peripheral Anionic Site
PBT	Persistence, Bioaccumulation, and Toxicity
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PDB	Protein Databank
PDMS	Polydimethylsiloxane
PEC	Predicted Environmental Concentration
PEG	Poly(ethylene glycol)
PNEC	Predicted No-Effect Concentrations
preCOL	Prepolymerized Collagens
PTMP	Proximal Thread Matrix Protein
PU	Polyurethane
ROV	Remote Operated Vehicles
rRNA	Ribosomal Ribonucleic Acid
SAR	Structure-Activity Relationship
SCaMC	Calcium-Binding Mitochondrial Carrier Protein
SDS	Sodium Dodecyl Sulphate
SiO ₂	Silicon dioxide
SPE	Solid Phase Extraction
SRC	Syracuse Research Corporation
TBT	Tributyltin
TLC	Thin-Layer Chromatography
TPBP	Triphenylborane Pyridine
TR	Toxic Ratio
Tris-HCl	Tris-hydrochloride

TRL	Technology Readiness Level
TYR	Tyrosinase
UCL	Upper Confidence Limits
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible
VLC	Vacuum Liquid Chromatography
VNSS	Våatanen Nine Salt Solution
vWF	von Willebrand Factor
WoRMS	World Register of Marine Species
ZFET	Zebrafish Embryo Acute Toxicity Test

1. Introduction

1.1. Marine biofouling

Marine biofouling refers to the undesired accumulation of marine organisms on artificial submerged surfaces. It results from the natural and rapid colonization of these surfaces by a diverse range of marine micro- and macroorganisms, with more than 4,000 species reported to occur in biofouling communities (Qiu *et al.*, 2024). Microfouling communities consist of bacteria, cyanobacteria, diatoms, microalgae, fungi, viruses, and protozoa (Qian *et al.*, 2022). Many of these microorganisms produce extracellular polymeric substances (EPS), which play a crucial role in biofilm formation. Macrofouling, in turn, is divided into two groups: (a) soft-fouling organisms such as sponges, soft corals, anemones, tunicates, bryozoans, macroalgae, and hydroids; and (b) hard-fouling organisms, including barnacles, mussels, hard corals, tubeworms and polychaetes (Gizer *et al.*, 2023). The diversity and richness of the biofouling communities depend on numerous abiotic and biotic, including salinity, pH, temperature, nutrient levels, flow rates, and the light intensity, all of which vary seasonally, spatially, and with depth. Biotic interactions such as competition and predation can further influence community structure. Additionally, the physicochemical properties of the substrate – including hydrophobicity, roughness, porosity, wettability, and mobility – strongly affect the adhesion and establishment of fouling organisms (Lejars *et al.*, 2012).

This natural process begins immediately after submersion, when organic macromolecules naturally present in seawater (e.g., glycoproteins, proteoglycans, and polysaccharides) adsorb to the surface, creating a conditioning film. This film facilitates the adhesion of bacteria and diatoms, promoting biofilm development through the production of EPS and quorum sensing, which together results in the modification of the physicochemical properties of the substrate (Gizer *et al.*, 2023). Within weeks, the biofouling community becomes increasingly complex with the settlement of protozoans, invertebrate larvae and algal spores, marking the mature stage of marine biofouling colonization (Figure 1).

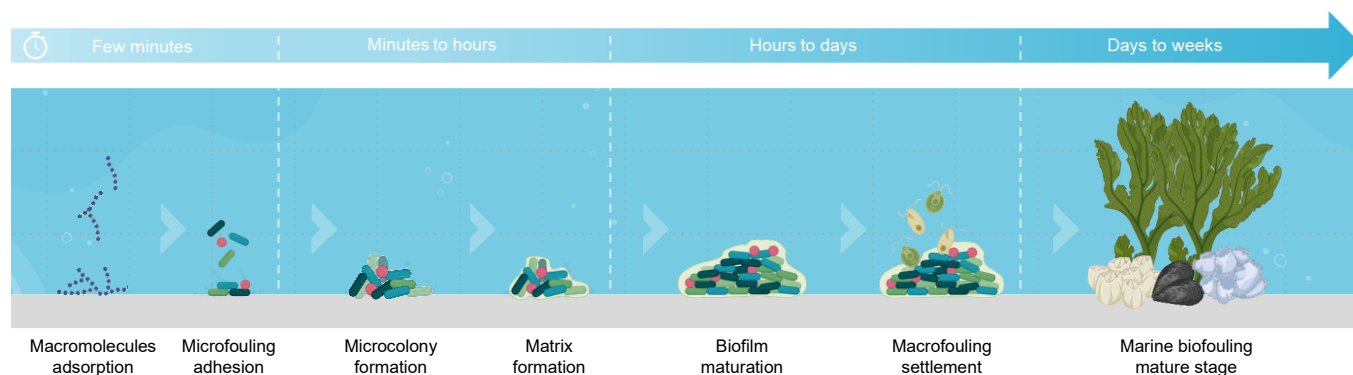


Figure 1. Marine biofouling formation stages. Created with Biorender.com.

Although this sequential colonization is largely accepted, in some cases the marine biofouling colonization process can be more dynamic and may not depend on the occurrence of previous stages (Vinagre *et al.*, 2020).

1.1.1. Implications in Maritime Industries

Submerged infrastructures are particularly susceptible to marine biofouling, which impacts a wide range of maritime sectors, including the oil and gas industry, marine renewable energy, desalination plants, aquaculture, the shipping sector, among others. Structural degradation is a major consequence of biofouling. Vessels and marine installations suffer material deterioration due to corrosion induced by microfouling communities, which is further accelerated by macrofouling through both chemical adhesion and the mechanical action of boring organisms (Bannister *et al.*, 2019; Hadžić *et al.*, 2022; Vinagre *et al.*, 2024). Operational inefficiencies are also a significant concern. In marine renewable energy systems, the accumulation of macrofouling organisms alters the dynamic properties of submerged structures by adding weight, increasing drag on moving components, and

ultimately reducing equipment functionality and performance (Vinagre *et al.*, 2024). Similarly, in the shipping industry, macrofouling-induced drag increases fuel consumption, leading to higher operational costs and greater greenhouse gas emissions (Hadžić *et al.*, 2022). In desalination facilities, microfouling can clog the membranes of reverse osmosis systems, impairing filtration efficiency and performance (Jamieson & Leterme, 2021). In aquaculture, the effects of marine biofouling extend beyond structural damage to cages, moorings, and buoyancy systems. Fouling organisms can directly harm cultured species through contact – for instance, by nematocysts causing skin or gill irritation – or indirectly, through the transmission of pathogens and parasites responsible for various diseases (e.g. by the presence of nematocysts) or by transmission of pathogens and parasitic organisms that cause various diseases (Bannister *et al.*, 2019). Thereby, the economic burden of marine biofouling is substantial. The U.S. Navy alone spends an estimated USD 100–200 million annually on maintenance and cleaning operations; in the oil and gas industry, a single cleaning cycle can cost between USD 30,000 and 100,000; and within Europe, the aquaculture industry spends approximately EUR 260 million per year on biofouling management (Santos-Simón *et al.*, 2025).

Beyond the economic impact, ecological consequences are equally critical. Marine biofouling plays a major role in the spread of invasive aquatic species (IAS) and pathogens (Table 1). IAS can outcompete native flora and fauna for space and resources, or predate upon native species, thereby disrupting local biodiversity and ecosystem balance. The introduction of invasive filter feeders can further alter nutrient cycling by filtering large water volumes, modifying particulate organic matter distribution, sedimentation rates, and nutrient availability. These shifts reduce plankton abundance and disrupt trophic dynamics within coastal food webs. Ultimately, such ecological disturbances cascade into socioeconomic effects, threatening fisheries, aquaculture productivity, and even the drinking water resources that sustain many coastal communities.

Table 1. List of high profile invasive aquatic species that are translocated via biofouling. Adapted from www.imo.org

Species	Common name	Native Area	Area introduced to	Impact
<i>Didemnum vexillum</i>	Colonial tunicate	North-west Pacific	North-east and north-west Atlantic, north-east Pacific, New Zealand	• Aggressive invader • Sexually or asexually reproduction • Fouls hydrotechnical constructions, ships, aquaculture infrastructure and cultured molluscs • Outcompetes for habitat • Grows over or smothers existing species
<i>Perna viridis</i>	Asian green mussel	Persian Gulf through to the Philippines, east Asian seas and up to eastern China	Caribbean, south Atlantic, south Pacific	• Tolerates wide fluctuations of salinity and temperature • Reaches high densities • Fouls hydrotechnical constructions, ships and aquaculture infrastructure • Affects the biodiversity of existing communities • Alter trophic levels
<i>Mytilopsis sallei</i>	Black striped mussel	North-west Atlantic, Caribbean and south Atlantic	India, east Asian seas (Malaysia, Singapore), south Pacific, north-west Pacific (Japan, China)	• Tolerates wide fluctuations of salinity and temperature • Highly fecund, grows and reaches maturity rapidly • Form dense aggregations, impacting biodiversity as they exclude most other species • Fouls hydrotechnical constructions, ships and aquaculture infrastructure • Causes corrosion, technical problems and loss of efficiency.
<i>Sabella spallanzanii</i>	European fan worm	North-east Atlantic, Mediterranean	South-west Atlantic, southern Australia, New Zealand, north-west Pacific	• Highly fecund and forms mat-like, dense populations on the seafloor • Tolerates wide ranges in salinity and successfully • Fouls hydrotechnical constructions, ships and aquaculture infrastructure • Competes with native filter-feeding organisms for habitat and food • Dense formations alter water flow, sediment stability and bacterial communities due to their efficiency filtering particulate matter from the water column

<i>Amphibalanus improvisus</i>	Bay barnacle	Thought to be the east coast of north-east and north-west Atlantic	South-west Atlantic, Caribbean Sea, Atlantic, Baltic Sea, Black Sea, Caspian Sea, north-west Pacific, east Asia seas	• Fast growing and gregarious • Sexually and asexually reproduction • Tolerates wide fluctuations of salinity and temperature • Fouls hydrotechnical constructions, ships and aquaculture infrastructure • Causes corrosion, technical problems and loss of efficiency • Affects biodiversity, change community structures and alter trophic levels
<i>Undaria pinnatifida</i>	Wakame seaweed	North-west Pacific	Mediterranean, north-east Atlantic, south-west Atlantic, north-east Pacific, south-east Australia, New Zealand	• Rapidly colonises temperate regions • Fouls hydrotechnical constructions, ships and aquaculture infrastructure • Affects biodiversity, change community structures and alter trophic levels

1.2. Management of Marine Biofouling

Several approaches – ranging from mechanical removal methods to chemical and physical interventions – have been developed to manage and minimize the impacts of marine biofouling on maritime structures and equipment. Among mechanical methods, grooming and in-water cleaning are widely applied to remove or reduce biofouling accumulation. In recent years, some conventional technologies have been adapted for preventive antifouling (AF) purposes, including ultrasound and ultraviolet (UV) radiation. Ultrasound technologies can disrupt the adhesion of early-stage microfouling on membranes through cavitation processes (Aghapour Aktij *et al.*, 2020). High-power ultrasonic transducers are also used to prevent biofilm initiation and detach existing biofouling from offshore structures (Salimi *et al.*, 2023). Similarly, ultraviolet-C (UVC) radiation, commonly used for sterilization in medical and industrial systems, has been shown to interfere with the adhesion and settlement of both micro- and macrofouling organisms (Richard *et al.*, 2021). Its application is particularly promising when integrated into remotely operated vehicles (ROVs), oceanographic instruments, or ship niche areas that are difficult to coat or maintain (Richard *et al.*, 2021). Nevertheless, both ultrasound and UV-based strategies are generally used as complementary techniques alongside other AF systems rather than standalone solutions. Some AF strategies have drawn inspiration from natural mechanisms, leading to the development of micro- and nanostructured surfaces that mimic textures found in nature, like shark skin or lotus leaves. These bioinspired surfaces physically deter the attachment of fouling organisms without the use of chemical biocides. While highly effective in laboratory conditions, their complex fabrication processes, high production costs, and limited scalability have so far constrained their practical application (Jin *et al.*, 2022).

Fouling-release coatings (FRCs) represent another more environmentally benign approach. Typically composed of silicone- or fluoropolymer-based materials, these coatings reduce adhesion strength of biofouling organisms due to their low surface free energy, allowing fouling to be removed through hydrodynamic forces or simple cleaning (Lejars *et al.*, 2012). However, this same low surface free energy characteristic weakens the adhesion between the FRC coating and the substrate, increasing application costs due to the need for tie-coat systems (Hu *et al.*, 2020). In addition, the poor mechanical properties of FRCs render them more susceptible to mechanical damage (Hu *et al.*, 2020). Although generally less toxic than copper, detrimental effects to marine organisms have been reported, particularly during the initial months of exposure and depending on formulations (Lagerström *et al.*, 2022).

Moreover, some leachable components from commercial silicone FRCs have been shown to be highly persistent in the marine environment (Lagerström *et al.*, 2022). Overall, while FRCs are considered non-toxic and effective at reducing biofouling accumulation, they remain costly, less durable than traditional biocidal coatings, less efficient on slow-moving or stationary structures and require further investigation regarding the environmental fate of their leachables.

Although the above mentioned methods offer valuable alternatives to traditional biocidal coatings, these approaches often face limitations related to cost, durability, and performance consistency under real marine conditions. Also, their real ecotoxicological and environmental health effects are underestimated. Therefore, chemically active-based coatings remain the dominant method for preventing marine biofouling.

1.2.1. Preventive Biocide-based Marine Coatings

Since the earliest days of maritime navigation, humans have recognized the need to coat the vessels to prevent the accumulation of fouling organisms. Ancient civilizations used various materials such as wax, tar, and asphalt as primary AF methods (Yebra *et al.*, 2004). Between 1500 and 300 BC, the Phoenicians and Carthaginians were reportedly among the first to employ pitch and copper sheathing on wooden vessels to deter marine biofouling (Dafforn *et al.*, 2011; Yebra *et al.*, 2004). Nowadays, the shipping industry has come to rely predominantly on biocide-based AF coatings (Table 2). Among these, organotin compounds – notably tributyltin (TBT) – were once the most widely used due to their long-lasting efficacy (up to ~5 years) and broad-spectrum activity against both micro- and macrofoulers (Batista-Andrade *et al.*, 2018; Dafforn *et al.*, 2011). However, their high octanol-water and organic carbon-water partition coefficients indicated persistence in sediments and strong potential for bioaccumulation in aquatic organisms. Severe toxic effects on non-target species, including imposex in gastropods, ultimately revealed the environmental hazards posed by these compounds (Batista-Andrade *et al.*, 2018; Dafforn *et al.*, 2011). Following the environmental hazards of organotins, copper-based coatings became the principal alternative. Copper is effective against a range of macrofouling invertebrates and is considered less toxic than tin-based formulations. However, its AF performance is strongly influenced by environmental factors such as temperature, pH, salinity, light, and water currents, which typically limit its efficacy to 5–12 months. Although copper ions released

from AF coatings can be toxic to algae and molluscs, their relatively low bioavailability generally allows compliance with environmental quality standards (Amara *et al.*, 2018; Dafforn *et al.*, 2011). To address the limited efficacy of copper – particularly against resistant macroalgae – herbicides, also known by booster biocides, were introduced as co-formulants. These include compounds such as chlorothalonil, dichlofluanid, cybutryne, thiocyanatomethylthio-benzothiazole (TCMTB), diuron, dichloro-octylisothiazolinone (DCOIT), zinc and copper pyrithione, and zineb (Dafforn *et al.*, 2011). However, many of these boosters have demonstrated ecotoxic effects on diverse marine organisms, including algae, invertebrates, crustaceans, and fish species (Amara *et al.*, 2018). Moreover, both these compounds and their transformation products (TPs) have been detected in surface waters and bottom sediments at very low concentrations, raising concerns about their persistence and long-term ecological impacts (Batista-Andrade *et al.*, 2018; Kevin V. Thomas *et al.*, 2002).

Table 2. Biocides used in preventive marine coatings. Information includes name of the active compound, chemical class, AF activity with short description of mode of action, half-life in seawater/ freshwater (DT_{50}), organic carbon-water partition coefficient ($\log K_{oc}$), and octanol-water partition coefficient ($\log K_{ow}$).

Active compound	Chemical Class	AF activity (mode of action)	DT_{50} in seawater/ freshwater (days)	$\log K_{oc}$	$\log K_{ow}$	Current Status	References
Chlorothalonil	Halogenated aromatic	Fungicide (inhibitor of electron transport)	1.8–8	5.7	2.6–4.4	Approved	(Amara <i>et al.</i> , 2018; Sakkas <i>et al.</i> , 2002; Kevin V. Thomas <i>et al.</i> , 2002)
Copper pyrithione (Copper Omadine®)	Cyclic organocopper complex	Bactericide and fungicide (multi-site inhibitor)	12.9–96	2.9–4.0	2.8	Approved until 2028	(Amara <i>et al.</i> , 2018; Turley <i>et al.</i> , 2005)
Dichlofluanid	Halogenated sulphamide	Fungicide (PSII inhibitor)	0.12–0.75	3.1	2.8–3.7	Approved	(Amara <i>et al.</i> , 2018; Cai, Koning, <i>et al.</i> , 2021)
Diuron	Halogenated arylurea	Herbicide (PSII inhibitor)	31.4–365	2.3–4.1	2.9	Under assessment	(Amara <i>et al.</i> , 2018; Negroni <i>et al.</i> , 2010)
Cybutryne (Irgarol 1051®)	S-Triazine	Herbicide (PSII inhibitor)	100–250	2.4–4.9	2.4–4.1	Banned since 2023	(Dyer <i>et al.</i> , 2006; H. Okamura <i>et al.</i> , 2000)
DCOIT (Sea-Nine 211®)	Isothiazolone	Herbicide, bactericide and fungicide (inhibitor of electron transport)	0.04–3	4.2	2.9	Approved	(Amara <i>et al.</i> , 2018; Negroni <i>et al.</i> , 2010)
TBT	Trialkyl organotin	Fungicide and molluscicide	67–152	4.6–5.3	3.7–4.4	Banned since 2008	(Antizar-Ladislao, 2008; Batista-Andrade <i>et al.</i> , 2018)

		(inhibitor of electron transport)					
TCMTB	Benzothiazole	Herbicide and fungicide (inhibitor of electron transport)	31–36	2.7	2.7–3.3	Under assessment	(Kevin V. Thomas <i>et al.</i> , 2002)
Zinc pyrithione (Zinc Omadine®)	Cyclic organocopper complex	Bactericide (multi-site inhibitor)	< 1	0.7	1.0	Under assessment	(Amara <i>et al.</i> , 2018; Turley <i>et al.</i> , 2005)
Zineb	Dithiocarbamate	Fungicide (multi-site inhibitor)	< 1 days	0.6	0.8	Approved until 2028	(Hunter & Evans, 1990)

Although modern AF coatings have evolved from simple physical barriers to complex chemical formulations, most remain reliant on biocidal mechanisms that pose environmental and ecological risks. The balance between maintaining efficacy against fouling and ensuring environmental safety remains a major challenge for the maritime industry.

1.2.2. Regulations and Guidelines

The environmental risks associated with marine biofouling and the biocides used to prevent it have been increasingly recognized worldwide, leading to significant international efforts to safeguard marine ecosystems. The United Nations, together with the International Maritime Organization (IMO) and its Marine Environment Protection Committee (MEPC), has established the global framework for both chemical safety and biological risk management in AF practices. A major milestone was the International Convention on the Control of Harmful Antifouling Systems on Ships (AFS Convention, 2001), which introduced a two-step control system banning the use of organotin compounds as biocides in AF coatings (IMO, 2001). These included TBT compounds – such as tributyltin oxide, tributyltin fluoride, tributyltin methacrylate, and tributyltin chloride – and triphenyltin (TPT) compounds like triphenyltin hydroxide and triphenyltin chloride. The phase-out process began in January 2003, prohibiting the application or re-application of organotin-based systems, followed by a January 2008 deadline for their removal or sealing with an effective barrier coating to prevent leaching. From 2008 onwards, ships were prohibited from operating with any active organotin biocides on their hulls. An amendment to the AFS Convention (MEPC.331(76)) later extended this ban to include cybutryne (marketed as Irgarol 1051®), effective from January 2023 (IMO, 2021). More recently, the Biofouling Guidelines (Resolution MEPC.378(80), 2023) introduced updated strategies to manage marine biofouling and

reduce the transfer of invasive aquatic species (IMO, 2023). Key recommendations include the implementation of approved AF systems on ship hulls and niche areas; Biofouling Management Plan, tailored to each vessel; Biofouling Record Book documenting inspections, cleaning, and maintenance activities; and periodic inspections of hulls and other submerged structures.

Within the European Union, several complementary regulatory instruments address the environmental and chemical safety of AF substances. The Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation (2007) obliges companies to register chemicals and evaluate their risks to human health and the environment. Annex XIII of REACH defines the criteria for identifying persistent, bioaccumulative, and toxic (PBT) substances (Table 3), which are directly applicable to AF agents.

Table 3. Criteria for the identification of persistent, bioaccumulative and toxic (PBT) substances, and very persistent and very bioaccumulative (vPvB) substances, present in the Annex XIII from REACH.

PBT Substances		vPvB Substances
Persistence		
	Degradation half-life	
Marine water	> 60 days	> 60 days
Fresh or estuarine water	> 40 days	> 60 days
Marine sediments	> 180 days	> 180 days
Fresh or estuarine water sediment	> 120 days	> 180 days
Soil	> 120 days	> 180 days
Bioaccumulation		
Aquatic species	BCF > 2000	BCF > 5000
Toxicity		
Marine or freshwater organisms	NOEC or EC ₁₀ < 0.01 mg L ⁻¹	
Carcinogenic (category 1A or 1B), Germ cell mutagenic (category 1A or 1B), Or toxic for reproduction (category 1A, 1B, or 2) according to Regulation EC No 1272/2008		
Chronic toxicity with specific target organ toxicity after repeated exposure (STOT RE category 1 or 2) according to Regulation EC No 1272/2008		

Furthermore, the European Chemicals Agency (ECHA) Guidance on Information Requirements and Chemical Safety Assessment Part C: PBT/vPvB provides additional screening information to outline if a chemical is persistent, bioaccumulative, and toxic, or

very persistent and very bioaccumulative, according to REACH Annex XIII (Table 4) (ECHA, 2017).

Table 4. Additional information for persistence, bioaccumulation, and toxicity according to ECHA (ECHA, 2017).

Persistence		
Biowin 2 (non-linear model prediction) and Biowin 3 (ultimate biodegradation time)	Does not biodegrade fast (probability <0.5) and ultimate biodegradation timeframe prediction: $\geq$ months (value < 2.25 (to 2.75))	Potentially persistence
Biowin 6 (MITI non-linear model prediction) and Biowin 3 (ultimate biodegradation time)	Does not biodegrade fast (probability <0.5) and ultimate biodegradation timeframe prediction: $\geq$ months (value <2.25 (to 2.75))	Potentially persistence
Bioaccumulation		
Octanol-water partitioning coefficient (experimentally determined or estimated by QSAR)	$\text{Log } K_{ow} \leq 4.5$	Not bioaccumulative in aquatic organisms
	$\text{Log } K_{ow} > 4.5$	Potentially bioaccumulative in aquatic organisms
Toxicity		
Short-term aquatic toxicity (algae, daphnia, fish)	EC_{50} or $\text{LC}_{50} < 0.01$ mg/L	Toxic
Short-term aquatic toxicity (algae, daphnia, fish)	EC_{50} or $\text{LC}_{50} < 0.1$ mg/L	Potentially toxic

In addition, the Biocidal Products Regulation BPR (EU 528/2012) ensures that all biocides and biocidal coatings marketed in the EU are authorized, effective, and environmentally safe (European Commission, 2024). Under the BPR, AF substances must undergo comprehensive data submission and risk assessment covering efficacy against target organisms and mode of action, toxicological and ecotoxicological effects on aquatic species, and environmental fate parameters, including degradation, bioaccumulation, and leaching potential. The BPR also introduces product authorization, environmental risk assessments, and post-market surveillance, making it one of the most stringent regulatory frameworks for AF systems worldwide.

Recently implemented European regulations (EU) 2019/677 and (EU) 2023/1783 banned the usage of chlorothalonil and diuron in any products (European Comission, 2019; European Commission, 2023).

Overall, these stricter bans and regulations have encouraged the surfacing of innovative and more sustainable approaches to marine biofouling prevention.

1.2.3. Emerging biocides

The increasingly restrictive regulatory framework governing the use of booster biocides has stimulated growing interest in emerging biocides, such as tralopyril, triphenylborane pyridine (TPBP), and medetomidine. The organohalogen compound tralopyril is the active ingredient in *ECONEA*®, a non-persistent, biodegradable, and metal-free biocide effective against barnacles, hydroids, mussels, oysters, and polychaetes (Thomas & Brooks, 2010). It was approved in the European Union in 2014 through the implementation of Regulation (EU) No. 1091/2014, under the BPR (EU 528/2012) (European Commission, 2014). Despite its rapid degradation in seawater, tralopyril has shown developmental toxicity in several aquatic organisms, including mussels, sea urchins, zebrafish, and marine medaka (B. Liu *et al.*, 2025; Oliveira *et al.*, 2014, 2017) and exhibits a high bioconcentration potential in mussel tissues (Oliveira, Groh, *et al.*, 2016). Another emerging compound, triphenylborane pyridine (TPBP), developed in Japan and commercialized as *Borocide*®, is an organoborane compound effective against both soft and hard fouling organisms. It is characterized by very low water solubility and long-lasting AF efficacy (Hideo Okamura & Mieno, 2006; X. Zhou *et al.*, 2007). TPBP undergoes chemical and photochemical degradation in aquatic environments (X. Zhou *et al.*, 2007), however, it has been reported to cause acute toxicity in *Daphnia magna*, sub-lethal effects in zebrafish, and is considered a potential threat to aquatic ecosystems (Oliveira *et al.*, 2014, 2017). The medetomidine, is the active ingredient in *Selektope*®, and acts through inhibition of presynaptic receptors, preventing the settlement of *Balanus improvisus* cyprids at nanomolar concentrations (Dahlström *et al.*, 2000). Medetomidine is biodegradable under sunlit water conditions, yet several of its photoproducts have shown potential persistence in aquatic environments (Cai, Apell, *et al.*, 2021). Although approved as an AF biocide under the BPR (EU 528/2012), medetomidine has been flagged for possible endocrine-disrupting properties (European Commission, 2025). Despite being promoted as environmentally friendlier alternatives to metal-based booster biocides, these emerging biocides still pose significant ecological risks, with some, such as TPBP, exhibiting toxicity levels comparable to TBT. Nevertheless, metal-based compounds containing copper (e.g., cuprous oxide, copper pyrithione, cuprous thiocyanate) and zinc (e.g., zinc pyrithione, zineb) remain the most widely used active ingredients in AF coatings (Paz-Villarraga *et al.*, 2022). Given these environmental concerns and regulatory pressures, research efforts have increasingly shifted towards natural products as a sustainable alternative. These compounds, derived from aquatic and terrestrial organisms,

offer a promising alternative for the development of non-toxic, biocide-free, and environmentally benign AF solutions.

1.2.4. Bio-based Alternatives to Biocides

The urgency to develop more environmentally sustainable alternatives to the metal-based and highly toxic biocides currently used in AF coatings has driven an increasing interest in natural products as a promising solution. Some well-known examples include capsaicin, a compound extracted from *Capsicum* species (chilli peppers), which exhibits strong AF activity with low environmental toxicity (Beyazkilic *et al.*, 2023; Oliveira *et al.*, 2014, 2017), and zosteric acid, a sulphated phenolic acid isolated from the seagrass *Zostera marina* and *Zostera noltii*. Zosteric acid is considered a “green” AF agent due to its simple chemical structure, high water solubility, low bioaccumulation potential, and lack of ecotoxicity, making it effective against a broad range of micro- and macrofouling organisms (Vilas-Boas *et al.*, 2017). However, its low natural abundance limits extraction yields, and although chemical synthesis can achieve high yields, it relies on hazardous reagents, rendering this approach environmentally harmful and economically inefficient (P. Zhang *et al.*, 2023). Although *de novo* biosynthesis of zosteric acid has been achieved through multilevel metabolic engineering in *Escherichia coli* (P. Zhang *et al.*, 2023), microbial production remains in its early stage. This limitation is largely attributed to the preservative properties of the zosteric acid, which can negatively affect microbial growth, thereby constraining production efficiency and scalability for commercial application (Shen *et al.*, 2025).

Marine ecosystems represent an extraordinary reservoir of chemical diversity, offering access to structurally unique metabolites evolved under selective pressures such as predation, competition, and surface colonization. More than 3,000 new compounds were isolated from marine sources, including microorganisms, phytoplankton, green, brown, and red algae, sponges, cnidarians, bryozoans, molluscs, tunicates, echinoderms, mangroves, and other intertidal organisms (Carroll *et al.*, 2021). Yet, only 182 marine natural products were investigated for AF purposes between 2014 and 2020 (L. L. Liu *et al.*, 2020), stressing how underexplored the potential of marine-derived AF agents remains. Several notable discoveries illustrate the value of this approach. For example, AF compounds isolated from a deep-sea sediment *Streptomyces* strain led to the synthesis of butenolide, a highly effective and non-toxic anti-settlement agent against multiple macrofouling organisms in

field trials (Ying Xu *et al.*, 2010). Similarly, the marine actinomycete *Streptomyces aculeolatus* produces napyradiomycins, madeirone, and neomadeirone, described as strong inhibitors of *Mytilus galloprovincialis* plantigrades settlement (F. Pereira *et al.*, 2020; Wissner *et al.*, 2024). Another remarkable example is portimine, isolated from the dinoflagellate *Vulcanodinium rugosum*, which effectively inhibited larval development and metamorphosis in several macrofouling species (Brooke *et al.*, 2018). Among the wide diversity of marine microorganisms able to produce interesting bioactive compounds, cyanobacteria stand out for their remarkable metabolic diversity and broad spectrum of bioactivities, positioning them as highly promising candidates for AF applications.

1.2.4.1. Cyanobacteria Biotechnological Potential for AF

Formerly known as “blue-green algae,” cyanobacteria are a phylogenetically diverse group of Gram-negative prokaryotes. This ancient lineage played a pivotal role in the oxygenation of Earth’s atmosphere and oceans during the Great Oxidation Event, around 2.4 billion years ago, which enabled the diversification of complex life forms (Demoulin *et al.*, 2019). Even today, marine cyanobacteria contribute to nearly 25% of the ocean’s net primary production (Flombaum *et al.*, 2013).

Through evolutionary time, cyanobacteria have adapted to an extraordinary range of habitats – from marine, freshwater, and estuarine systems to terrestrial and extreme environments such as geothermal springs, frozen tundra, and hypersaline lagoons (Allaf & Peerhossaini, 2022). Their remarkable adaptability and resistance to environmental stressors derive from distinctive structural and physiological features. Cyanobacteria perform oxygenic photosynthesis within thylakoid membranes using chlorophyll *a* and accessory phycobiliproteins (e.g., phycocyanin, allophycocyanin, and phycoerythrin), which allow them to exploit a wide range of light environments. Their complex Gram-negative cell envelope consists of an outer lipopolysaccharide membrane, a thin peptidoglycan layer, and an inner plasma membrane (Allaf & Peerhossaini, 2022). Most cyanobacteria are also surrounded by a sheath of EPS – complex heteropolymers rich in heteropolysaccharides – that play major roles in protection against desiccation, UV radiation, predation, and chemical stress, as well as in biofilm and colony formation and nutrient concentration under scarcity (Mota *et al.*, 2022). Certain filamentous cyanobacteria differentiate specialized cells (heterocysts and akinetes) under nitrogen-deprived conditions. Heterocysts fix atmospheric

nitrogen and transfer the fixed nitrogen to adjacent vegetative cells (Zeng & Zhang, 2022), while akinetes are thick-walled dormant cells containing nutrient reserves that ensure survival under adverse conditions such as cold, desiccation, or nutrient deprivation (Ho *et al.*, 2024). Many aquatic species also possess gas vesicles – proteinaceous, gas-filled nanostructures that confer buoyancy and allow vertical migration in the water column. This adaptation promotes population stratification and influences bloom dynamics (Huber & Jakobi, 2024). Moreover, cyanobacteria also participate in diverse symbiotic associations with protozoans, macroalgae, invertebrates (e.g., sponges, corals, ascidians), plants (e.g., seagrasses, cycads, mosses), and fungi (e.g., lichens) (Álvarez *et al.*, 2023; Mutalipassi *et al.*, 2021).

Cyanobacterial diversity is also reflected in their morphological variability, ranging from unicellular to colonial and multicellular filamentous forms. The classical taxonomy of cyanobacteria, established by Rippka *et al.* (1979), categorized them into five subclasses based on morphology: (I) unicellular forms dividing by binary fission, (II) unicellular forms dividing by multiple fission, (III) filamentous forms dividing in a single plane, (IV) filamentous forms with specialized cells, and (V) filamentous branched forms with heterocysts and akinetes. These subclasses corresponded to the historical orders *Chroococcales*, *Pleurocapsales*, *Oscillatoriales*, *Nostocales*, and *Stigonematales*, respectively. Nowadays, modern cyanobacterial taxonomy now follows a polyphasic approach, integrating morphological, molecular, physiological, and ecological data to better represent this complex group (Komárek *et al.*, 2014). With the exponential increase in genomic data, cyanobacterial classification continues to undergo frequent revisions (Strunecký *et al.*, 2023).

Currently, around 5.000 cyanobacterial species have been described based on molecular and morphological analyses, though estimates suggest up to 8.000 may exist (Allaf & Peerhossaini, 2022). Interestingly, this translates into cyanobacteria being a prolific source of natural products. This taxonomic diversity parallels an exceptional chemical diversity, positioning cyanobacteria among the most prolific producers of natural products. Since the 1970s, 1,630 unique metabolites from 260 families have been reported from cyanobacteria (Demay *et al.*, 2019). The order *Oscillatoriales* accounts for the largest number of metabolite families (153), followed by *Nostocales* (98), *Chroococcales* (34), and *Synechococcales* (31). Reported compounds span diverse chemical classes, including alkaloids, peptides (linear, cyclic, and depsipeptides), lipopeptides, macrolides/lactones, terpenes, polysaccharides, lipids, and polyketides. Peptides represent the largest class (66 families) (Demay *et al.*,

2019). Cyanobacterial metabolites exhibit a wide range of biological activities, including cytotoxic, neurotoxic, hepatotoxic, dermatotoxic, and antimicrobial effects, as well as anti-inflammatory, antioxidant, antiviral, and enzyme inhibitory activities (Demay *et al.*, 2019). Among these, cytotoxicity is the most reported (110 families), followed by lethality and antibacterial activity (54 and 43 families, respectively).

Despite the vast metabolic potential of cyanobacteria, their application in AF research remains relatively limited compared to other fields (reviewed in Dobretsov *et al.*, 2013; Fusetani, 2011; Gademann, 2007; L. L. Liu *et al.*, 2020; Qian *et al.*, 2015; K. L. Wang *et al.*, 2017). Most cyanobacterial strains studied for AF potential to date belong to *Nostocales*, *Oscillatoriales*, and *Synechococcales*. Among these, *Lyngbya majuscula* (Oscillatoriales) is the most prolific, yielding at least six AF-bioactive compounds (Dobretsov *et al.*, 2010; Levert *et al.*, 2018; L. T. Tan *et al.*, 2010). Other notable examples include *Okeania* sp., from which 3 compounds and several analogues with AF activities were reported (Fathoni *et al.*, 2020; Petitbois *et al.*, 2017). Reported AF cyanobacterial metabolites include diverse chemical classes – peptide derivatives (linear and cyclic), lactones, macrolides, alkaloids, diterpenoids, fatty acid amides, and non-peptidic linear compounds.

Bioactivities such as the inhibition of bacterial, cyanobacterial, and algal growth, quorum sensing modulation, molluscicidal effects, and the prevention of marine invertebrate settlement are particularly relevant in terms of AF applications and are summarized in Table 5. For instance, molluscicidal activity has been reported for compounds such as palmyrrolinone (A. R. Pereira *et al.*, 2011), cyanolide A (A. R. Pereira *et al.*, 2010), comnostin B (Jaki *et al.*, 2000), and thiopalmyrone (A. R. Pereira *et al.*, 2011) against the snail *Biomphalaria glabrata*. Settlement inhibition was investigated against *Amphibalanus amphitrite* cyprids and against *Mytilus galloprovincialis* plantigrades. Cyanobacterial metabolites such as hantupeptin C, isomalyngamide A, majusculamide A, all isolated from *Lyngbya majuscula*, were active against the barnacles cyprids ($EC_{50} = 0.54\text{--}10.6 \mu\text{g mL}^{-1}$) (L. T. Tan *et al.*, 2010). Interestingly, the macrolide dolastin 16, exhibited a high potency against barnacles cyprids at EC_{50} value of $0.003 \mu\text{g mL}^{-1}$, was isolated from two different cyanobacterial strains: *Lyngbya majuscula* and *Okeania* sp. (Petitbois *et al.*, 2017; L. T. Tan *et al.*, 2010). The same model organism was used to investigate the anti-settlement activity of the cyclodepsipeptides lyngbyabellin B, G, O and P ($EC_{50} = 0.09\text{--}7.4 \mu\text{M}$), and the fatty acid amides serinolamides C and D ($<1 \mu\text{g mL}^{-1}$), all isolated from *Okeania* sp. (Fathoni *et al.*, 2020; Petitbois *et al.*, 2017). On the other hand, portoamides inhibited the settlement of

mussel plantigrades at an EC_{50} value of 3.16 μM (Antunes *et al.*, 2019). Maculalactone A, a lactone isolated from *Kyrtuthrix maculans*, was the only cyanobacterial compound submitted to field trials. Maculalactone A was incorporated at 10 mg g^{-1} on commercially available household paint and in the coated PVC plates was able to significantly decrease the settlement of bivalves after a submersion period of 12 weeks (Brown *et al.*, 2004). Growth inhibition was investigated against several microorganisms (e.g. microalgae, cyanobacteria, marine bacteria). Several cyanobacterial metabolites were able to inhibit the growth of microalgae and cyanobacteria, as is the case of the alkaloids fisherellin, from *Fisherella muscicola* (Gross *et al.*, 1991), nostocarboline from *Nostoc* 78-12A (Blom *et al.*, 2006), cyanobacterin LU-1 from *Nostoc linckia* (Gromov *et al.*, 1991) and nostocine A from *Nostoc spongiaeforme* TISTR 8169 (Hirata *et al.*, 2003) as well as the other linear non-peptide cyanobacterin from *Scytonema hofmanni* (Abarzua *et al.*, 1999). Aside for the anti-settlement activity, growth inhibition of marine biofilm-forming bacteria was also reported for portoamides (Antunes *et al.*, 2019). Others, like the cyclodepsipeptides tiahuramides A, B and C, isolated from *Lyngbya majuscula*, inhibited the growth of marine fouling bacteria *Aeromonas salmonicida*, *Vibrio anguillarum* and *Shewanella báltica* (MIC = 6.7- 50 μM) (Levert *et al.*, 2018). The quorum sensing inhibition were reported for the lactones malyngolide, from *Lyngbya majuscula*, against *Chromobacterium violaceum* (EC_{50} = 0.11 mM) (Dobretsov *et al.*, 2010) and the honaucins A, B and C, from *Leptolyngbya crossbyana* against *Lactone Vibrio harveyi* (IC_{50} = 5.6-17.6 μM) (Choi *et al.*, 2012).

Table 5. Bioactive metabolites with AF bioactivities isolated from cyanobacteria. EC₅₀: Half-maximal effective concentration; IC₅₀: Half-maximal inhibitory concentration; MIC: Minimum inhibitory concentration; MED: Minimum effective dose.

Species	Order	Bioactive compound	Class	Bioactivity	Target organism (active concentration)	Reference
cf. <i>Oscillatoria</i> and <i>Hormoscilla</i> spp.	Oscillatoriales	Thiopalmirone	Other linear non-peptide	Molluscicidal	<i>Biomphalaria glabrata</i> (LC ₅₀ = 8.3 µM)	(A. R. Pereira <i>et al.</i> , 2011)
		Palmyrolinone	Linear lipopeptide	Molluscicidal	<i>Biomphalaria glabrata</i> (LC ₅₀ = 6.0 µM)	
<i>Fisherella muscicola</i>	Nostocales	Fischerellin	Alkaloid	Anticyanobacterial	<i>Anabaena variabilis</i> <i>Anabaena</i> sp. <i>Phormidium</i> sp. <i>Synechococcus</i> sp. <i>Synechocystis</i> sp.	(Gross <i>et al.</i> , 1991)
				Antialgal	<i>Ankistrodesmus falcatus</i> <i>Nannochloris</i> <i>Scenedesmus armatus</i> <i>Scenedesmus communis</i> <i>Scenedesmus falcatus</i> <i>Scenedesmus obliquus</i>	
<i>Kyrtuthrix maculans</i>	Nostocales	Maculalactone A	Lactone	Anti-settlement	Bivalves (10 mg g ⁻¹)	(Brown <i>et al.</i> , 2004)
<i>Leptolyngbya crossbyana</i>	Synechococcales	Honaucins A, B, C	Lactone	Quorum Sensing	<i>Vibrio harveyi</i> (IC ₅₀ = 5.6-17.6 µM)	(Choi <i>et al.</i> , 2012)
<i>Lyngbya bouillonii</i>	Oscillatoriales	Cyanolide A	Macrolide	Molluscicidal	<i>Biomphalaria glabrata</i> (LC ₅₀ = 1.2 µM)	(A. R. Pereira <i>et al.</i> , 2010)
<i>Lyngbya majuscula</i>	Oscillatoriales	Dolastin 16	Cyclodepsipeptide	Anti-settlement	<i>Amphibalanus amphitrite</i> (EC ₅₀ = 0.003 µg mL ⁻¹)	(L. T. Tan <i>et al.</i> , 2010)
		Hantupeptin C	Cyclodepsipeptide	Anti-settlement	<i>Amphibalanus amphitrite</i> (EC ₅₀ = 10.6 µg mL ⁻¹)	(L. T. Tan <i>et al.</i> , 2010)
		Isomalyngamide A	Fatty acid amides	Anti-settlement	<i>Amphibalanus amphitrite</i> (EC ₅₀ = 2.6 µg mL ⁻¹)	(L. T. Tan <i>et al.</i> , 2010)
		Majusculamide A	Other linear peptide	Anti-settlement	<i>Amphibalanus amphitrite</i> (EC ₅₀ = 0.54 µg mL ⁻¹)	(L. T. Tan <i>et al.</i> , 2010)
		Malyngolide	Lactone	Quorum sensing	<i>Chromobacterium violaceum</i> (EC ₅₀ = 0.11 mM)	(Dobretsov <i>et al.</i> , 2010)
		Tiahuramides A, B, C	Cyclodepsipeptide	Antibacterial	<i>Aeromonas salmonicida</i> (MIC = 6.7-27 µM) <i>Vibrio anguillarum</i> (MIC = 7.4-33 µM) <i>Shewanella baltica</i> (MIC = 16- >50 µM)	(Levert <i>et al.</i> , 2018)
<i>Nodularia harveyana</i>	Synechococcales	Norharmane	Alkaloid	Anticyanobacterial	<i>Arthrospira laxissima</i> (24 µg mL ⁻¹) <i>Chroococcus minutus</i> (16 µg mL ⁻¹) <i>Nostoc carneum</i> (24-56 µg mL ⁻¹) <i>Nostoc insulare</i> (8-80 µg mL ⁻¹) <i>Synechocystis aquatilis</i> (32 µg mL ⁻¹)	(R.-B. B. Volk & Furkert, 2006)

<i>Nostoc</i> 78-12A	Nostocales	Nostocarboline	Alkaloid	Anticyanobacterial	<i>Microcystis aeruginosa</i> (MIC = 1 µM) <i>Synechococcus</i> (MIC = 1 µM)	(Blom <i>et al.</i> , 2006)
				Antialgal	<i>Kirchneriella contorta</i> (MIC = 1 µM)	
<i>Nostoc commune</i>	Nostocales	Comnostin B	Diterpenoid	Molluscicidal	<i>Biomphalaria glabrata</i> (MIC = 20 ppm)	(Jaki <i>et al.</i> , 2000)
<i>Nostoc insulare</i>	Nostocales	4,4' dihydroxybiphenyl	Other cyclic peptide	Anticyanobacterial	<i>Arthrospira laxissima</i> (32-80 µg mL ⁻¹) <i>Chroococcus minutus</i> (48 µg mL ⁻¹) <i>Nostoc carneum</i> (56 µg mL ⁻¹) <i>Nostoc insulare</i> (80 µg mL ⁻¹) <i>Synechocystis aquatilis</i> (16 µg mL ⁻¹)	(R.-B. B. Volk & Furkert, 2006)
				Anticyanobacterial	<i>Anabaena variabilis</i> (MED = 0.5 µg mL ⁻¹) <i>Anabaena</i> sp. (MED = 0.3 µg mL ⁻¹) <i>Synechococcus</i> sp. (MED = 1.0 µg mL ⁻¹) <i>Aphanizomenon</i> sp. (MED = 0.6 µg mL ⁻¹) <i>Aphanocapsa</i> sp. (MED = 1.0 µg mL ⁻¹) <i>Microcystis aeruginosa</i> (MED = 0.08 µg mL ⁻¹) <i>Nostoc punctiforme</i> (MED = 0.5 µg mL ⁻¹) <i>Nostoc</i> sp. (MED = 0.7 µg mL ⁻¹) <i>Nostoc boryanum</i> (MED = 5.6 µg mL ⁻¹) <i>Phormidium tenuissimum</i> (MED = 1.4 µg mL ⁻¹) <i>Synechococcus</i> sp. (MED = 1.0 µg mL ⁻¹) <i>Synechocystis aquatilis</i> (MED = 5.6 µg mL ⁻¹)	
<i>Nostoc linckia</i>	Nostocales	Cyanobacterin LU-1	Alkaloid	Anticyanobacterial	<i>Ankistrodesmus acicularis</i> (MED = 1.0 µg mL ⁻¹) <i>Chlamydomonas gloeogama</i> (MED = 20 µg mL ⁻¹) <i>Chlorella pyrenoidosa</i> (MED > 85.0 µg mL ⁻¹) <i>Chlorella vulgaris</i> (MED = 2.0 µg mL ⁻¹) <i>Chlorella intermedium</i> (MED = 10.0 µg mL ⁻¹) <i>Coelastrum proboscideum</i> (MED = 5.0 µg mL ⁻¹) <i>Euglena gracilis</i> (MED > 66.0 µg mL ⁻¹) <i>Cosmarium</i> sp. (MED = 4.5 µg mL ⁻¹) <i>Klebsormidium fiacidum</i> (MED > 60.0 µg mL ⁻¹) <i>Kirchneriella obesa</i> (MED = 2.0 µg mL ⁻¹) <i>Scenedesmus obliquus</i> (MED = 2.0 µg mL ⁻¹)	(Gromov <i>et al.</i> , 1991)
				Antialgal	<i>Nostoc spongiaeforme</i> (MIC > 50 µM) <i>Nostoc muscorum</i> (MIC > 50 µM) <i>Nostoc commune</i> (MIC = 20 µM) <i>Anabaena variabilis</i> (MIC = 30 µM) <i>Anabaena cylindrica</i> (MIC = 30 µM) <i>S. schmidlei</i> (MIC > 50 µM) <i>Oscillatoria</i> sp. (MIC > 50 µM)	
<i>Nostoc spongiaeforme</i> TISTR 8169	Nostocales	Nostocine A	Alkaloid	Anticyanobacterial	<i>Chlamydomonas reinhardtii</i> (MIC = 30 µM) <i>Chlamydomonas</i> sp. (MIC > 50 µM) <i>Chlorella pyrenoidosa</i> (MIC = 30 µM) <i>Chlorella fusca</i> (MIC = 5 µM) <i>Dunaliella tertiolecta</i> (MIC = 20 µM) <i>Dunaliella salina</i> (MIC = 15 µM)	(Hirata <i>et al.</i> , 2003)
				Antialgal		

<i>Okeania</i> sp.	Oscillatoriales	Lyngbyabellin B, G, O, P	Cyclodepsipeptide	Anti-settlement	<i>Amphibalanus amphitrite</i> (EC ₅₀ = 0.09-7.4 µM)	(Fathoni <i>et al.</i> , 2020; Petitbois <i>et al.</i> , 2017)
		Serinolamides C, D	Fatty acid amides	Anti-settlement	<i>Amphibalanus amphitrite</i> (<1 µg mL ⁻¹)	(Petitbois <i>et al.</i> , 2017)
		Dolastatin 16	Cyclodepsipeptide	Anti-settlement	<i>Amphibalanus amphitrite</i> (EC ₅₀ = 0.09 µM)	
<i>Phormidium</i> sp. LEGE 05292	Oscillatoriales	Portoamides	Cyclic peptides	Anti-settlement	<i>Mytilus galloprovincialis</i> (EC ₅₀ = 3.16 µM)	(Antunes <i>et al.</i> , 2019)
				Antibacterial	<i>Cobetia marina</i> (EC ₃₀ = 14.56 µM) <i>Halomonas aquamarina</i> (EC ₃₀ = 14.83 µM) <i>Pseudoalteromonas atlantica</i> (EC ₃₀ = 13.59 µM)	
<i>Scytonema hofmanni</i>	Nostocales	Cyanobacterin	Other linear non-peptide	Anticyanobacterial	<i>Synechococcus</i> (MIC = 1.5 µg mL ⁻¹)	(Mason <i>et al.</i> , 1982)
				Antialgal	<i>Nitzschia pusilla</i> (MIC = 0.000002 mg mL ⁻¹)	(Abarzua <i>et al.</i> , 1999)

Aside from their AF activity, some cyanobacterial compounds were also investigated for their environmental impact. Compounds like dolastin 16, hantupeptin C, isomallyngamide A and majusculamide A were described as non-lethal towards *A. amphithrite* cyprids (L. T. Tan *et al.*, 2010). Although no other organism was tested to assess their general toxicity against non-target species. Portoamides were reported as non-lethal on target (e.g. mussel plantigrades, marine biofilm-forming bacteria and diatoms) and non-target species (*Artemia salina* nauplii) (Antunes *et al.*, 2019). Conversely, lyngbyabellin B was reported as highly toxic to barnacles cyprids, since high mortality among the cyprids was observed at 1 µg mL⁻¹ (Fathoni *et al.*, 2020). On the same note, maculalactone A reported high toxicity towards several naupliar larvae from non-target organisms, such as *A. amphithrite*, *Tetraclita japonica*, *Ibla cumingii*, and *A. salina* (Brown *et al.*, 2004). These findings emphasize the importance of evaluating ecotoxicity across multiple organisms and addressing criteria such as bioaccumulation and persistence to identify truly sustainable AF candidates.

1.3. Rational and Aims of the Thesis

The prevention of marine biofouling impact on maritime industries and marine-associated activities remains a major challenge, as currently available strategies often rely on the application of harmful biocides. Although effective, these compounds raise significant environmental and regulatory concerns, which has prompted the search for sustainable and environmentally compatible alternatives. Biotechnological alternatives to these biocides based on natural compounds with AF activity represent a promising avenue. However, to be considered viable bio-based alternatives, new AF compounds must comply with increasingly stringent regulatory guidelines (REACH (Annex XIII), and BPR (EU 528/2012)), ensuring both efficacy and environmental safety. In line with this framework, the present research aimed to discover promising bioactive and environmentally compatible compounds with desirable AF profiles within current regulatory requirements, by exploring the biological and chemical diversity of selected cyanobacterial strains hosted in the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) at CIIMAR.

To accomplish this aim, the following specific objectives and an integrated research approach were defined:

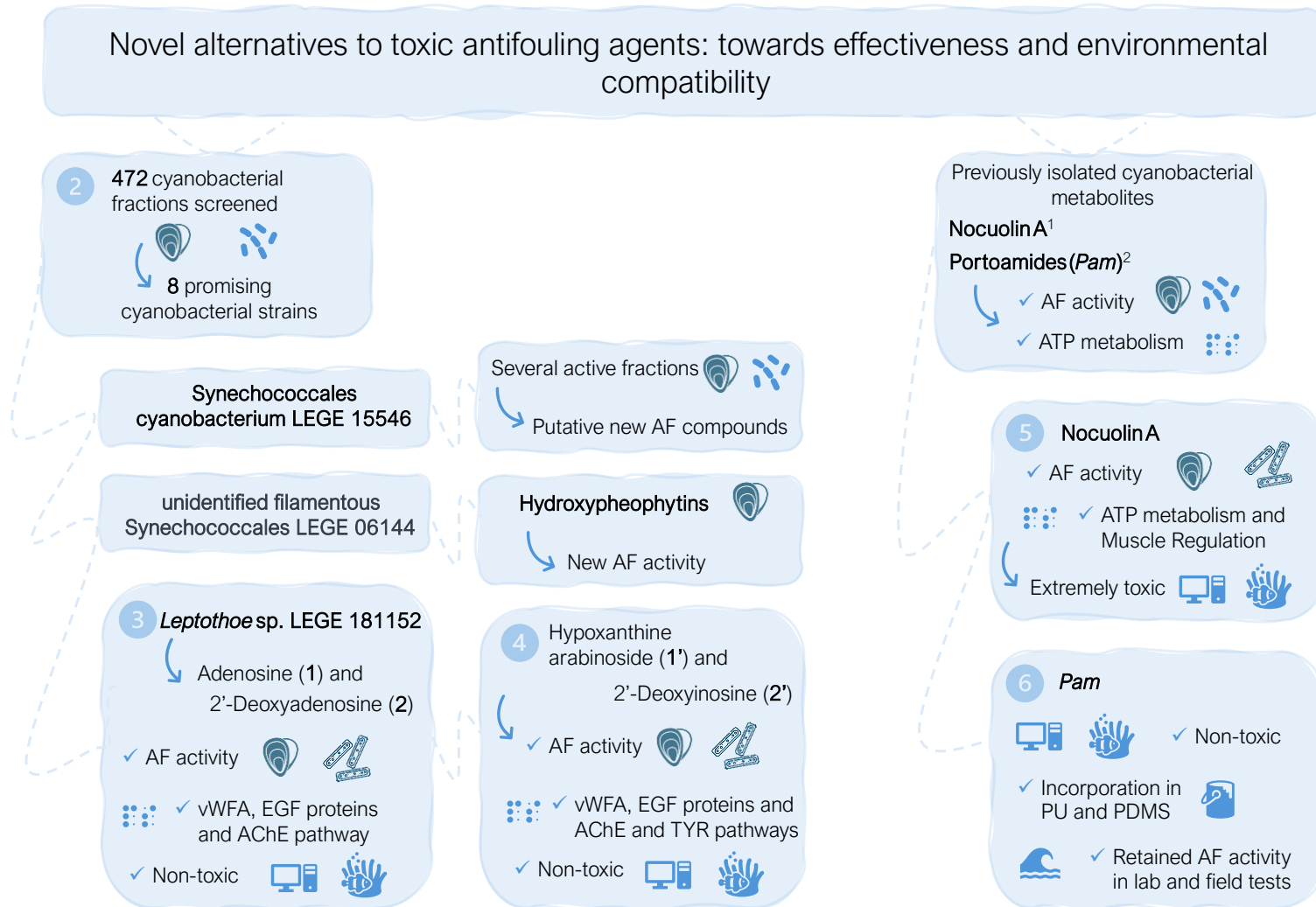
- I. To screen a wide selection of morpho-taxonomically diverse cyanobacterial strains, as well as previously isolated pure cyanobacterial compounds for AF properties, based on their diversity and unexplored AF bioactivity, against micro- and macrofouling organisms, using bacterial growth inhibition and settlement inhibition bioassays.
- II. To up-scale the most promising cyanobacterial strains to: (a) perform a bioassay-guided isolation and characterization of novel bioactive compounds, using mussel plantigrades settlement inhibition bioassay, and chemical elucidation through LC-MS, UV-Vis and NMR analyses; (b) obtain previously isolated promising compounds in high amounts for further AF profiling tests regarding complementary bioactivity, ecotoxicity parameters and coating affinity.
- III. To evaluate the AF effectiveness of the selected bioactive compounds against microfouling organisms by bacterial and diatom growth inhibition bioassays.
- IV. To characterize the molecular mechanisms of the bioactive compounds through *in vitro*, *in silico* and *in vivo* methodologies such as enzymatic assays (TYR and AChE), docking studies of AChE, and untargeted shotgun proteomics.
- V. To assess the environmental compatibility of the bioactive compounds, by comparing *in silico* predictions (EPI Suite™, ECOSAR, MAMPEC) with *in vivo* lethality bioassays (*Artemia salina* and *Amphibalanus amphitrite* nauplii) and acute toxicity tests (*Danio rerio* embryos).
- VI. To incorporate environmentally compatible bioactive compounds in coating formulations and evaluation of their AF performance at lab-scale (settlement inhibition bioassays, leaching tests, anti-biofilm bioassays) and real marine scenarios (morpho-taxonomic analysis of biofouling community) advancing technology readiness.

The objectives of this thesis align with the United Nations 2030 Agenda for Sustainable Development by addressing the urgent need to reduce the release of hazardous chemicals into the marine environment. This research fosters the improvement of ocean water quality by minimizing the input of harmful biocides widely employed in conventional AF systems, in line with **SDG 6** (Clean Water and Sanitation). In compliance with the **SDG 9** (Industry, Innovation and Infrastructure) this research will contribute to enhance scientific research and innovation upgrade the technological capabilities of maritime industrial sectors, including shipping industry, aquaculture and others by developing sustainable bioinspired AF coating

technologies. Furthermore, it promotes the technological valorisation of marine-derived resources, particularly marine cyanobacteria, through their application in the development of efficient, environmentally compatible, and value-added marine AF technologies, supporting **SDG14** (Life Below Water).



1.4. Thesis Outline



¹(M. L. Sousa *et al.*, 2019); ²(Antunes *et al.*, 2019)

Chapter 1. Introduction

The first chapter provides an overview of marine biofouling, its economic and environmental impacts, and the limitations of conventional AF strategies. It introduces the search for eco-friendly alternatives inspired by natural defence mechanisms, particularly those produced by marine microorganisms. Special attention is given to cyanobacteria, highlighting their ecological versatility, metabolic richness, and potential as sources of bioactive compounds. The progress in the identification of cyanobacterial metabolites with AF activity is also featured in this chapter.

Chapter 2. Harnessing Cyanobacterial Diversity for the Discovery of Antifouling Natural Products

The second chapter explores the AF potential of fifty-nine cyanobacterial strains from LEGE-CC, through a fractionation process. Fractions were tested against five strains of marine biofilm-forming bacteria and *M. galloprovincialis* plantigrades. Eight promising strains were selected for bioassay-guided isolation procedures. The outputs of this chapter were further explored in Chapters 3 and 4.

Chapter 3. Bioassay-Guided Discovery of Antifouling Nucleosides from the cyanobacterium *Leptothoe* sp. LEGE 181152: Efficacy, Molecular Targets, and Ecotoxicological Assessment

The third chapter reports the bioassay-guided isolation of two nucleosides with AF activity – adenosine (**1**) and 2'-deoxyadenosine (**2**) – from the cyanobacterium *Leptothoe* sp. LEGE 181152, that was highlighted as a promising strain in Chapter 2. The molecular targets on mussel plantigrades were evaluated through enzymatic assays, docking studies, mussel plantigrade untargeted shotgun proteomics and functional enrichment analysis. Additionally, ecotoxicity were assessed through multi-specific lethality bioassays and *in silico* predictions.

The content of this chapter is published as an original research article entitled “Bioassay-Guided Discovery of Antifouling Nucleosides from the cyanobacterium *Leptothoe* sp. LEGE 181152: Efficacy, Molecular Targets, and Ecotoxicological Assessment”, in the journal *Ecotoxicology and Environmental Safety* (DOI: 10.1016/j.ecoenv.2025.119175).

Chapter 4. Structure-Antifouling Activity Relationship, Molecular Mechanisms, and Ecotoxicological Assessment of Nucleosides Hypoxanthine arabinoside and 2'-Deoxyinosine

The fourth chapter investigates the potential, structure–activity relationships, molecular mechanisms, and ecotoxicological evaluation underlying nucleoside-mediated antifouling activity by analysing two analogues – hypoxanthine arabinoside (1') and 2'-deoxyinosine (2') – of the nucleosides isolated in Chapter 3. AF efficacy was assessed in micro and macrofouling species. The AF molecular mechanisms on mussel plantigrades were evaluated through enzymatic assays, docking studies, whole-organism untargeted shotgun proteomics and functional enrichment analysis. Additionally, ecotoxicity were assessed through lethality bioassays and *in silico* predictions.

The content of this chapter is submitted to publication as an original research article entitled “Structure–Activity Relationships, Molecular Mechanisms, and Ecotoxicological Evaluation Underlying Nucleoside-Mediated Antifouling Activity” in the journal *Ecotoxicology and Environmental Safety*.

Chapter 5. Antifouling activity and ecotoxicological profile of the cyanobacterial oxadiazine Nocuolin A

The fifth chapter addresses the often-contradictory status of supposedly 'ecofriendly' natural product AF agents by studying AF activity and ecotoxicological profile of the cyanobacterial compound nocuolin A. AF efficacy was assessed in micro and macrofouling species. The molecular targets on mussel plantigrades were evaluated through enzymatic assays, docking studies, whole-organism untargeted shotgun proteomics and functional enrichment analysis. Additionally, ecotoxicity were assessed through lethality and embryo acute toxicity tests coupled with *in silico* predictions.

The content of this chapter is published as an original research article entitled “Antifouling activity and ecotoxicological profile of the cyanobacterial oxadiazine Nocuolin A”, in the journal *Chemosphere* (DOI: 10.1016/j.chemosphere.2024.143318).

Chapter 6. Engineered coatings containing cyclic peptides from cyanobacteria delay the development of a stable macrofouling community

The sixth chapter explores the potential of portoamides A and B (**1**), a previously described cyanobacterial cyclic peptide with AF activity, to be a sustainable AF agent. Studies of environmental compatibility, coating affinity and lab-scale and field validation were conducted.

The content of this chapter is published as an original research article entitled “Engineered coatings containing cyclic peptides from cyanobacteria delay the development of a stable macrofouling community”, in the journal *Trends in Biotechnology* (DOI: 10.1016/j.tibtech.2025.11.018)

1.4.1. Thesis Highlights

This work contributed to highlight cyanobacterial strains with bioactive potential for AF purposes for future development. From the fifty-nine cyanobacterial strains tested, thirty-six fractions from twelve different strains exhibited AF potential, revealing several new opportunities for exploring natural AF compounds in future studies. Novel promising AF activity was firstly described for four nucleosides (adenosine, 2'-deoxyadenosine, hypoxanthine arabinoside, and 2'-deoxyinosine). A strong AF activity was described for the cyanobacterial oxadiazine, nocuolin A, although it was proved to be harmful to the marine environment. Insights on the molecular targets of these compounds helped advancing our knowledge of the pathways underlying their AF activity on mussel plantigrades. A significant technological advance (TRL 3 to 6) was reached for the cyanobacterial portoamides A and B, successful incorporation in marine coatings was achieved, while maintaining its AF activity, and validation in real marine conditions showed compounds ability to modulate macrofouling community using different AF coating matrices.

2. Harnessing Cyanobacterial Diversity for the Discovery of Antifouling Natural Products

2.1. Introduction

Nature-based solutions, increasingly considered for biotechnological applications, may offer promising alternatives for AF agents that are both effective and environmentally compatible. Cyanobacteria are recognized for possessing chemically diverse secondary metabolites with a range of bioactive properties (Ali Shah *et al.*, 2017; Demay *et al.*, 2019). Several studies have already reported cyanobacterial compounds exhibiting AF activity against various marine organisms, including bacteria, microalgae, barnacles, mussels, and algae, have already been reported (Fathoni *et al.*, 2020; Levert *et al.*, 2018; Petitbois *et al.*, 2017). Nonetheless, their underlying potential as a source of AF agents remains largely underexplored.

The Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) (<https://lege.ciimar.up.pt/>), housed at CIIMAR in Matosinhos, Portugal, comprises a vast taxonomic and genetic diversity of cyanobacteria, with over 700 isolates obtained from a variety of ecosystems (Ramos *et al.*, 2018). LEGE-CC serves as a valuable biological repository, being explored for diverse biotechnological applications including nutraceuticals, cosmeceuticals, and environmental purposes. In recent years, LEGE-CC strains have demonstrated a broad range of bioactivities, including anticancer (Avalon *et al.*, 2024), antiviral (Raja *et al.*, 2025), antiprotozoal and anti-helminthic (Vieira *et al.*, 2025), lipid reducing (Ribeiro *et al.*, 2023; N. G. Silva *et al.*, 2024), insulin mimetic (Ribeiro *et al.*, 2024), antioxidant and anti-inflammatory (Morone *et al.*, 2025; Rodrigues *et al.*, 2024). However, few studies explore the biotechnological potential of LEGE-CC cyanobacterial strains for AF applications (Almeida *et al.*, 2015). In fact, only two cyanobacterial compounds were reported with AF activity: portoamides A-B, isolated from *Phormidium* sp. LEGE 05292 (Antunes *et al.*, 2019) and nocuolin A, isolated from *Nodularia* sp. LEGE 06071 (S. Pereira *et al.*, 2024). Therefore, LEGE-CC cyanobacterial strains represent a relatively untapped resource with significant potential for the discovery of novel AF agents. Taking into account the extensive number of available strains, Ferreira *et al.*, (2021) developed the Cyanobacterial Natural Products Library (LEGE-NPL) - a strategic tool to accelerate the

screening process and facilitate the identification of bioactive compounds. This platform enables the rapid and automated fractionation of cyanobacterial extracts, producing eight uniformly classified fractions (A–H) per strain, each with comparable mass. The standardized process ensures the availability of sufficient material for LC-MS analysis, streamlining dereplication and facilitating the efficient prioritization of bioactive fractions.

Building on this strategy, this work aimed to investigate the biotechnological potential of a wide diversity of cyanobacterial strains housed in LEGE-CC as a source of novel AF agents. Using the LEGE-NPL approach for preliminary screening and bioassay-guided discovery, AF activity was evaluated against the growth of five marine biofilm-forming bacteria, and the settlement of an important macrofouler species, the mussel *Mytilus galloprovincialis*.

2.2. Materials and Methods

2.2.1. General Experimental Procedures

High-performance liquid chromatography (HPLC) was performed in a Waters Alliance e2695 Separations Module instrument, coupled to a photodiode array detector (Waters 2998 PDA) and an automatic Waters Fraction Collector III (Waters, Milford, MA, USA), using an ACE 10 C8 column (50×10 mm, ACE, Reading, UK), and HPLC grade solvents (VWR).

The liquid chromatography-high resolution electrospray ionization tandem mass spectrometry (LC-HR-ESI-MS/MS) data were acquired on a system composed of a Dionex UltiMate 3000 HPLC with an MWD-3000RS UV/VIS detector, coupled to a Q Exactive Focus mass spectrometer controlled by Xcalibur 4.1 software (Thermo Fisher Scientific, Waltham, MA, USA), and using an ACE UltraCore 2.5 SuperC18 column (75 × 2.1 mm, ACE, Reading, UK). The UV absorbance was monitored at 254 nm. HR-ESI-MS-MS was obtained in positive mode using a capillary temperature of 262.5 °C, spray voltage of 3.5 kV, full MS scan at the resolution of 70,000 FWHM (m/z range of 150–2000), and data dependent MS2 (ddMS2, Discovery mode) at the resolution of 17,500 FWHM (isolation window used was 3.0 amu and normalized collision energy was 35).

2.2.2. Preliminary screening for AF Activity

2.2.2.1. Selection and Culture of Cyanobacterial Strains

Twelve cyanobacterial strains were selected from the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) (<https://lege.ciimar.up.pt/>) (Ramos *et al.*, 2018) representing a broad phylogenetic and ecological diversity (Figure 3). The selected strains belonged to the orders *Synechococcales*, *Oscillatoriales*, *Pleurocapsales*, and *Nostocales*, and were

primarily isolated from marine intertidal zones, environments characterized by extreme and fluctuating conditions. According to their characteristics, strains were cultured in Z8 medium or Z8 medium supplemented with 25‰ of synthetic sea salts (Tropic Marin, Berlin, Germany) along with 1‰ of Vitamin B₁₂ (Kotai, 1972). Cultures were maintained at 25 °C, with constant aeration, under a 14 h /10 h (10–30 µmol photons s⁻¹ m⁻²) light/dark cycle and cultured up to 4 L. Cyanobacterial biomass was harvested after 90 to 160 days of growth, using either centrifugation or filtration, and subsequently freeze-dried (LyoQuest, Telstar, Terrassa, Spain).

2.2.2.2. Preparation of crude extracts and fractions

For the extraction and fractionation process of the cyanobacterial strains, the methodology outlined by Ferreira *et al.* (2021) was followed. Briefly, the freeze-dried biomass (approximately 2 g per stain) was extracted three times with methanol, with a 5-minute ultrasound treatment applied between each extraction. After being filtered and concentrated on a rotary evaporator, 40 mg mL⁻¹ of the crude extracts were used in the fractionation process, in a reverse-phase HPLC with a water: acetonitrile gradient, yielding eight fractions (A–H) (Table 6). Fractions were then dried on a CentriVap Concentrator (LabConco, Kansas City, MO, USA), solubilized in 500 µL of Dimethyl Sulfoxide (DMSO) (≥ 99.9%, Sigma–Aldrich, USA), and stored at -80 °C.

Table 6. HPLC conditions for the fractionation of the twelve cyanobacterial strains.

Time (min)	Flow (mL min ⁻¹)	MeCN (%)	H ₂ O (%)	Collection Time (min)	Fraction
0.0	3.0	10	90	1.00-2.30	A
2.0	3.0	80	20	2.30-3.60	B
3.0	3.0	80	20	3.60-4.90	C
4.0	3.0	100	0	4.90-6.20	D
8.9	3.0	100	0	6.20-7.50	E
9.2	3.5	100	0	7.50-8.80	F
12.0	3.5	100	0	8.80-10.36	G
12.3	3.0	100	0	10.36-11.50	H
14.0	3.0	100	0	-	-
15.0	3.0	10	90	-	-
18.0	3.0	10	90	-	-

2.2.3. Bioactivity screening

In the preliminary screening for AF activity against micro- and macrofouling species, the twelve selected cyanobacterial strains were included, along with forty-seven more from the

LEGE-NPL, performing a total of 472 tested fractions from fifty-nine strains. All fifty-nine cyanobacterial strains used during the screening are listed in the Supplementary Table S1.

2.2.3.1. Settlement inhibition bioassay

The settlement inhibition bioassay using *M. galloprovincialis* plantigrades was performed following the protocol described by Almeida *et al.* (2017). Briefly, juvenile mussel aggregates were collected during low neap tides from intertidal pools at Memória Beach, Matosinhos, Portugal (41° 13' 59" N; 8° 43' 28" W). In the laboratory, samples were examined using a binocular magnifier (Olympus SZX2-ILLT) to identify mussel plantigrades ranging from 0.5 to 2 mm in size. Plantigrades exhibiting exploratory behavior were then transferred to a petri dish containing filtered seawater for gentle cleaning of adhered organic particles prior to their utilization in the bioassays.

The selected mussel plantigrades were exposed to test solutions in 24-well microplates with four replicates per condition, each with five plantigrades per well, for a duration of 15 hours at a temperature of 18 ± 1 °C in the darkness. Test solutions were prepared with filtered seawater, and fractions were evaluated at a concentration of $10 \mu\text{g mL}^{-1}$. Bioassays included negative control (0.1% DMSO) and positive control ($5 \mu\text{M CuSO}_4$). Following exposure, anti-settlement activity was assessed based on the presence or absence of efficiently attached byssal threads produced by each plantigrade under all tested conditions.

2.2.3.2. Bacterial growth inhibition bioassays

The bacterial growth inhibition bioassay (Almeida *et al.*, 2017) was performed on five strains of marine biofilm-forming bacteria *Cobetia marina* CECT 4278, *Vibrio harveyi* CECT 525, *Halomonas aquamarina* CECT 5000, *Pseudoalteromonas atlantica* CECT 570, and *Roseobacter litoralis* CECT 5395, selected from the Spanish Type Culture Collection (CECT). After inoculation in Marine Broth (Condalab), bacteria were exposed to cyanobacterial fractions at a concentration of $10 \mu\text{g mL}^{-1}$, in a 96-well flatbottom microtiter plates and incubated for 24 h (72 h for *Roseobacter litoralis*) at 26 °C. Four replicates were used for each condition. Bioassays included a negative (Marine broth with 1% DMSO) and a positive control (1:100 penicillin-streptomycin-neomycin stabilized solution (Sigma P4083)). Initial optical density was ensured to be at 0.1 (OD600). Bacterial growth inhibition was determined at 600 nm through the microplate reader (Biotek Synergy HT, Vermont, USA).

2.2.4. Exploring promising cyanobacterial strains for AF activity

2.2.4.1. Large-scale culture

Settlement inhibition results from the initial screening led to the selection of eight cyanobacterial strains: cf. *Oxynema acuminatum* LEGE 06072, unidentified filamentous Synechococcales LEGE 06144, unidentified unicellular Synechococcales LEGE 08333, Oscillatoriales cyanobacterium LEGE 12541, Synechococcales cyanobacterium LEGE 15546, *Gloeothoece* sp. LEGE 16572, *Leptothoe* sp. LEGE 181152, and *Limnorphis robusta* LEGE 18358 (Figure 2). As detailed in 2.2.2.1, this cyanobacterial strains were cultivated on a large-scale, using the appropriate medium under the standard growth conditions, for further fractioning and testing. The cultures were maintained in 20 L Nalgene polycarbonate containers with aeration. Biomasses were harvested through centrifugation or filtration, depending on whether the strain was filamentous or unicellular, respectively, followed by freeze-drying. This process was repeated until approximately 40 g of freeze-dried biomass was obtained for each strain to facilitate the bio-guided isolation of AF compounds.

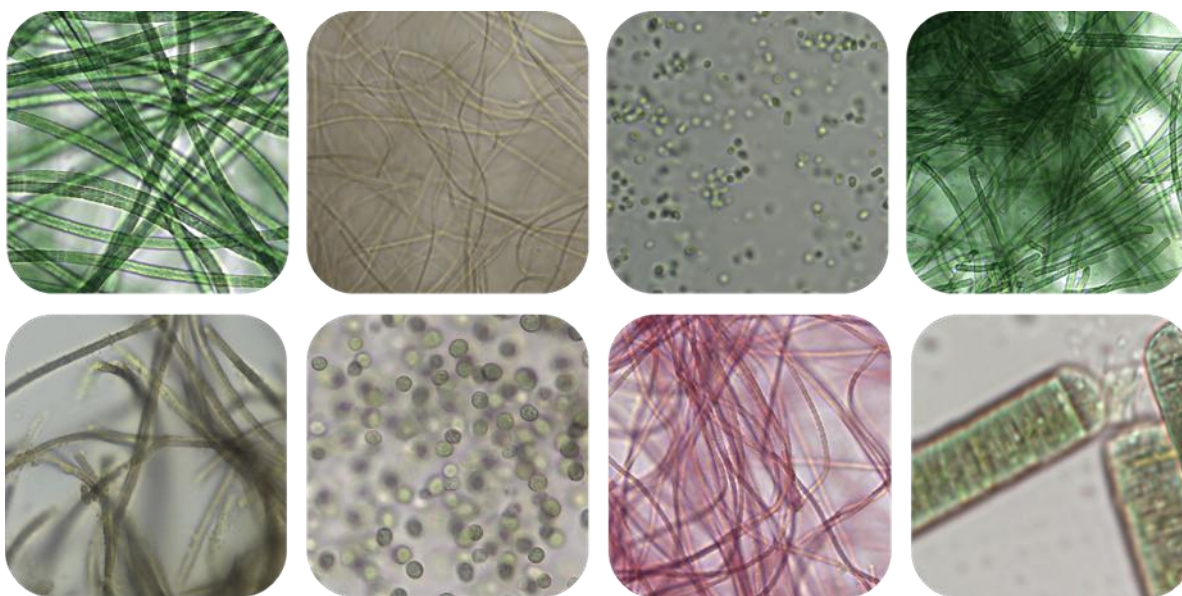


Figure 2. Cyanobacterial strains selected for AF study. From top to bottom: cf. *Oxynema acuminatum* LEGE 06072, unidentified filamentous Synechococcales LEGE 06144, unidentified unicellular Synechococcales LEGE 08333, *Geitlerinema* sp. LEGE 12541, Synechococcales cyanobacterium LEGE 15546, *Gloeothoece* sp. LEGE 16572, *Leptothoe* sp. LEGE 181152, and *Limnorphis robusta* LEGE 18358. Microphotography by LEGE-CC.

2.2.4.2. Bio-guided Fractionation

Five cyanobacterial strains – cf. *Oxynema acuminatum* LEGE 06072, unidentified filamentous Synechococcales LEGE 06144, unidentified unicellular Synechococcales LEGE

08333, *Synechococcales* cyanobacterium LEGE 15546, and *Leptothoe* sp. LEGE 181152 – were obtained in sufficient biomass to proceed to the organic extraction. Freeze-dried biomass was repeatedly extracted with methanol, using 5 min ultrasound intervals between extractions. The crude extracts were submitted to a Vacuum Liquid Chromatography (VLC) using silica gel 60 as the stationary phase and a mobile phase gradient from hexane: ethyl acetate (9:1 to 3:7, v/v, 0.5 L each), to ethyl acetate: methanol (1:0 to 0:1, v/v, 0.5 L each) (Avalon *et al.*, 2024). VLC conditions for cf. *Oxynema acuminatum* LEGE 06072 were adapted as shown in Table 7. Thin-layer chromatography (TLC) was used to analyse the chemical composition of the fractions, and those showing similar TLC profiles were combined. The fractions were dried, resuspended in DMSO at 50mM, and stored at -20°C.

Table 7. VLC conditions for the different cyanobacterial strains. (A) Conditions used on unidentified filamentous *Synechococcales* LEGE 06144, unidentified unicellular *Synechococcales* LEGE 08333, *Synechococcales* cyanobacterium LEGE 15546, and *Leptothoe* sp. 181152; and (B) Conditions adapted for cf. *Oxynema acuminatum* LEGE 06072.

A				B			
Fraction	Hexane	Ethyl Acetate	Methanol	Fraction	Hexane	Ethyl Acetate	Methanol
A	90%	10%	-	A	50%	50%	-
B	80%	20%	-	B	30%	70%	-
C	70%	30%	-	C	-	100%	-
D	60%	40%	-	D	-	70%	30%
E	40%	60%	-	E	-	-	100%
F	30%	70%	-				
G	-	100%	-				
H	-	70%	30%				
I	-	-	100%				

During each fractionation step, bioassay trials were conducted against a panel of marine biofilm-forming bacteria and mussel plantigrades. Fractions that resulted in $\leq 30\%$ of plantigrades settlement were selected for further investigation. All bioassays were performed as outlined in section 2.2.3, with fractions tested at a concentration of $10 \mu\text{g mL}^{-1}$.

To identify the compounds responsible for the AF activity against mussel plantigrades, bioactive fractions were analysed using LC-HR-ESI-MS/MS. Samples were prepared in 1 mg mL^{-1} in isopropanol, from which $5 \mu\text{L}$ was separated on an ACE UltraCore 2.5 SuperC18 column, at 40°C , using a gradient from 99.5 to 10% water: methanol: formic acid (95:5:0.1, v/v) to 0.5 to 90% isopropanol: methanol: formic acid (95:5:0.1, v/v) for 9.5 min, maintaining the last mixture until 15.5 min before returning to the initial conditions, with a flow rate of

0.35 mL min⁻¹ (L. Ferreira *et al.*, 2021). Manual dereplication was done by using the Dictionary of Natural Products 34.1 Chemical Search (<https://dnp.chemnetbase.com>), CyanoMetDB Version 3 (Janssen *et al.*, 2024; Jones *et al.*, 2021), and Natural Products Atlas 3.0 (Poynton *et al.*, 2025; Van Santen *et al.*, 2019).

2.2.5. Statistical Analysis

All data from the settlement and bacterial growth inhibition bioassays were verified by Shapiro-Wilk test for normality and by Levene's test for homogeneity of variances ($p < 0.05$). One-way analysis of variance (ANOVA) followed by Dunnett's test were used to identify significant differences between the fractions and the negative control ($p < 0.01$). Kruskal-Wallis' test was used for data exhibiting non-parametric distribution. Analyses were conducted using IBM SPSS Statistics 30. Data visualization was done using heatmaply package (Galili *et al.*, 2018) from R: a language and environment for statistical computing version 4.4.1 (R Core Team, 2021).

2.3. Results and Discussion

2.3.1. Bioactivity screening of the cyanobacterial strains

In this work, we analysed cyanobacterial strains from our in-house biological resource LEGE-CC for AF purposes. Figure 3 presents an overview of the diversity of cyanobacterial strains. Fifty-nine cyanobacteria from different environments (63% marine, 29% freshwater and 8% brackish) and distributed by five orders (10% Chroococcales, 14% Nostocales, 19% Oscillatoriales, 5% Pleurocapsales, and 53% Synechococcales) were included. According to Demay *et al.* (2019), from the more than 1600 unique cyanobacterial metabolites recently described, most were produced by Oscillatoriales, followed by Nostocales, Chroococcales and Synechococcales. Pleurocapsales are among the least reported as bioactive metabolite producers. Regarding the cyanobacterial metabolites with AF activity, Oscillatoriales and Nostocales are also the most studied cyanobacterial orders, with compounds active against marine bacteria, microalgae, barnacles, mussels, and algae (Antunes *et al.*, 2019; Brown *et al.*, 2004; Jaki *et al.*, 2000; Petitbois *et al.*, 2017; L. T. Tan *et al.*, 2009, 2010). By comparison, Synechococcales is less explored for its biotechnological AF potential, despite being an abundant order, with more than 70 genera and significant ecological roles (Allaf & Peerhossaini, 2022). For instance, honaucins A-C, isolated from the cyanobacterium *Leptolyngbya crossbyana* (Synechococcales), inhibited the bacterial quorum sensing bioluminescence in *Vibrio harveyi* BB120 and *Escherichia coli* JB525 (Choi *et al.*, 2012). Compounds that inhibit bacterial quorum sensing may have potential application as a

strategy to control the growth of microfouling communities (Muras, Parga, *et al.*, 2021), though this potential remained unexplored for these compounds. To our best knowledge, there is scarce information about the AF potential from Chroococcales and Pleurocapsales strains.

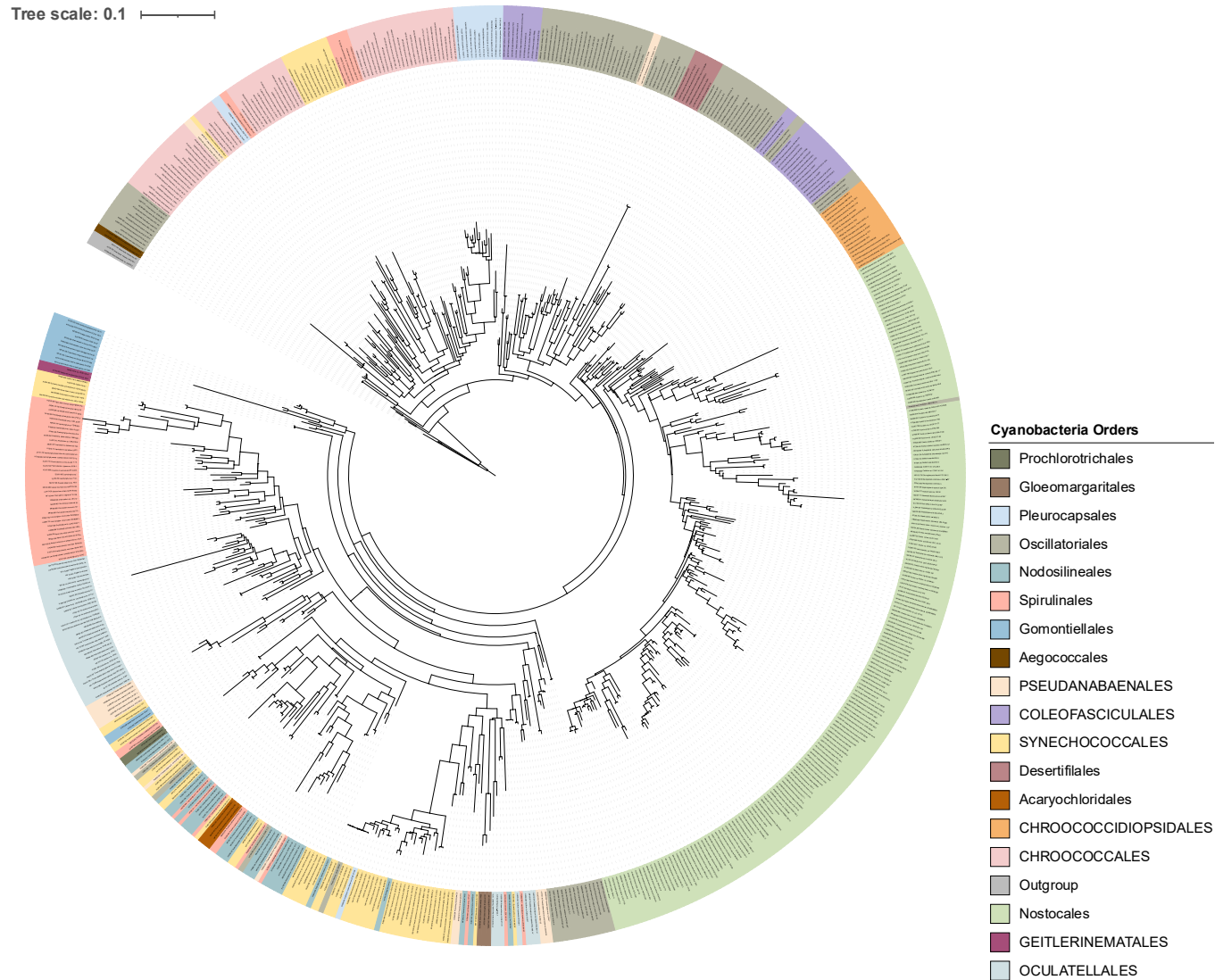


Figure 3. Cyanobacterial diversity in the screening. Maximum likelihood phylogenetic tree (GTR+G evolutive model) inferred from 16S rRNA gene fragments of cyanobacterial strains. Only bootstraps higher than 80 are shown. Cyanobacterial strains used in the study are marked with (*).

For the initial bioactivity screening, the fifty-nine strains were extracted and fractionated, following the methodology of the LEGE-NPL. Crude extracts are chemically highly heterogeneous; thus, this strategy facilitates the separation of samples into eight fractions of decreasing polarity. This simplification of the samples enables a more efficient search for bioactivities, as was demonstrated by Ferreira *et al.*, (2021) for cytotoxic activity against 2D and 3D models of human carcinoma (HCT 116), and non-cancerous cell line hCMEC/D3. The preliminary screening included bioassays against micro and macrofouling organisms, to assess general AF properties. The settlement inhibition bioassay used *M. galloprovincialis* plantigrades, which are commonly used as a macrofouling model organism in various studies (Neves, Vilas Boas, *et al.*, 2022; S. Pereira *et al.*, 2024; Resende *et al.*, 2021; Vilas-Boas *et al.*, 2023) due to their prevalence as a biofouling species worldwide. Figure 4 presents the results of the settlement inhibition assay conducted on mussel plantigrades using 432 fractions derived from 54 selected cyanobacterial strains. Notably, 79.63% of the tested cyanobacterial strains produced at least one fraction that effectively inhibited the settlement of mussel plantigrades (threshold was set at $\leq 30\%$ settlement), indicating a high prevalence of bioactive potential among the evaluated strains. Settlement inhibition was observed for all fractions; however, fraction H emerges as the most promising, with 31.48% exhibiting a settlement of $\leq 30\%$, followed by fraction D at 27.78%. In contrast, fraction F exhibited the lowest activity, with only 7.41% being active. Cyanobacterial strains such as *Synechocystis* sp. LEGE 06005, cf. *Oxynema acuminatum* LEGE 06072, *Nodosilinea* sp. LEGE 07085, *Leptothoe* sp. LEGE 181152, cf. *Oculatella* sp. LEGE 06141, *Dolichospermum* sp. LEGE 00263, *Cyanobium* sp. LEGE 06015, *Synechococcus* sp. LEGE 07172, *Cyanobium* sp. LEGE 07183, unidentified unicellular *Synechococcales* LEGE 10387, *Leptolyngbya saxicola* LEGE 06131, and *Synechococcales* cyanobacterium LEGE 15546 exhibited more than three fractions with settlement inhibition of mussel plantigrades. Of these twelve cyanobacterial strains that showed more active fractions against mussel plantigrades, *Synechococcales* was the most represented order with 66.67%, followed by *Oscillatoriales* (16.67%), and by *Chroococcales* and *Nostocales*, both with 8.33%. Most of these cyanobacterial strains were from the marine environment (66.67%), while freshwater and brackish was also represented, with 16.67% each.

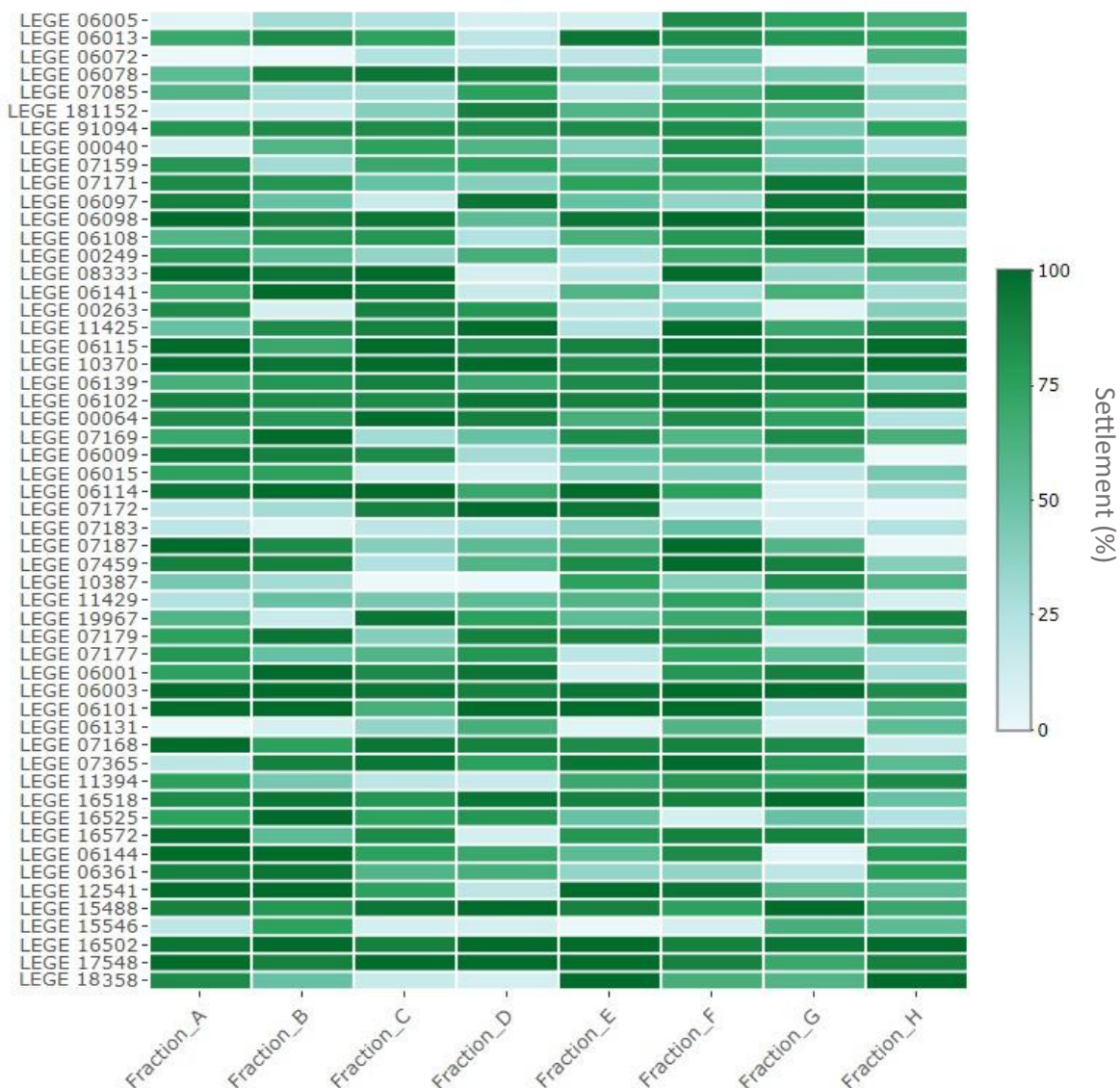


Figure 4. Screening results against *M. galloprovincialis* plantigrades. A total of 432 fractions (A-H) from 54 cyanobacterial strains were screened at $10 \mu\text{g mL}^{-1}$. Heatmap demonstrates the activity of the fractions through a colour gradient. Scale bar represents the percentage of settlement. Rows represent the cyanobacterial strains. Columns represent the fractions.

The adhesion of settling larvae and spores from macrofoulers is influenced by the chemical cues emitted from biofilms, which can induce, inhibit, or have a neutral effect on their adherence (Qian *et al.*, 2022). Consequently, growth inhibition bioassays performed against five strains of marine biofilm-forming bacteria were included. Among the fifty-nine cyanobacterial strains, 49.15% had fractions that inhibited the bacterial growth (threshold of $<70\%$ for bacterial growth). Growth inhibition was observed for all fractions against *C. marina* and *R. litoralis*, with fraction A (16.95%), and fraction B (27.12%) being the most active,

respectively. Overall, fractions demonstrated weak activity against *H. aquamarina*, with only fractions G (6.78%) along with E and F (3.39%); against *P. atlantica*, where only fraction A, C and E were active (1.69%); and against *V. harveyi* in which fraction D was the most active (5.08%), and no activity reported for fractions A, F and G.

The cyanobacteria *Synechococcus nidulans* LEGE 07171, *Cyanobium* sp. LEGE 06097, *Cyanobium* sp. LEGE 06098 showed fractions that inhibited the growth of *C. marina*, *H. aquamarina* and *R. litoralis*. Particularly, fractions A, B and C were active against both *C. marina* and *R. litoralis*. Other cyanobacteria such as *Toxifilum mysidocida* LEGE 06108, Aphanizomenonaceae cyanobacterium LEGE 00249, unidentified unicellular *Synechococcales* LEGE 08333, and cf. *Oculatella* sp. LEGE 06141 exhibited fractions active against *C. marina* and *R. litoralis*. The growth inhibition of *R. litoralis* and *V. harveyi* was observed on fractions from *Cyanobium* sp. LEGE 07183 and *Hyella patelloides* LEGE 07179 against, with highlight for fractions that were able to inhibit both bacteria (fractions B, D and E for *Cyanobium*, and fractions C and D for *Hyella*). On the other hand, there were cyanobacterial strains with fractions only active against *R. litoralis* such as cf. *Oculatella* sp. LEGE 06141, *Dolichospermum* sp. LEGE 00263, *Cyanobium* sp. LEGE 06015, *Synechococcus* sp. LEGE 07172, *Chroococcopsis* sp. LEGE 07187, *Geminobacterium atlanticum* LEGE 07459, *Synechocystis salina* LEGE 00038, *Nodosilinea* sp. LEGE 06001 and cf. *Romeria* sp. LEGE 06003. A total of seventeen cyanobacterial fractions showed promising activity mainly against marine bacteria *C. marina* and *R. litoralis* (Figure 5), although some bacterial inhibition was observed against *H. aquamarina* and *V. harveyi*. Heatmaps with the results from *H. aquamarina*, *P. atlantica*, and *V. harveyi* are presented on the Supplementary Material Figure S1. Of the seventeen active cyanobacterial strains, 64.71% belonged to *Synechococcales* order, followed by *Chroococcales*, *Pleurocapsales* and *Nostocales* with 11.76% each. These strains were mostly from the marine environment (82.35%), with only 17.65% from the freshwater ecosystems. No strains from the brackish environment were found among the seventeen active. In summary, preliminary screening results indicated that more cyanobacterial strains were effective against mussel plantigrades than marine bacteria, with cyanobacteria from *Synechococcales* order being the most active in both bioassays. This may be partially attributed to the larger presence of cyanobacterial strains from this order within the study. In contrast, none of the *Oscillatoriales* cyanobacterial strains, the second most represented order in this study, showed activity against marine bacteria. Notably, although previously identified compounds such as tiahuramides A-C

isolated from an Oscillatoriales cyanobacterium *Lyngbya majuscula* have been reported to inhibit marine bacterial growth (Levert *et al.*, 2018), similar activity was not observed here. Furthermore, the marine bacterial strains *C. marina* and *R. litoralis* appeared particularly susceptible to the cyanobacterial fractions.

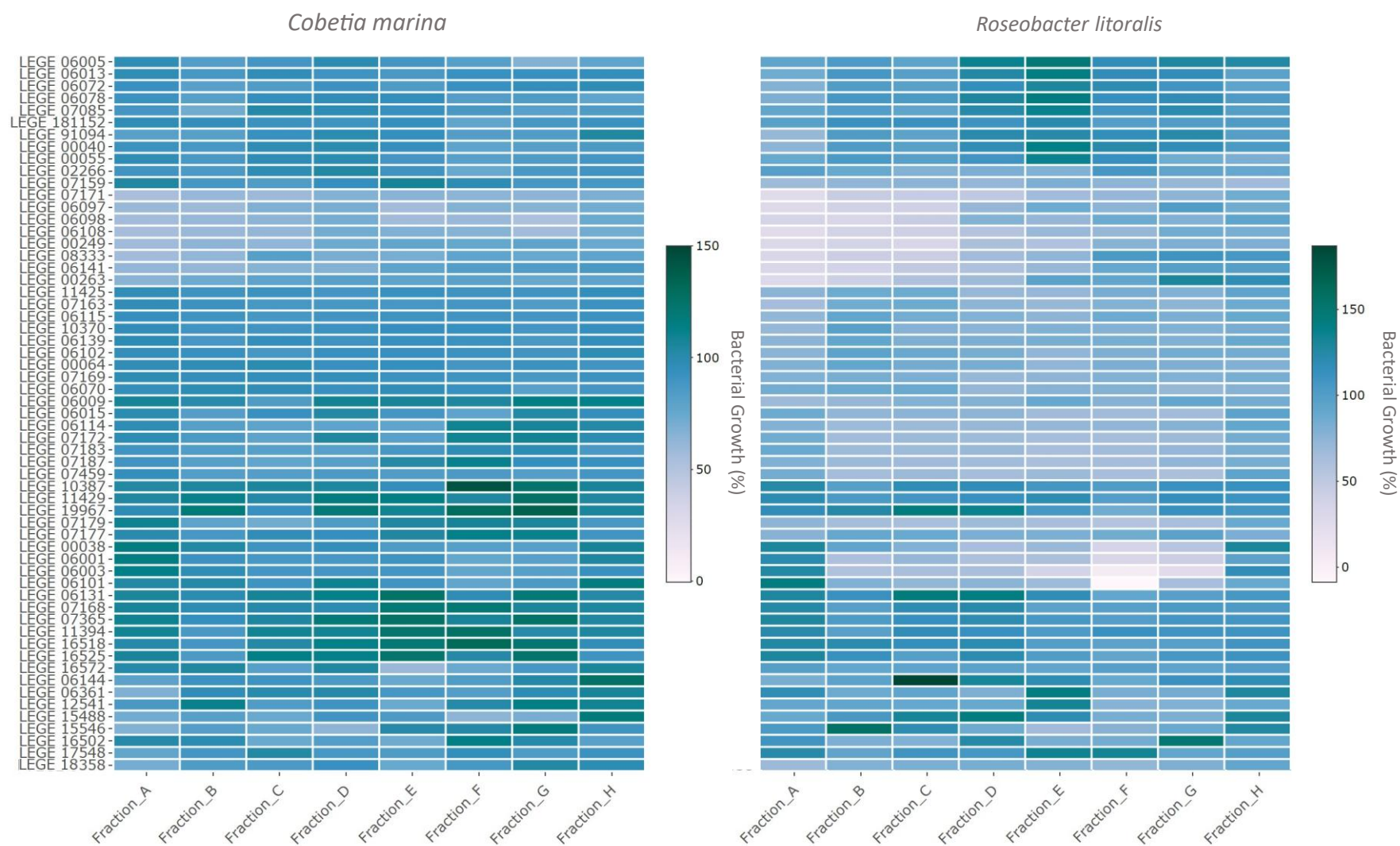


Figure 5. Screening results against *C. marina* and *R. litoralis*. A total of 472 fractions (A-H) from 59 cyanobacterial strains were screened at $10 \mu\text{g mL}^{-1}$. Heatmap demonstrates the activity of the fractions through a colour gradient. Scale bar represents the percentage of bacterial growth. Rows represent the cyanobacterial strains. Columns represent the fractions.

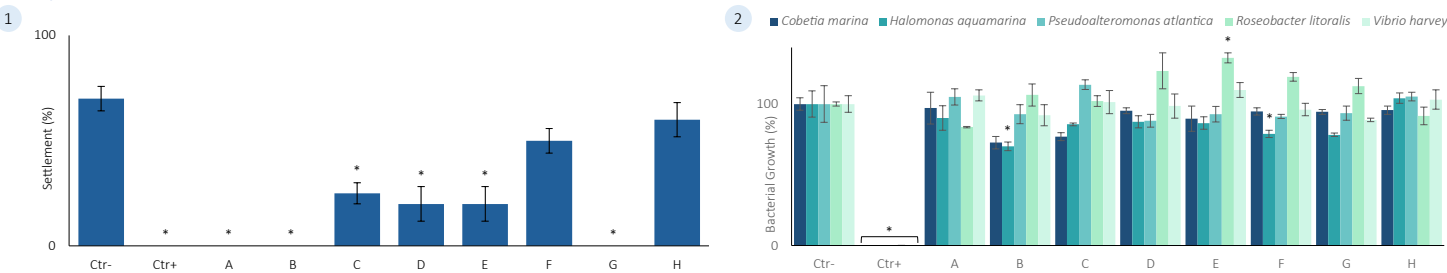
2.3.2. Selection and chemical processing of promising AF cyanobacterial strains

The preliminary screening led to the selection of eight promising cyanobacterial strains for further investigation. The selection was based on this criteria: bioactive hits against mussel plantigrades as is the case of cf. *Oxynema acuminatum* LEGE 06072, unidentified filamentous Synechococcales LEGE 06144, unidentified unicellular Synechococcales LEGE 08333, *Geitlerinema* sp. LEGE 12541, Synechococcales cyanobacterium LEGE 15546, *Leptothoe* sp. LEGE 181152, and *Limnoraphis robusta* LEGE 18358; and *Gloethece* sp. LEGE 16572 which has been recognized for its potential in the discovery of novel chemical structures (L. Ferreira *et al.*, 2021). The detailed results from these eight cyanobacterial strains are shown in Figure 6.

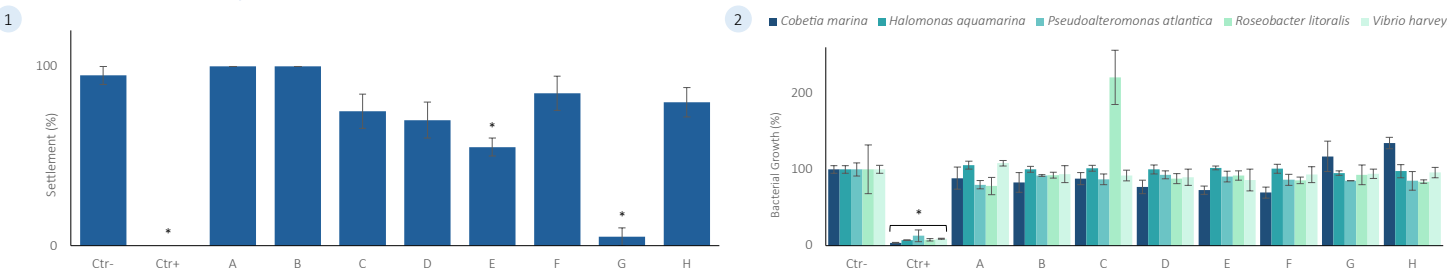
Six fractions (A, B, C, D, E, and G) from cf. *Oxynema acuminatum* LEGE 06072 significantly inhibited the settlement of mussel plantigrades ($p < 0.01$). However, the same promising AF activity was not observed during the preliminary screening against the growth of biofilm-forming bacteria, with only fractions B and F significantly inhibited *H. aquamarina* ($p < 0.01$). On the other hand, the unidentified filamentous Synechococcales LEGE 06144 cyanobacterium showed two fractions (E and G) able to significantly inhibit the settlement of mussel plantigrades ($p < 0.01$), while no significant activity was found against the five strains of marine bacteria, when compared to the negative control ($p > 0.01$). Fractions D, E, G and H from the unidentified unicellular Synechococcales LEGE 08333 significantly inhibited the settlement of mussel plantigrades, and all the eight fractions significantly inhibited the growth of some of the tested marine bacteria ($p < 0.01$), highlighting fractions C and E, that significantly inhibited the growth of three out of the five bacterial strains (*C. marina*, *H. aquamarina* and *P. atlantica*). The cyanobacterium *Geitlerinema* sp. LEGE 12541 exhibited three active fractions (D, G and H) significantly active against mussel plantigrades ($p < 0.01$). Interestingly, fraction G was also significantly active against *H. aquamarina* and *V. harveyi*, as was fraction F ($p < 0.01$). Another strain, the Synechococcales cyanobacterium LEGE 15546, also showed six fractions (A, C, D, E, F and H) that significantly inhibited the settlement of mussel plantigrades ($p < 0.01$). All the fractions except A, showed significant growth inhibition against *P. atlantica* ($p < 0.01$). *Gloethece* sp. LEGE 16572 only exhibited one significantly active fraction, D, against mussel plantigrades ($p < 0.01$). However, significant growth inhibition of *P. atlantica* was observed for fractions A and B, and fraction E significantly inhibited the growth of *C. marina* ($p < 0.01$). The marine cyanobacterium *Leptothoe* sp. 181152 showed five fractions (A, B, C, E and H) with significant activity against mussel plantigrades, and fractions F and G significantly inhibited the growth of *C. marina*, *H. aquamarina*, *P. atlantica* and *V. harveyi* ($p < 0.01$). Five fractions (A, B, C, E and H) from *Limnoraphis robusta*

LEGE 18358 significantly inhibited the settlement of mussel plantigrades ($p < 0.01$). All fractions except A, showed significant bacterial growth inhibition, particularly fractions B, C and D that inhibited more than one bacterial strain ($p < 0.01$).

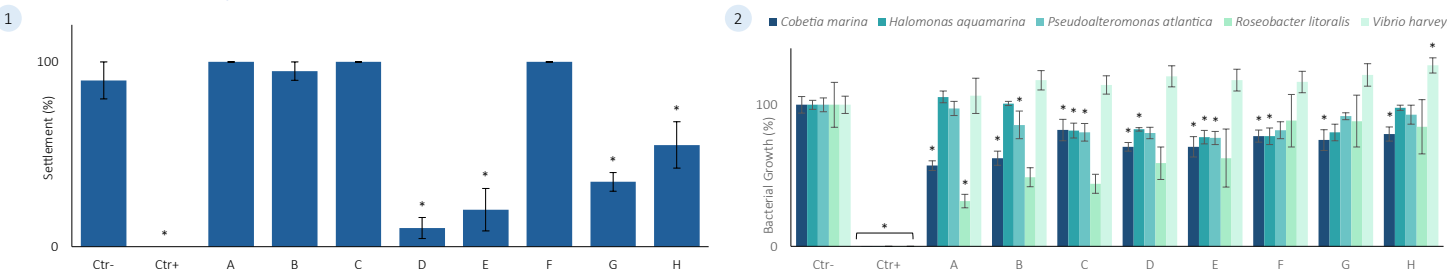
cf. *Oxynema acuminatum* LEGE 06072



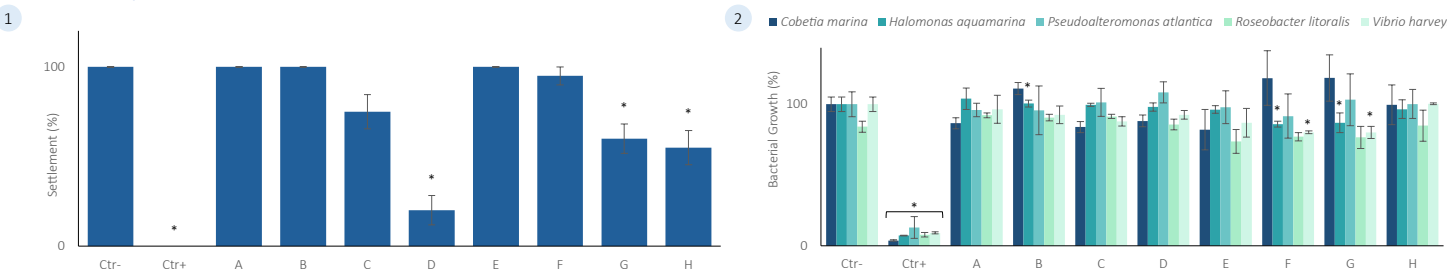
unidentified filamentous Synechococcales LEGE 06144



unidentified unicellular Synechococcales LEGE 08333



Geitlerinema sp. LEGE 12541



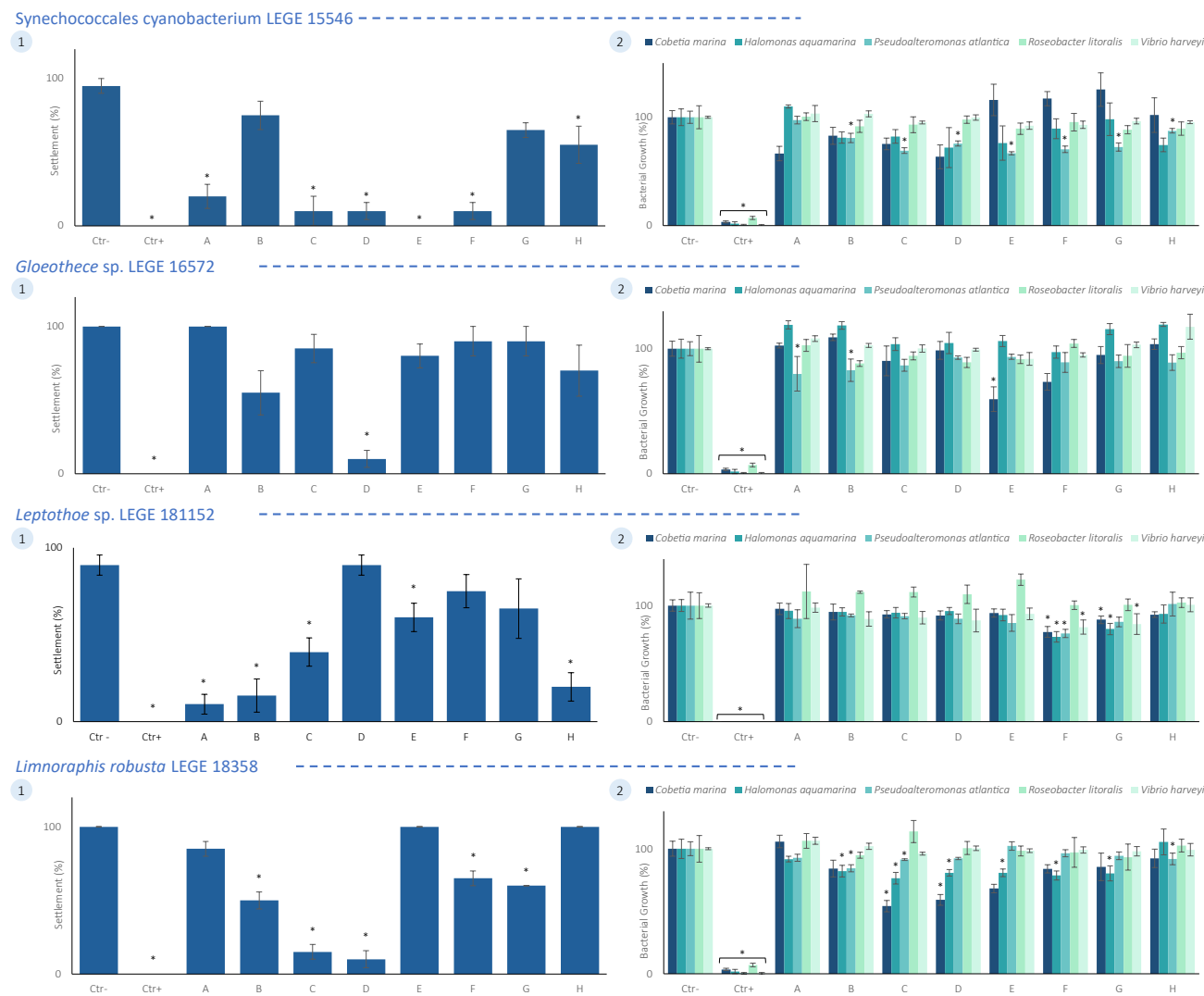


Figure 6. Detailed screening results for the eight selected cyanobacterial strains. All fractions were screened at $10 \mu\text{g mL}^{-1}$. (1) Settlement inhibition assays against the *M. galloprovincialis* plantigrades. Ctr-: DMSO (0.1 %); Ctr+: CuSO_4 (5 μM) (2) Growth inhibition assays against the five-marine biofilm-forming bacteria. Ctr-: DMSO (1%); Ctr+: 1:100 penicillin-streptomycin-neomycin stabilized solution.*Significant differences compared to the negative control ($p < 0.01$).

These strains were cultured continuously on a large scale until achieving the desired amount of biomass (ideally ≥ 40 g) necessary to proceed in the study. This threshold was defined (a) to compensate cumulative mass losses across extraction/fractionation steps considering mass losses during the extraction and fractionation, and (b) the possibility of a low natural abundance of the active metabolite. The total amount of freeze-dried biomass collected from each of the eight cyanobacterial strains is presented in Figure 7. Three strains exhibited limited growth rates and did not achieve the required biomass threshold. These include the marine cyanobacteria *Geitlerinema* sp. LEGE 12541, belonging to the Oscillatoriales order, which was cultured for 490 days and yielded 31 g of dried biomass; the freshwater cyanobacteria *Gloeothoe* sp. LEGE 16572 (from the Chroococcales order), cultured for 413 days and produced only 12.1 g of dried biomass; and *Limnoraphis robusta* LEGE 18358, a freshwater cyanobacteria from Oscillatoriales order, grown for 605 days and yielded 33.8 g of dried biomass. Consequently, these three cyanobacteria were not further investigated for AF compounds in the present study.

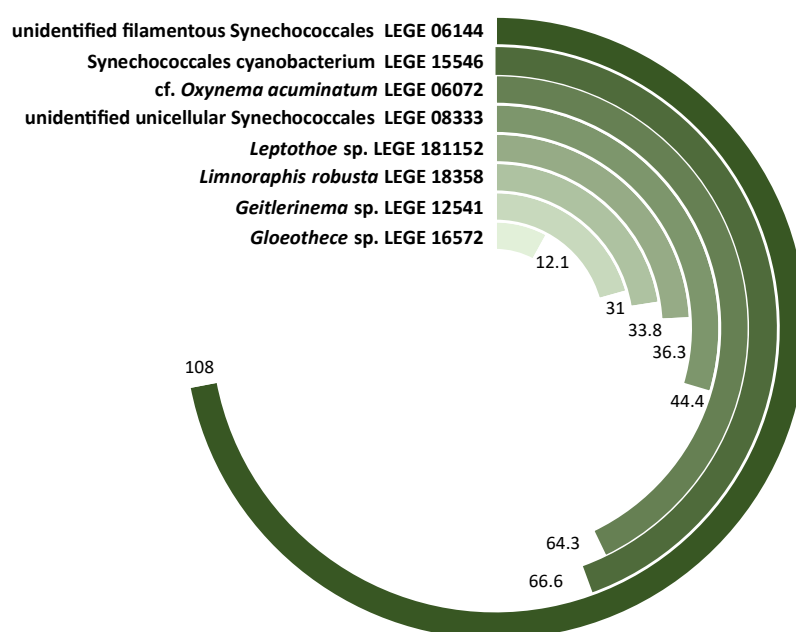


Figure 7. Biomass (g dw) of each of the selected cyanobacterial strains obtained after culture scale-up. Values are expressed in grams.

The investigation for AF bioactivity proceeded with the remaining five bacterial strains – cf. *Oxynema acuminatum* LEGE 06072, unidentified filamentous Synechococcales LEGE 06144, unidentified unicellular Synechococcales LEGE 08333, Synechococcales cyanobacterium LEGE 15546, and *Leptothoe* sp. LEGE 181152. Biomasses from these

strains were extracted and fractionated through VLC. The overall extraction yield was average; however, the unidentified filamentous *Synechococcales* LEGE 06144 and *Leptothoe* sp. LEGE 181152 exhibited the highest yields, both exceeding 30% (Table 8). According to VLC conditions, each crude extract should yield 9 fractions (A-I) as was for unidentified unicellular *Synechococcales* LEGE 08333 and *Synechococcales* cyanobacterium LEGE 15546. However, other cyanobacterial strains yielded different numbers of fractions. For instance, since the unidentified filamentous *Synechococcales* LEGE 06144 had the highest amount of crude extract, 38.2 g, volumes of 1 L were used for each solvent mixture, instead of the standard 0.5 L, yielding 14 fractions (A1-G2). Due to the high activity against mussel plantigrades shown during the preliminary study of cf. *Oxynema acuminatum* LEGE 06072, VLC conditions were adapted to start at hexane: ethyl acetate (1:1, v/v) instead of the standard hexane: ethyl acetate (9:1, v/v), which yielded 5 fractions (A-E) for this cyanobacterial strain. *Leptothoe* sp. LEGE 181152 yielded 10 fractions (A-H2). All VLC fractions were tested against marine bacteria and mussel plantigrades in search for promising AF hits.

Table 8. Details for the five cyanobacterial strains that were submitted to VLC.

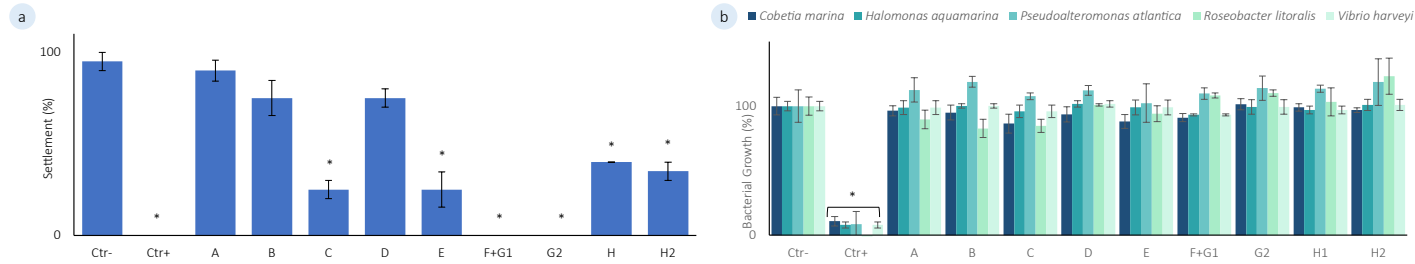
Strain ID	Time in culture (days)	Biomass (g dw)	Crude extract (g)	Extraction yield (%)	Fractions
cf. <i>Oxynema acuminatum</i> LEGE 06072	137	47.1	12	25.5	A, B, C, D, E
unidentified filamentous <i>Synechococcales</i> LEGE 06144	257	108	38.2	35.4	A1, A2, B1, B2, C1, C2, D1, D2, E1, E2, F1, F2, G1, G2
unidentified unicellular <i>Synechococcales</i> LEGE 08333	351	44.4	10	22.5	A, B, C, D, E, F, G, H, I
<i>Synechococcales</i> cyanobacterium LEGE 15546	550	66.6	15.2	22.8	A, B, C, D, E, F, G, H, I
<i>Leptothoe</i> sp. LEGE 181152	140	36.3	12.5	34.4	A, B, C, D, E, F+G1, G2, H1, H2

2.3.2.1. Cyanobacterial strains with AF hits

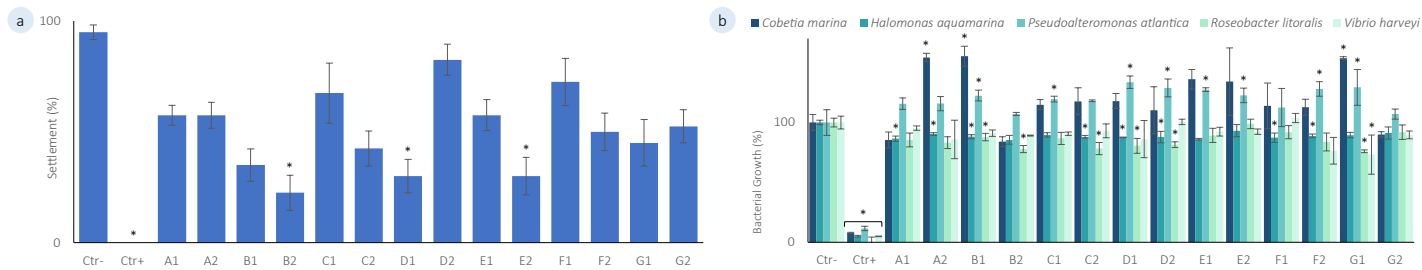
After the separation through VLC, promising results were obtained for three cyanobacterial strains: *Leptothoe* sp. LEGE 181152, unidentified filamentous *Synechococcales* LEGE 06144 and *Synechococcales* cyanobacterium LEGE 15546. Results from their AF activity against micro and macrofouling organisms are represented in Figure 8. *Leptothoe* sp. LEGE 181152 significantly inhibited the settlement of mussel plantigrades in six of the ten tested fractions ($p < 0.01$), with fractions F+G1 and G2 exhibiting an inhibition of 100%. However, no significant effect was observed on the bacterial growth ($p > 0.01$). Three fractions (B2, D1 and E2) from the marine cyanobacterium unidentified filamentous *Synechococcales* LEGE 06144, significantly inhibited the settlement of the mussel plantigrades ($p < 0.01$). In bioassays against marine bacteria, most fractions of this strain demonstrated significant growth inhibition.

Specifically, fractions B1, C2, D1, and D2 inhibited the growth of *H. aquamarina* and *R. litoralis*, while fraction G1 inhibited the growth of *R. litoralis* and *V. harveyi*. The freshwater Synechococcales cyanobacterium LEGE 15546 exhibited five fractions (B, C, D, H and I) that significantly inhibited the settlement of mussel plantigrades ($p < 0.01$). All fractions exhibited significant inhibition of bacterial growth ($p < 0.01$), particularly fractions C and H which inhibited *R. litoralis* and *V. harveyi*, and additionally *H. aquamarina* (fraction C) and *C. litoralis* (fraction H).

Leptothoe sp. LEGE 181152



unidentified filamentous *Synechococcales* LEGE 06144



Synechococcales cyanobacterium LEGE 15546

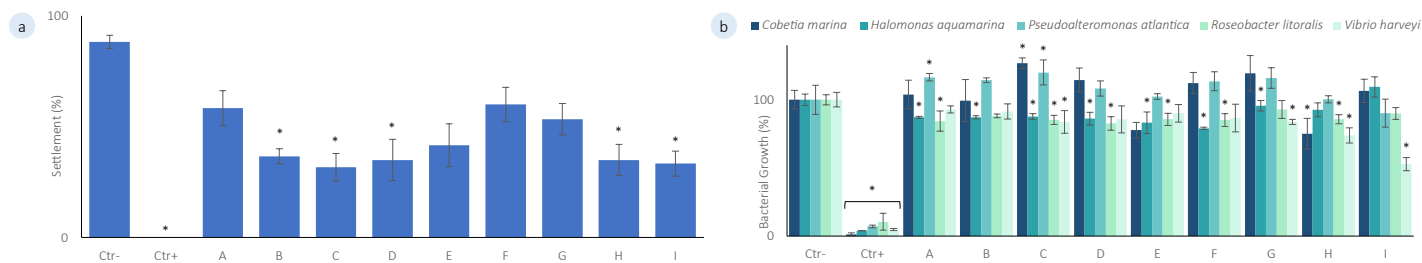


Figure 8. Antifouling activity of *Leptothoe* sp. LEGE 181152, unidentified filamentous *Synechococcales* LEGE 06144 and *Synechococcales* cyanobacterium LEGE 15546. VLC fractions were screened at 10 µg mL⁻¹. (a) Settlement inhibition bioassays against the *M. galloprovincialis* plantigrades, depicting the mean results of two bioassays. Ctr-: DMSO (0.1%); Ctr+: CuSO₄ (5 µM). (b) Bacterial growth inhibition against five marine biofilm-forming bacteria. Ctr-: DMSO (1%); Ctr+: 1:100 penicillin-streptomycin-neomycin stabilized solution. *Significant differences compared to the negative control (p < 0.01).

Leptothoe sp. LEGE 181152 fractions showed the strongest activity against mussel plantigrades, reaching 100% of settlement inhibition in two active fractions (F+G1 and G2). Fraction G2 was selected to proceed in the bio-guided isolation leading to the discovery of two primary metabolites with novel AF activity. Chapter 3 encompasses a comprehensive AF profiling of these metabolites with described AF efficacy, putative molecular targets, and environmental compatibility. Future work could explore the AF properties of the remaining active fractions of this promising cyanobacterium.

Based on the results obtained for unidentified filamentous *Synechococcales* LEGE 06144, a LC-HR-ESI-MS/MS analysis was conducted on fractions B1, B2, C1, C2 and D1, in positive mode, to analyse their chemical profile and predict which compound may be responsible for the AF activity, according to their relative abundance in the samples. The presence of the mass features m/z 887.5661 $[M + H]^+$ and m/z 917.5772 $[M + H]^+$ were consistently found across all the analysed fractions, only varying in terms of relative abundance: m/z 887.5661 $[M + H]^+$ was the most abundant in fractions B2 and C1, while m/z 917.5772 $[M + H]^+$ was the most abundant in fractions B1 and C2. In fraction D1, the mass feature m/z 903.5616 $[M + H]^+$ was the most abundant. Manual dereplication of these mass features on databases dedicated to natural products correlated them to hydroxypheophytin A (m/z 887.5661 $[M + H]^+$) and respective derivatives. Since another group at CIIMAR (manuscript under submission) was working with ^{13}C -R-hydroxypheophytin A and its isomeric form, ^{13}C -S-hydroxypheophytin A, isolated from cyanobacteria these compounds were used to confirm previous data. As such, the compounds were evaluated in the settlement inhibition assay, and results showed a significant settlement inhibition for mussel plantigrades (Figure 9).

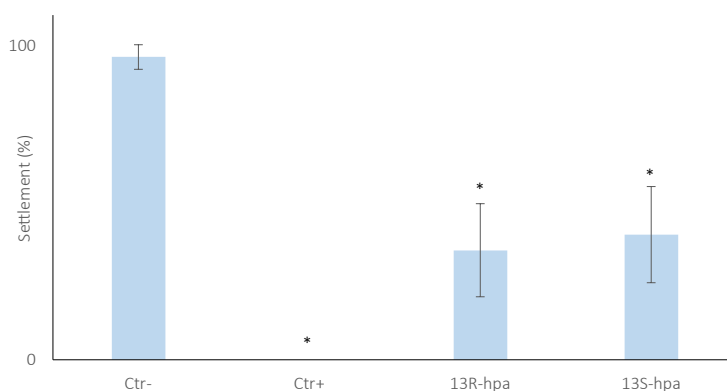


Figure 9. Settlement inhibition bioassay for ^{13}C -R-hydroxypheophytin A and its isomeric form, ^{13}C -S-hydroxypheophytin A. Compounds were tested at 11.3 μM against *M. galloprovincialis* plantigrades. Represented are the mean results of three bioassays. Ctr-: DMSO (0.1%); Ctr+: $CuSO_4$ (5 μM). *Significant differences compared to the negative control ($p < 0.01$).

Hydroxypheophytins, isolated from cyanobacteria and green algae, have demonstrated notable bioactivities relevant to human health applications, including lipid-reducing (Freitas *et al.*, 2019) and anticancer effects (X. Huang *et al.*, 2007). Del Amor *et al.* (2019) further highlighted the nutraceutical potential of 13²-hydroxypheophytin A by identifying its molecular targets on the hepatic cell line HepG2, linked to obesity and diabetes. However, no studies have reported biotechnological activities with environmental applications for these chlorophyll derivatives, emphasizing the relevance of investigating their AF potential. Future work should extend AF testing to other microfouling organisms (marine bacteria and diatoms), elucidate their mode of action on mussel plantigrades, and evaluate ecotoxicological effects to determine their suitability for incorporation into preventive marine coatings.

The Synechococcales cyanobacterium LEGE 15546 yielded several fractions with activity against mussel plantigrades; however, unlike *Leptothoe* sp. 181152, none achieved 100% settlement inhibition, making it challenging to select a single fraction for further study. Therefore, future work should adopt an approach similar to that used for the unidentified filamentous Synechococcales LEGE 06144, performing untargeted metabolomics on the active fractions to support the selection process for bio-guided isolation of AF compounds.

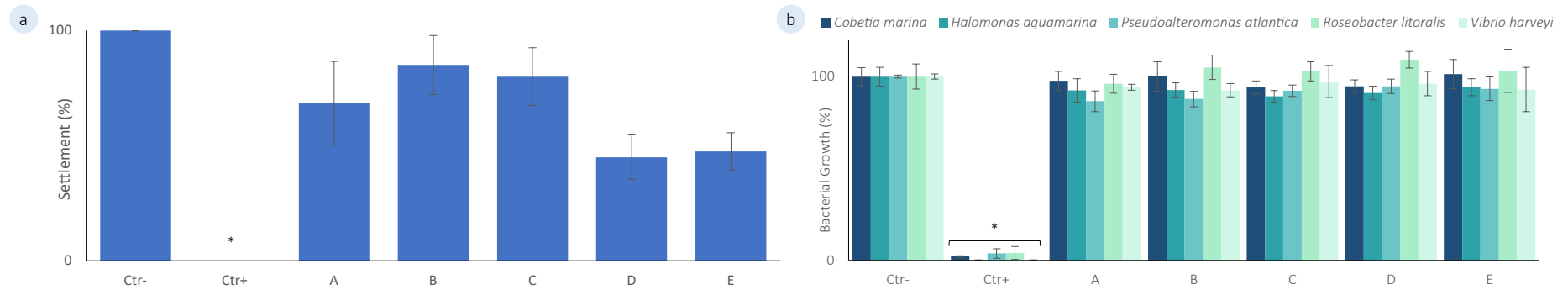
In summary, all three cyanobacterial strains showed several AF hits against mussel plantigrades, with some of them additionally have effects on marine bacteria. The development of new AF that effectively target diverse fouling species while remaining environmentally safe it has been proved to be quite challenging task (Cahill *et al.*, 2024). Hence, the broad-spectrum AF activity displayed by these cyanobacterial strains make them of high AF biotechnological value, supporting their further exploration.

2.3.2.2. Cyanobacterial strains with activity loss

On the other hand, cf. *Oxynema acuminatum* LEGE 06072, and unidentified unicellular Synechococcales LEGE 08333 seemed to have lost their activity on mussel plantigrades, since none of the fractions showed significant settlement inhibition against these organisms ($p > 0.01$) (Figure 10). Loss of activity during bio-guided fractionation is a common occurrence and can result from (a) degradation of the bioactive compounds during the process, (b) low concentrations of the bioactive compounds on the fractions, and (c) synergistic effects that contributed to overall bioactivity (Nothias *et al.*, 2018). An alternative explanation for the observed activity loss in cf. *Oxynema acuminatum* LEGE 06072 could be that the VLC conditions selected for this strain may not be adequate. Moreover, no activity was also registered against marine bacteria for the cf. *Oxynema*

acuminatum LEGE 06072 fractions ($p > 0.01$). In contrast, all fractions from the unidentified unicellular Synechococcales LEGE 08333 significantly inhibited bacterial growth ($p < 0.01$). Specifically, fractions B, C, D, E and F inhibited the growth of both *H. aquamarina* and *R. litoralis*. However, since the study's primary focus was on bioactive hits identified from the settlement inhibition bioassay to explore AF activity, further investigation of these cyanobacterial strains was concluded.

cf. *Oxynema acuminatum* LEGE 06072



unidentified unicellular *Synechococcales* LEGE 08333

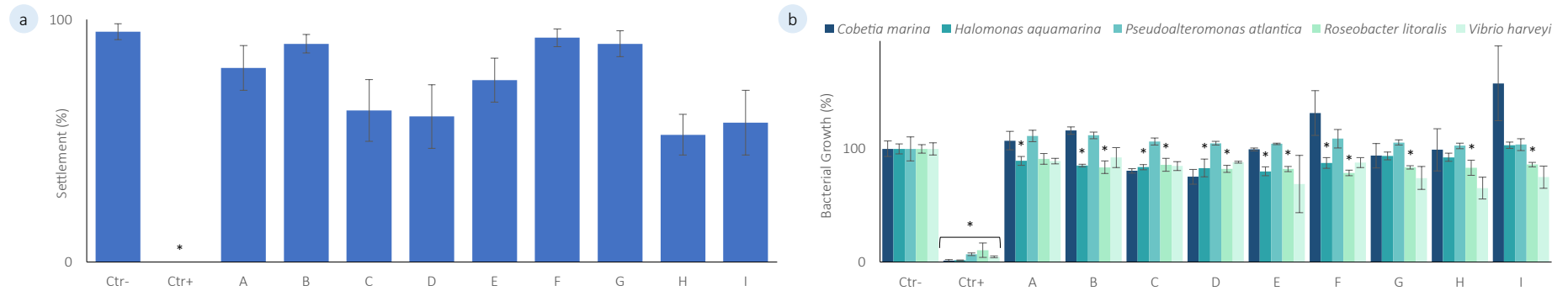


Figure 10. Antifouling activity of cf. *Oxynema acuminatum* LEGE 06072 and unidentified unicellular *Synechococcales* LEGE 08333. VLC fractions were screened at 10 µg mL⁻¹. (a) Settlement inhibition bioassays against the *M. galloprovincialis* plantigrades. Ctr-: DMSO (0.1%); Ctr+: CuSO₄ (5 µM). (b) Bacterial growth inhibition against five marine biofilm-forming bacteria. Ctr-: DMSO (1%); Ctr+: 1:100 penicillin-streptomycin-neomycin stabilized solution. *Significant differences compared to the negative control (p < 0.01).

2.4. Conclusions

This study broadens the understanding of the AF potential of LEGE-CC cyanobacteria, identifying promising bioactive leads in three strains - *Leptothoe* sp. LEGE 181152, unidentified filamentous Synechococcales LEGE 06144, and Synechococcales cyanobacterium LEGE 15546. These findings highlight cyanobacterial hydroxypheophytins as potential AF agents worthy of further investigation. Although cyanobacteria remain largely underexplored for AF biotechnological applications, this work demonstrates their great promise as sources of natural compounds for the development of effective, environmentally responsible AF solutions.

3. Bioassay-Guided Discovery of Antifouling Nucleosides from the cyanobacterium *Leptothoe* sp. LEGE 181152: Efficacy, Molecular Targets, and Ecotoxicological Assessment

The content of this chapter corresponds to a published original research article:

Pereira, S., Ferreira, L., Oliveira, I. B., Gonçalves, C., Palmeira, A., Turkina, M., Vasconcelos, V., Campos, A., Reis, M., & Almeida, J. R. (2025). Bioassay-guided discovery of antifouling nucleosides from the cyanobacterium *Leptothoe* sp. LEGE 181152: Efficacy, molecular targets, and ecotoxicological assessment. *Ecotoxicology and Environmental Safety*, 305 (15), 119175. <https://doi.org/10.1016/j.ecoenv.2025.119175>.

Sandra Pereira performed the chromatographic procedures (flash chromatography, TLC, solid-phase extraction (SPE), and HPLC) for the compounds' isolation and structural elucidation. All the bioassays (*M. galloprovincialis*, *Navicula* sp., marine biofilm-forming bacterial strains, and *Artemia salina*), enzymatic assays, filter aided sample preparation (FASP), protein identification, quantification and functional classification as well as *in silico* environmental predictions. All was conducted at Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR).

Culture of the cyanobacterial strain, methanolic extraction and VLC were carried out by Leonor Ferreira at CIIMAR/CIMAR LA.

The *Amphibalanus amphitrite* bioassay was performed by Isabel Oliveira at CIIMAR.

Pre-screening study was conducted by Catarina Gonçalves at CIIMAR.

Acetylcholinesterase docking studies were conducted by Andreia Palmeira at Laboratório de Química Orgânica e Farmacêutica da Faculdade de Farmácia da Universidade do Porto (LQOF/FFUP).

Peptides LC-MS/MS analysis was conducted by Maria Turkina at Department of Biomedical and Clinical Sciences from Faculty of Medicine and Health Sciences of the Linköping University (BKV/MEDFAK.LiU).

Bioassay-Guided Discovery of Antifouling Nucleosides from the cyanobacterium *Leptothoe* sp. LEGE 181152: Efficacy, Molecular Targets, and Ecotoxicological Assessment

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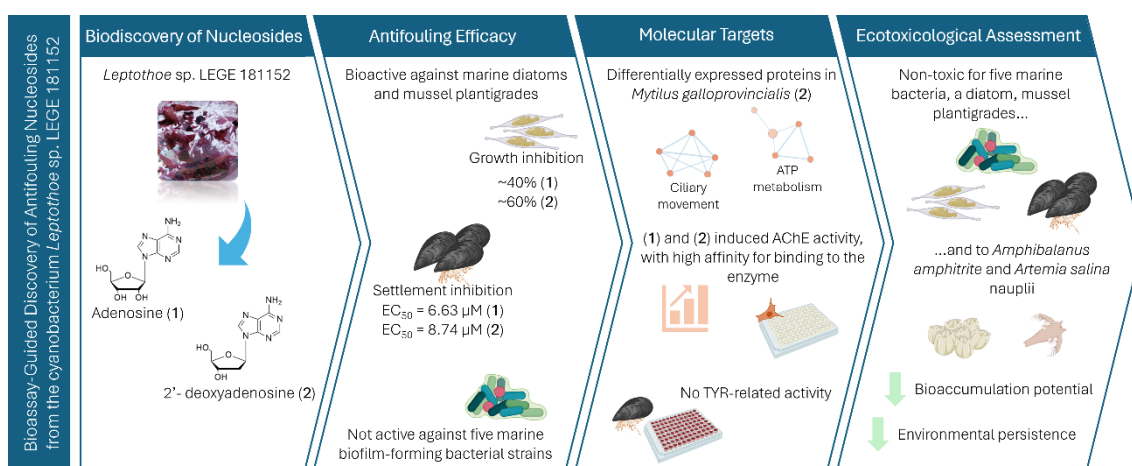
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Abstract

The prevention of marine biofouling remains a global challenge due to environmental concerns associated with current biocidal AF agents and increasing regulatory pressure to ban such biocides. Natural products present promising alternatives as effective and eco-friendly AF solutions, with cyanobacteria emerging as a rich source of bioactive compounds due to their remarkable biosynthetic potential. In this study, the AF bio-guided discovery of natural products from the cyanobacterium *Leptothoe* sp. LEGE 181152 led to the isolation of two nucleosides - adenosine (**1**) and 2'-deoxyadenosine (**2**), which were for the first time explored as innovative AF agents. A comprehensive approach was undertaken, including AF efficacy across biological levels, ecotoxicity and ecological risk assessment, and molecular targets elucidation. Compounds **1** and **2** successfully inhibited mussel settlement (EC_{50} = 6.63; 8.74 μ M), without exhibiting lethal effects (LC_{50} > 200 μ M). At these concentrations, both compounds increased the *in vitro* acetylcholinesterase activity. Functional enrichment analysis revealed effects on

proteins associated to ciliary motility and ATP metabolism. Additionally, both compounds inhibited the growth of *Navicula* sp. (~40% for **1**, and 60% for **2**). In addition to showing no effect on marine biofilm-forming marine bacteria, ecotoxicological assays with nauplii of *Artemia salina* and *Amphibalanus amphitrite* revealed no acute toxicity in these species. Furthermore, simulated environmental data indicated a low potential for bioaccumulation and low environmental persistence. Overall, these findings identify adenosine and 2'-deoxyadenosine as novel, low-risk natural AF agents, with real-world applicability as promising candidates for incorporation into marine coatings, thereby contributing to the development of more sustainable solutions for biofouling management.

Graphical Abstract



Keywords

Natural antifouling alternatives, Cyanobacteria, Nucleosides, Adenosine, 2'-Deoxyadenosine

3.1. Introduction

Marine biofouling, the natural accumulation of microorganisms, invertebrates and algae on submerged surfaces (X. Liu *et al.*, 2024), poses a major challenge to maritime industries, increasing drag on ships, fuel consumption, and the deterioration of marine structures. Although biocidal coatings (e.g. copper or metal-based, booster biocides) are the primary method for managing biofouling (Weber & Esmaeili, 2023), their effectiveness comes with environmental costs, as they release toxic compounds into marine ecosystems, raising concerns for both environmental and human health (Paz-Villarraga *et al.*, 2022; Vilas-Boas *et al.*, 2023). Efforts to minimize biocidal use in maritime industries have intensified the search for effective and environmentally sustainable AF solutions (European Commission: Deltares *et al.*, 2021). The Biocidal

Products Regulation (BPR) (EU 528/2012) was established to ensure that newly developed AF biocides comply with stringent environmental and safety requirements. These regulations mandate that AF agents exhibit target-specific efficacy, a well-defined mode of action, minimal toxicity to non-target species, and a low potential for bioaccumulation and environmental persistence (European Commission, 2024). Natural products are viewed as promising alternatives due to their broad biotechnological potential, offering AF solutions that may be both effective and environmentally safe. Among natural sources, cyanobacteria-ubiquitous photosynthetic prokaryotes - are a rich source of secondary metabolites with diverse bioactivities, making them strong candidates for AF applications. Several species from the genera *Okeania* (Petitbois *et al.*, 2017), *Lyngbya* (Levert *et al.*, 2018), *Kyrtuthrix* (Brown *et al.*, 2004), *Nostoc* (Blom *et al.*, 2006), *Phormidium* (Antunes *et al.*, 2019), *Leptolyngbya* (Choi *et al.*, 2012), *Anabaena* (R. B. Volk *et al.*, 2009), and *Nodularia* (S. Pereira *et al.*, 2024) have been reported to produce AF compounds effective against a range of biofouling organisms, including marine bacteria, microalgae, barnacles, mussels, and algae. More recently, the cyanobacterium genus *Leptothoe*, first described in sponge-associated cyanobacteria by Konstantinou *et al.* (2019), has emerged for its biotechnological potential. Strains of *Leptothoe* have demonstrated high potential for biofilm formation (Romeu *et al.*, 2023), inhibition of metalloproteinases (Morone *et al.*, 2022), anti-inflammatory activity (Rodrigues *et al.*, 2024), and cytotoxicity against human cancer cell lines (Konstantinou *et al.*, 2020). Interestingly, three geographically diverse *Leptothoe* strains were found to produce novel metallophores, the leptochelins A-C, which share homologous biosynthetic gene clusters with genetic signatures associated with this class of secondary metabolites (Avalon *et al.*, 2024). Here we explored the metabolic diversity of *Leptothoe* sp. LEGE 181152 as a source of AF agents. Using a bioassay-guided isolation approach, two primary metabolites, adenosine (**1**) and 2'-deoxyadenosine (**2**), were identified with notable AF activity. Nucleosides isolated from marine organisms have been reported with diverse bioactivities, including antibacterial, antiviral, anticancer, and vasodilator effects (M. Chen *et al.*, 2014; R. M. Huang *et al.*, 2014; Jiao *et al.*, 2007). However, despite their natural abundance and promising biotechnological potential, nucleosides remain largely unexplored as AF agents. To date, only kipukasin derivatives have been tested for AF activity showing low effectiveness in inhibiting settlement of *Amphibalanus amphitrite* cyprids (C. Y. Wang *et al.*, 2016) and *Bugula neritina* larvae (Wu *et al.*, 2020).

Here, after a bioguided-discovery and structural elucidation of two bioactive nucleosides, their AF profiles were further investigated by evaluating efficacy, molecular targets, and

predicted marine ecotoxicity, in accordance with the BPR EU 528/2012 requirements for new AF agents. Representative micro and macrofouling organisms were used to assess their effects on biofilm formation and larval settlement. In addition, to gain insight into underlying molecular mechanisms, we examined the impact of compounds **1** and **2** on mussel plantigrades through *in vitro* enzymatic assays targeting tyrosinase and acetylcholinesterase (AChE), two enzymes implicated in larval adhesion and settlement. Furthermore, an untargeted shotgun proteomics analysis was performed to identify potential molecular targets at the whole-organism level. To investigate environmental safety, marine ecotoxicity was also assessed through lethal endpoint assays across various marine species, complemented by *in silico* toxicity predictions to evaluate their ecological impact.

3.2. Materials and Methods

3.2.1. Bioguided-discovery of AF compounds

The marine cyanobacterial strain *Leptothoe* sp. LEGE 181152 was identified by Avalon *et al.* (2024) and is deposited at the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) (<https://lege.ciimar.up.pt/>) as a unialgal culture. The process of cultivation and extraction of the dry biomass was previously described in Avalon *et al.* (2024). Briefly, large-scale cultures of the cyanobacterium, up to 40 L, were grown in Z8 medium supplemented with 25‰ synthetic sea salts (Tropic Marin, Berlin, Germany) and 1‰ vitamin B₁₂, under standard conditions. The freeze-dried biomass was extracted with methanol, and the resulting extract was fractionated as described below and illustrated in Figure 11. The general chemical experimental procedures used for compounds isolation and characterization are described in detail in Supplementary Text S1.

Fractionation was initiated with vacuum liquid chromatography (VLC), using silica gel-60 (0.015-0.040 mm, Merck). The mobile phase consisted of a stepwise gradient of hexane: ethyl acetate (9:1 to 0:1, v/v), followed by mixtures of ethyl acetate and methanol (7:3 to 0:1, v/v). This chromatography yielded ten fractions (A-H2). The chemical composition of the fractions was evaluated using thin-layer chromatography (TLC), and fractions with similar TLC profiles were pooled together. Fraction G2 (1.68 g), eluted at ethyl acetate: methanol (3:7, v/v), showed promising AF activity in the settlement inhibition assay (Figure 11.b); therefore, it was subjected to flash chromatography. A stationary phase SiO₂ (Silicycle 25 g cartridge) was used, as well as a mobile phase with a mixture of hexane: ethyl acetate (from 1:1 to 0:1, v/v) and ethyl acetate: methanol (from 1:0 to 0:1, v/v), yielding 15 fractions. Fraction G2_11, eluted at ethyl acetate: methanol (19:1, v/v), was active in the settlement inhibition assay, and because of their similar TLC profile,

fractions G2_10-12 (160.38 mg) were pooled together and further fractionated through flash chromatography in a SiliaSep 4 g cartridge (Silicycle). This sample was eluted in ethyl acetate and isopropyl alcohol (from 1:0 to 0:1, v/v, in increasing gradients of 10%), yielding five subfractions (A-E). The active fraction D (41.14 mg), eluted at ethyl acetate: isopropyl alcohol (9:1, v/v), was further separated using an SPE C-18 cartridge (Strata® C18-E 55 µm, 70 Å, 1 g / 6 mL, Phenomenex). A stepwise elution was performed with water: methanol (1:1, v/v, 12 mL), methanol (12 mL), and dichloromethane (12 mL), yielding two fractions: D1 (33.88 mg), eluted with water: methanol, and D2 (6.12 mg), eluted with methanol and dichloromethane. Fraction D1 was purified by HPLC using a reverse-phase C18-AR column (ACE Excel 3 µm C18-AR, 75 x 4.6 mm) with water: acetonitrile in a gradient from 99:1 to 19:1 (v/v) in 20 min (1 mL min⁻¹), yielding 1.85 mg of compound **1** (*t_R* = 10.07 min) and 0.84 mg of compound **2** (*t_R* = 11.45 min).

Adenosine (1): Amorphous white powder; $[\alpha]_D^{23}$ -16.66 (c 0.1, CD₃OD); UV (MeOH) λ_{max} (log ϵ) 255.5 (3.42), 213.5 (3.39), 194.5 (2.99) nm; ¹H NMR (400.14 MHz, MeOD): δ 8.34 (1H, s, H-8), 8.22 (1H, s, H-2), 6.00 (1H, d, *J* = 6.4 Hz, H-1'), 4.75 (1H, m, H-2'), 4.36 (1H, dd, *J* = 5.1, 2.6 Hz, H-3'), 4.20 (1H, q, *J* = 2.6 Hz, H-4'), 3.90 (1H, dd, *J* = 12.5, 2.5 Hz, H-5a'), 3.78 (1H, dd, *J* = 12.6, 2.7 Hz, H-5b') ppm. ¹³C NMR (100.63 MHz, MeOD): δ 157.6 (C-4), 153.5 (C-2), 150.03 (C-6), 142.0 (C-8), 121.0 (C-5), 91.2 (C-1'), 88.1 (C-4'), 75.4 (C-2'), 72.6 (C-3'), 63.4 (C-5') ppm. LR-ESI-MS *m/z* 267 [M + H]⁺

2'-Deoxyadenosine (2): Amorphous white powder; $[\alpha]_D^{23}$ +5.42 (c: 0.1, MeOH); UV (MeOH) λ_{max} (log ϵ) 258.5 (3.39), 214.5 (3.42), 195.0 (2.98), 194.0 (2.98) nm; ¹H NMR (400.14 MHz, CD₃OD): δ 8.34 (1H, s, H-8), 8.20 (1H, s, H-2), 6.43 (1H, dd, *J* = 8.0, 6.0 Hz, H-1'), 4.08 (1H, q, *J* = 3.0 Hz, H-4'), 3.85 (1H, dd, *J* = 12.3, 3.0 Hz, H-5a'), 3.75 (1H, dd, *J* = 12.4, 3.3 Hz, H-5b'), 2.81 (1H, ddd, *J* = 13.6, 8.0, 5.8 Hz, H-2a'), 2.42 (1H, ddd, *J* = 13.6, 6.0, 2.7 Hz, H-2b') ppm. LR-ESI-MS *m/z* 251 [M + H]⁺

3.2.1.1. Macrofouling settlement inhibition bioassay

The settlement inhibition of mussel (*Mytilus galloprovincialis*) plantigrades was used for the bio-guided discovery of bioactive compounds. Bioassay trials were used for each step of fractionation, and bioactive hits were selected and pursued accordingly.

For the bioassays, small aggregates of juvenile mussels (*M. galloprovincialis*) were collected during low neap tides in intertidal pools at Memória Beach, Matosinhos, Portugal (41° 13' 59" N; 8° 43' 28" W). In the laboratory, the samples were screened in a binocular magnifier (Olympus SZX2-ILLT) for mussel plantigrades (0.5 – 2 mm). Mussel plantigrades displaying exploratory behaviour were transferred to a petri dish

with filtered seawater and gently cleaned with adhered organic particles before being used in the bioassays.

According to Almeida *et al.* (2017), the selected active mussel plantigrades were exposed to test solutions in 24-well microplates with four replicates (five plantigrades per well) for 15 h at 18 ± 1 °C in the dark. Fractions were assessed at a concentration of $10 \mu\text{g mL}^{-1}$. All the test solutions were prepared in filtered seawater. Bioassays included negative (0.1% DMSO) and positive controls ($5 \mu\text{M CuSO}_4$). After exposure, settlement activity was evaluated by the presence of byssal threads - the adhesion structures produced by mussel plantigrade larvae - firmly attached to the substrate. Plantigrades lacking these structures, or failing to attach, were considered unsettled.

Pure compounds were tested at 25 and $50 \mu\text{M}$ by diluting the stock solution in DMSO in filtered seawater. The half-maximal effective concentration that inhibited 50% larval settlement (EC_{50}) and median lethal dose (LC_{50}) were also determined for each compound.

3.2.2. Anti-microfouling evaluation of hit compounds **1** and **2**

To assess the compounds' activity against other microfouling organisms, five strains of marine biofilm-forming bacteria *Cobetia marina* CECT 4278, *Vibrio harveyi* CECT 525, *Halomonas aquamarina* CECT 5000, *Pseudoalteromonas atlantica* CECT 570, and *Roseobacter litoralis* CECT 5395 were selected from the Spanish Type Culture Collection (CECT), and the antibacterial bioassay was performed as described in Almeida *et al.*, (2017). After inoculation in Marine Broth (Condalab), bacteria were exposed to 25 and $50 \mu\text{M}$ in 96 well flatbottom microtiter plates and incubated for 24 h (72 h for *Roseobacter litoralis*) at 26 °C. Four replicates were used for each condition. Marine broth with 1% DMSO was used as the negative control and 1:100 penicillin-streptomycin-neomycin stabilized solution (Sigma P4083) was used as the positive control. The optical density was ensured to be at 0.1 (OD_{600}). Bacterial growth inhibition at the tested concentrations was determined at 600 nm using a microplate reader (Biotek Synergy HT, Vermont, USA).

A strain of the benthic marine diatom *Navicula* sp. was acquired from the Spanish Collection of Algae (BEA-Banco Español de Algas, Las Palmas, Spain) for anti-microalgal screening, performed according to Antunes *et al.*, 2019. Diatoms, inoculated in Guillard's (F/2) medium with silica (Sigma G9903) at an initial concentration of $2 - 4 \times 10^5 \text{ cells mL}^{-1}$, were exposed to compounds at 25 and $50 \mu\text{M}$ for the first screening, in 96 well flat-bottom microtiter plates for 10 days at 20 °C. Subsequently, a range of

dilutions was tested on the diatoms, with concentrations from 0.8 to 200 μM . Each condition was assessed in quadruplicate. The negative control was constituted by medium F/2, with 1% DMSO and cycloheximide (3.55 μM) as a positive control. Growth inhibition by diatoms was quantified by the difference in cell densities among conditions. Optical microscopy checks were performed at the start and end of the bioassay to confirm cell motility and structural integrity of the diatoms across all tested concentrations for both compounds.

3.2.3. AF Mechanism of Action of compounds **1** and **2**

3.2.3.1. *In silico* docking study for AChE activity

The crystal structure of *Electrophorus electric* AChE (PDB code: 1C2O) (Bourne *et al.*, 1999) was obtained from the protein databank (PDB) and it was used as the target in the *in silico* docking studies. The structures of the test compounds, and two known AF agents with AChE inhibitory capacity, pulmonarins A and B (Trepos *et al.*, 2014), were drawn and minimized using the Austin Model 1 (AM1) semi-empirical quantum mechanics force field until the gradient between any two successive steps in the geometry search was less than $10^{-1} \text{ kcal mol}^{-1} \text{ \AA}^{-2}$, using Hyperchem 7.5 (Hypercube, Florida, USA) (Froimowitz, 1993). Autodock Vina (Molecular Graphics Lab, The Scripps Research Institute, San Diego, CA, USA) was employed in the docking study (Trott & Olson, 2010), using an exhaustiveness of 8 and a grid box with the dimensions of $15.0 \times 20.0 \times 15.0 \text{ \AA}$ encircling the acetylcholine active site. Nine docking poses were obtained for each ligand. The lowest docking score poses were selected for detailed analysis of binding mode and visual inspection using PyMol 1.3 (Schrödinger, New York, NY, USA) (Lill & Danielson, 2011).

3.2.3.1.1. *In Vitro* AChE Activity

AChE activity was assessed for the compounds using *electrophorus electric* AChE Type V-S (SIGMA C2888, E.C. 3.1.1.7) according to Ellman *et al.* (1961) and modified by Almeida *et al.* (2015). Succinctly, the preparation of the reaction solution involved 30 mL of phosphate buffer (0.1 M, pH = 7.2), 1 mL of the reagent dithiobisnitrobenzoate (DTNB) at 10 nM (5,5'-Dithiobis(2-nitrobenzoic acid) and sodium hydrogen carbonate dissolved in phosphate buffer at 0.1 M, pH = 7.2) and 200 μL of acetylthiocholine iodide at 0.075 M. To perform the assay, 50 μL of pure AChE (0.25 U mL^{-1}) was added to 250 μL of the reaction solution, for each condition. The compounds were assessed at concentrations of 25 μM and 50 μM . The negative and positive controls were DMSO (0.2%) and eserine (100 μM), respectively, and a blank containing 50 μL of phosphate buffer. All conditions

were assessed in quadruplicates. AChE activity was measured at 412 nm for 5 min at 25 °C.

3.2.3.2. *In Vitro* Tyrosinase Activity

Tyrosinase *in vitro* assay was conducted using *Agaricus bisporus* tyrosinase (EC 1.14.18.1) according to Adhikari *et al.*, (2008) with appropriate adaptations (Almeida *et al.*, 2017). Briefly, 50 µL of tyrosinase (25 U mL⁻¹) in phosphate buffer (50 mM, pH 6.5) was added to each condition. Compounds were screened at 25 and 50 µM. The enzymatic activity was initiated by L-dopa (25 mM) also prepared in phosphate buffer (50 mM, pH 6.5). Negative (0.2% DMSO) and positive controls (100 µM kojic acid) were included. All conditions were assessed in quadruplicate. Tyrosinase activity was detected at 475 nm for 5 minutes at 25 °C.

3.2.4. Proteomic profiling of *M. galloprovincialis* plantigrades

3.2.4.1. Sample preparation

M. galloprovincialis plantigrades were exposed to the compounds' concentrations (12.5 µM and 50 µM) as described previously for the settlement inhibition assay. The concentration of 12.5 µM was selected to represent a closer concentration to the EC₅₀ value observed for the mussel larvae while the 50 µM concentration purpose was to explore the differences in the proteomic expression of the larvae when exposed to higher concentrations. After the exposure, mussel plantigrades from negative control (0.1% DMSO) and tested compounds' concentrations were collected (10 larvae/replicate, 4 replicates/condition). Protein extraction and subsequent proteome analysis were performed as described by Campos *et al.*, (2016). Briefly, after defrosting in ice, samples were solubilized in lysis buffer (composed of 2% (w/v) SDS, 100 mM Tris-HCl at pH 7.6, 0.1 M DTT, and 1:50 protease inhibitor). Proteins were then digested through filter-aided sample preparation (FASP) (Wiśniewski *et al.*, 2009).

3.2.4.2. LC-MS/MS Analysis

Obtained peptides were reconstituted in formic acid (0.1%, v/v) and analysed by nano-LC coupled to a hybrid Ion trap-Orbitrap mass spectrometer (LTQ Orbitrap Velos Pro, Thermo Scientific, Waltham, EUA). Full scans were performed at 30,000 resolution with scan ranges of 380–2000 m/z and the top 20 most intense ions isolated and fragmented. Collision-induced fragmentation (CID) was used to fragment precursor ions. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analysed using Sequest HT (Thermo Fisher Scientific, San Jose, CA, USA; version IseNode in Proteome Discoverer 2.5.0.400). Sequest HT was set up to search the Mollusca UniProt Knowledgebase protein sequence database (The UniProt Consortium,

2023), Taxon ID6447 downloaded March 2023 comprising 753986 entries, and assuming the digestion enzyme trypsin. Sequest HT was searched with a fragment ion mass tolerance of 0.080 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine was specified in Sequest HT as a fixed modification. Oxidation of methionine was specified in Sequest as a variable modification.

3.2.4.3. Protein identification and quantification

Scaffold (version Scaffold_5.3.2, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Percolator posterior error probability calculation (Käll *et al.*, 2008). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii *et al.*, 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Protein abundances were determined by label-free method, using normalized spectral abundance factors (NSAFs) in Scaffold program.

3.2.4.4. Functional classification of protein and pathways analysis

The response of *M. galloprovincialis* to the compounds was evaluated by investigating molecular functions involved in mussel adhesive process. For this purpose, differentially expressed proteins (DEPs) were analysed for the presence of adhesion-related proteins previously described in the literature. Additionally, a functional enrichment analysis was performed to infer the putative biological functions of the affected proteins. Given its higher level of genome annotation comparatively to *M. galloprovincialis*, the homologous *Magallana gigas* (pacific oyster, previously *Crassostrea gigas*) proteins were selected for this analysis. Protein sequences were compared (blast) against the *M. gigas* sequences (97,078 sequences, downloaded from UniProt Knowledgebase release 2025_01, and *Crassostrea gigas*.cgigas_uk_roslin_v1 peptide sequences release 2024_08, downloaded from Ensembl Metazoa), using the local BLASTp function from Blast2GO version 6.0.3 (basic) (Conesa *et al.*, 2005; Conesa & Götz, 2008; Götz *et al.*, 2008), with a cut-off e-value of 1×10^{-3} . The g:OSt function in g:Profiler (version e112_eg59_p19_25aa4782, database updated on 03/02/2025) grouped the homologous *M. gigas* sequences into functional classes (Gene Ontology classes). Statistically significant enriched biological processes, molecular functions, and cellular components were detected using a hypergeometric test. Results were validated with

Benjamini-Hochberg's false discovery rate correction ($p < 0.05$) (Kolberg *et al.*, 2023). The functional networks were constructed on Cytoscape version 3.10.3 (Shannon *et al.*, 2003).

3.2.5. Ecotoxicity assessment

3.2.5.1. *Amphibalanus amphitrite* bioassay

The barnacle *A. amphitrite* nauplii lethality test was performed as described in S. Pereira *et al.* (2024). Briefly, active nauplii (<3 h post-release) were exposed in triplicate to 0.125–8 $\mu\text{g mL}^{-1}$ (0.5–32 μM) test solutions (3 mL; ~30–60 larvae/glass tube) for 24 h at 28 °C in darkness, without feeding. Mortality was recorded as immotile nauplii. Negative controls (0.1% DMSO) were included, and assays were repeated twice with pooled data for analysis.

3.2.5.2. *Artemia salina* bioassay

The brine shrimp (*A. salina*) nauplii lethality test was used to assess the general marine ecotoxicity of the compounds (Almeida *et al.*, 2017). Nauplii hatched in nutrient-enriched seawater (24 h, 26 °C) were exposed to compounds (25 and 50 μM) in 96-well plates (15–20 nauplii/well; 8 replicates) at 25 °C in darkness for 48 h. Negative (1% DMSO) and positive control (13.6 μM $\text{K}_2\text{Cr}_2\text{O}_7$) were included. Mortality percentage was calculated after 48 h.

3.2.5.3. *In silico* predictions of environmental fate parameters

A quick screening for the physicochemical properties and environmental fate of the compounds was performed using several models in the Estimation Programs Interface (EPI) Suite™, developed by the US Environmental Protection Agency's Office of Pollution Prevention and Toxics and Syracuse Research Corporation (SRC) (US EPA, 2024). The compounds' quantitative structure–activity relationship was used to estimate key criteria in ecotoxicology, such as bioconcentration (BCFBAF™ v3.01), bioaccumulation (KOWWIN™ v1.68), biodegradability (BIOWIN™ v4.10), and soil adsorption (KOCWIN™ v2.00). The compounds' names, molecular weights, chemical formulas, and SMILES notations were used as input values.

3.2.6. Data analysis

All data were first checked for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test), and appropriate transformations were made when necessary. Data from the compounds screening activities across all bioassays were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's test ($p < 0.01$) or Welch's ANOVA, followed by Dunnett's T3 test ($p < 0.01$) when necessary. For the diatom assay, Tukey HSD ($p < 0.01$) was used to assess differences between compounds'

concentrations. The half-maximal effective concentrations (EC_{50}) were determined for the settlement inhibition of *M. galloprovincialis* plantigrades. Probit regression analysis was employed to calculate EC_{50} values. The Pearson Goodness-of-Fit Chi-Square test ($p < 0.01$) was applied to the Probit regression analysis, with 95% lower and upper confidence limits (95% LCL; UCL). ANOVA and Probit analyses were conducted using IBM SPSS Statistics 30. The therapeutic ratio (LC_{50}/EC_{50}) was used to evaluate the compounds' effectiveness *versus* toxicity for the mussel plantigrades (Almeida *et al.*, 2017; Dan Rittschof *et al.*, 2003). For proteomic data, differentially expressed proteins (DEPs) were assessed using the "DEP" R package (Xiaofei Zhang *et al.*, 2018), with R: a language and environment for statistical computing version 4.4.1 (Team, 2021). Data with missing values were filtered, and only proteins identified in all replicates of at least one condition were considered. Subsequently, any remaining missing values were imputed. Proteins were classified as DEPs between compounds and control conditions by applying a $\log_2$ fold change ($lfc = \log_2(1.5)$) as a threshold.

3.3. Results and Discussion

3.3.1. Bio-guided isolation and structure elucidation of adenosine (1) and 2'-deoxyadenosine (2)

The cyanobacterium *Leptothoe* sp. LEGE 181152 was selected from an AF pre-screening study involving 59 different cyanobacterial strains (data not shown). Given its promising AF potential (Figure 11.a), this activity was protected through a patent (Reis *et al.*, 2022) and the strain was subsequently cultured on a large scale for the isolation and characterization of the AF active compounds. The normal-phase VLC performed on the organic extract yielded ten fractions of increasing polarity, which were tested at $10 \mu\text{g mL}^{-1}$ in the settlement inhibition bioassay. Of these, six fractions significantly inhibited mussel plantigrades settlement compared to the negative control (Dunnett's, $p < 0.01$), with fractions F+G1 and G2 achieving 100% inhibition (Figure 11). Fraction G2 was selected and subjected to a series of chromatographic procedures combined with bioassay testing (Figure 11.b-d). Subsequently, compounds were obtained from fraction G2_10-12_D (Figure 11.d), appearing in the LC-MS/MS analysis as well-defined peaks with reproducible fragmentation patterns.

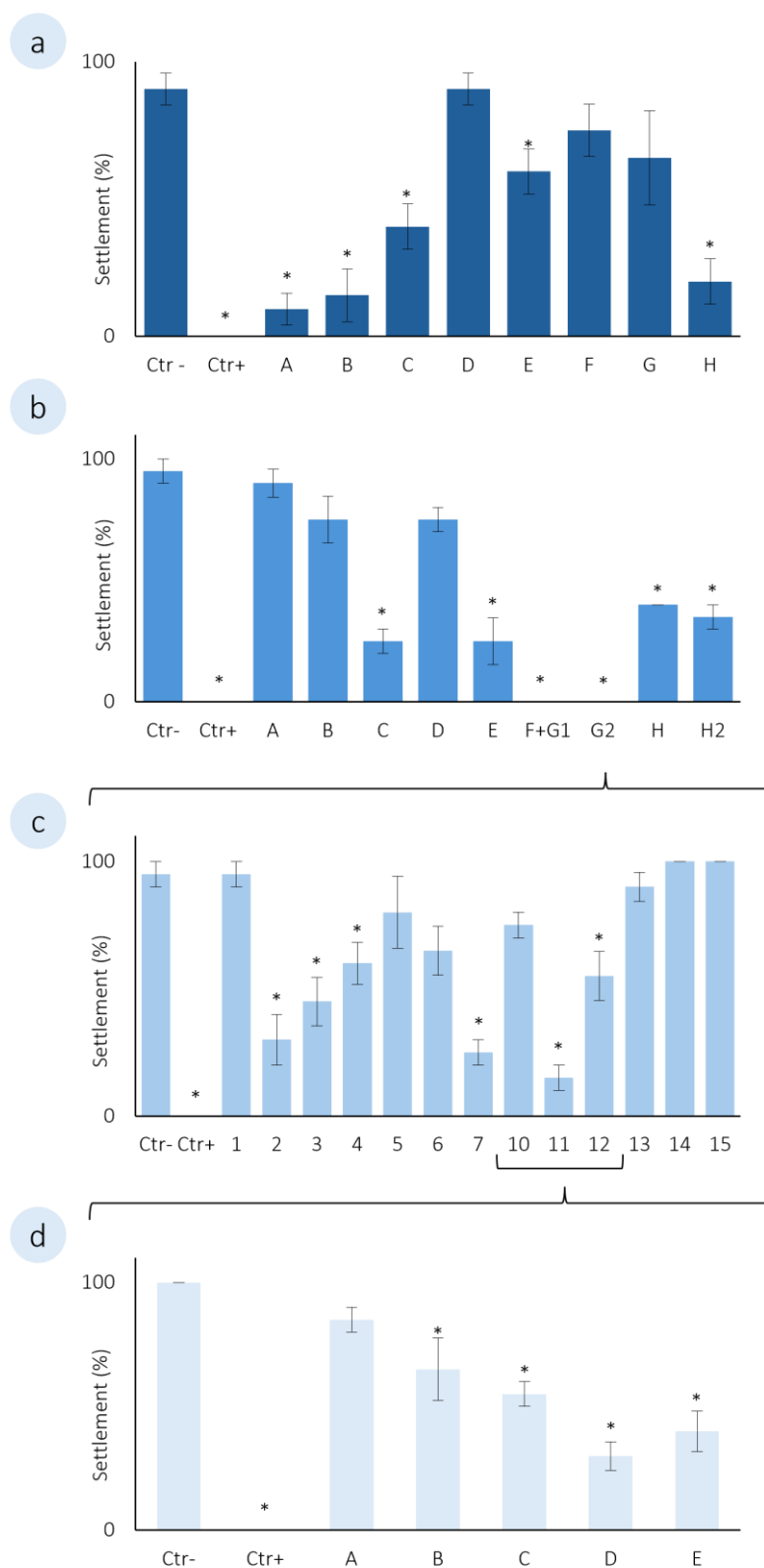


Figure 11. Bio-guided isolation of the compounds **1** and **2**. Results from the settlement inhibition assays against the *M. galloprovincialis* planigrades. **(a)** Pre-screening fractions; **(b)** VLC fractions; **(c)** Subfractions from the flash chromatography of VLC fraction G; **(d)** Fractions from the flash chromatography of subfraction G₁₀₋₁₂. All fractions were screened at 10 µg mL⁻¹. Ctrl-: DMSO (0.1 %); Ctrl+: CuSO₄ (5 µM). *Significant differences compared to the negative control (Dunnett's test, p < 0.01).

Compound **1** was identified as adenosine (Figure 12) by comparing its spectroscopic and spectrometric data with the literature (R. M. Huang *et al.*, 2014; Searle & Molinski, 1994). Specifically, the 1D (^1H and ^{13}C) and 2D (COSY, HMBC, and HSQC) NMR experiments revealed that compound **1** was a ribonucleoside, with the purine base identified as adenine (R. M. Huang *et al.*, 2011) and the glycosidic residue as β -D-ribose (Biabani *et al.*, 2002). The ESI-MS spectrum of compound **1** displayed a protonated molecule at m/z 268 $[\text{M}+\text{H}]^+$, consistent with the molecular formula $\text{C}_{10}\text{H}_{12}\text{N}_4\text{O}_5$ of adenosine. Furthermore, MS^2 fragmentation spectrum showed a dominant diagnostic fragment at m/z 136, corresponding to the adenine moiety (Price *et al.*, 2014). Compounds **1** and **2** exhibited similar proton spectra. Analysis of the ESI-MS spectrum revealed a $[\text{M}+\text{H}]^+$ ion at m/z 252 for compound **2**. The difference of 16 mass units between compounds **1** and **2** suggested that compound **2** lacks an oxygen atom. This difference was attributed to the glycosidic residue, as the MS^2 fragmentation spectrum also displayed the diagnostic fragment for adenine at m/z 136. This observation was further corroborated by the ^1H NMR spectrum, which confirmed that the structural variation between the two compounds lies in the sugar moiety. Namely, the multiplicity of the anomeric proton changed from a doublet in compound **1** (δ_{H} 6.00 d; $J = 6.4$ Hz) to a doublet of doublets in **2** (δ_{H} 6.43 dd; $J = 8.0, 6.0$ Hz). Additionally, the proton signals at position C-2' appeared as two diastereotopic protons in compound **2**: δ_{H} 2.81 (ddd, $J = 13.6, 8.0, 5.8$ Hz, H-2a') and δ_{H} 2.42 (ddd, $J = 13.6, 6.0, 2.7$ Hz, H-2b'), confirming the loss of the hydroxyl group at this position (in compound **1**: δ_{H} 4.75 m, H-2'). Thus, the glycosidic part was identified as a β -D-2-deoxyribose (R. M. Huang *et al.*, 2011) and the compound **2** was identified as 2'-deoxyadenosine (Figure 12).

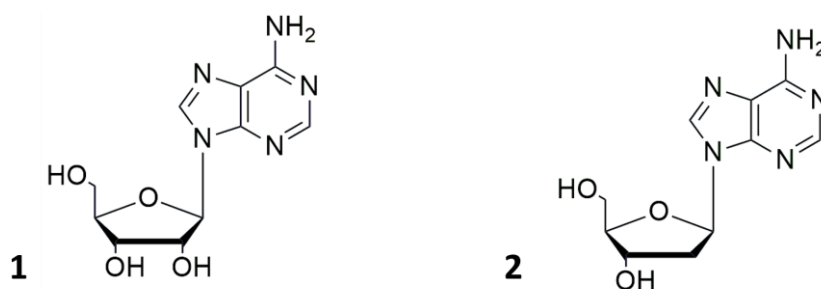


Figure 12. Chemical structures of the isolated compounds adenosine (**1**); 2'-deoxyadenosine (**2**).

3.3.2. Antifouling activity against micro and macrofouling organisms

This study is the first to report the isolation of adenosine (**1**) and 2'-deoxyadenosine (**2**) from the cyanobacterium *Leptothoe* sp. LEGE 181152, guided by their novel AF activity. Following purification and structure elucidation, their efficacy was characterized through

settlement inhibition assays against *M. galloprovincialis* plantigrades. Mussel plantigrades were exposed to the compounds for 15 hours, resulting in significant settlement inhibition (Dunnett's, $p < 0.01$) for **1** ($55\% \pm 5\%$) and **2** ($50\text{--}70\% \pm 5.8\%$) at initial screening concentrations of 25 and 50 μM . Hence, a dose-response bioassay was conducted, revealing an EC_{50} of $1.77 \mu\text{g mL}^{-1}$ ($6.63 \mu\text{M}$) for **1** and $2.20 \mu\text{g mL}^{-1}$ ($8.74 \mu\text{M}$) for **2**, both below the U.S. Navy's reference threshold for effective AF compounds ($\text{EC}_{50} < 25 \mu\text{g mL}^{-1}$) (Table 9).

Kipukasins, nucleosides isolated from marine invertebrates and their symbiotic microorganisms (C. Y. Wang *et al.*, 2016), and also from the basidiome of *Gandoderma australe* (Jiao *et al.*, 2007), were previously tested for AF efficacy. Specifically, kipukasin D, E and H along with synthesized derivative diacetylkipukasin D showed low effectiveness against the settlement of *Amphibalanus amphitrite* cyprids, with EC_{50} values higher than 49.40 to 61.30 μM , while diacetylkipukasin E showed also low AF activity ($\text{EC}_{50} = 44.50 \mu\text{M}$) (C. Y. Wang *et al.*, 2016). Kipukasins K and L inhibited larval settlement of the bryozoan *Bugula neritina* at $25.00 \mu\text{g mL}^{-1}$ (Wu *et al.*, 2020). Compared to these, the compounds **1** and **2** exhibited considerably stronger AF activity on mussel plantigrades. The AF effectiveness of the compounds **1** and **2** against *M. galloprovincialis* was as potent as the natural compounds portoamides ($\text{EC}_{50} = 4.86 \mu\text{g mL}^{-1}$) (Antunes *et al.*, 2019) and napyradiomycins ($\text{EC}_{50} = 0.1\text{--}6.34 \mu\text{g mL}^{-1}$) isolated from the marine actinomycetes *Streptomyces aculeolatus* (F. Pereira *et al.*, 2020). The potency of compound **1** is comparable to that of nocuolin A (EC_{50} of $1.17 \mu\text{g mL}^{-1}$) (S. Pereira *et al.*, 2024), the marinone derivative madeirone ($\text{EC}_{50} = 1.76 \mu\text{g mL}^{-1}$) (Wissner *et al.*, 2024) and the commercial AF agent ECONEA® ($\text{EC}_{50} = 1.40 \mu\text{g mL}^{-1}$) (Almeida *et al.*, 2017). Interestingly, He *et al.* (He *et al.*, 2021) demonstrated that the adenosine nucleoside induces settlement and metamorphosis in *Mytilopsis sallei* pediveligers. Therefore, assessing the AF activity of these compounds during early developmental stages would provide a more comprehensive AF profile and enable the evaluation of their effects across life stages with varying susceptibilities. For instance, several cyanobacterial compounds, such as lyngbyabellins O and P ($\text{EC}_{50} = 0.24$ and $0.62 \mu\text{g mL}^{-1}$, respectively) (Petitbois *et al.*, 2017) and majusculamide A and dolastatin 16 ($\text{EC}_{50} = 0.54$ and $0.003 \mu\text{g mL}^{-1}$, respectively) (L. T. Tan *et al.*, 2010), have shown greater potency against *A. amphitrite* cyprids than the isolated compounds demonstrated with mussel plantigrades. This increased sensitivity may be attributed to the early developmental stage, which could make cyprids more vulnerable to external stressors.

Table 9. Antifouling effectiveness versus toxicity of **1** and **2** against biofouling species. The half maximum effective concentration (EC₅₀), the median lethal concentration (LC₅₀), and the therapeutic ratio (LC₅₀/EC₅₀) are presented. The EC₅₀ and LC₅₀ are provided in both μM and $\mu\text{g mL}^{-1}$ for comparative purposes. Confidence intervals (CI) for EC₅₀ values are in μM (in parentheses). **1**: adenosine; **2**: 2'-deoxyadenosine. n.a. – not assessed.

Compound	Target species	EC ₅₀	LC ₅₀	LC ₅₀ / EC ₅₀
		μM (CI: μM); $\mu\text{g mL}^{-1}$	μM ; $\mu\text{g mL}^{-1}$	
1	<i>M. galloprovincialis</i>	6.63 (95% CI: 3.97-9.59); 1.77	> 200.00; > 53.45	> 30.17
	<i>Navicula</i> sp.	n.a.		n.a.
2	<i>M. galloprovincialis</i>	8.74 (95% CI: 5.43–12.52); 2.20	> 200.00; > 50.25	> 22.89
	<i>Navicula</i> sp.	n.a.		n.a.

Ideally, an AF agent should be effective against multiple species involved in the biofouling colonization cascade. Natural marine biofilms, formed by microfouling organisms such as bacteria and diatoms, can influence the adhesion of marine invertebrate larvae and macroalgal spores on submerged surfaces, thereby modulating the biofouling colonization process (Qian *et al.*, 2022; Vinagre *et al.*, 2020). However, developing a broad-spectrum, non-toxic AF agent remains a significant challenge due to the difficulty of designing compounds that effectively target diverse fouling species across multiple biological pathways (Cahill *et al.*, 2024). Therefore, the AF activity of **1** and **2** was evaluated against a panel of microfouling species, including five strains of marine biofilm-forming bacteria and a strain of a marine biofilm-forming diatom. A threshold of $\geq 30\%$ of growth inhibition was established to consider a compound active against these microorganisms. Following exposure to the compounds **1** and **2** at 25 and 50 μM , no significant growth inhibition nor lethality was observed in any of bacterial strains (Dunnett's, $p > 0.01$) (Figure 13.b). For the microalgae assay, the marine biofilm-forming diatom *Navicula* sp. was selected. In the initial screening, both compounds resulted in $\geq 30\%$ growth inhibition at 25 and 50 μM (Dunnett's, $p < 0.01$). To further evaluate potential dose-response effects, a concentration range from 200 μM to 0.8 μM was tested. Most concentrations significantly inhibited diatom growth compared to the negative control (Dunnett's, $p < 0.01$), with **2** exhibiting stronger inhibition than **1** ($\approx 60\%$ vs. 40%). This difference in activity may derive from structural differences between the nucleosides: adenosine has a hydroxyl group (-OH) at the 2' position of the ribose, whereas 2'-deoxyadenosine lacks this group. This may increase compound **2** lipophilicity and likely enhancing cell permeability in diatoms, contributing to its increased effectiveness. However, a dose-response model could not be established, suggesting

that the inhibitory effect of the compounds may follow a threshold-like pattern (Figure 13.c). Possible explanations include (a) saturation of biological targets (e.g. enzymes, receptors, or binding sites) at low concentrations; (b) limited availability of receptors, substrates, or cofactors; or (c) saturation of compounds uptake (Y. Tan *et al.*, 2022). These remain hypothesis, and further studies are required to clarify the mechanisms underlying this nonlinear inhibition. In contrast, cyclic peptides known as portoamides, isolated from the cyanobacterial strain *Phormidium* sp. LEGE 05292, were previously reported to inhibit the growth of three strains of marine bacteria (*C. marina*, *H. aquamarina*, and *P. atlantica*) with EC_{30} values ranging from 13 to 15 μM , but had no inhibitory effect on *Navicula* sp. (Antunes *et al.*, 2019). Other compounds, such as the resorcylic acid lactone cochliomysin G ($EC_{50} = 0.61 \mu\text{g mL}^{-1}$ for *Navicula exigua*) and its analogue zeaenol ($EC_{50} = 3.20$ and $8.71 \mu\text{g mL}^{-1}$ for *Navicula levissima* and *Navicula exigua*, respectively) (W. Xu *et al.*, 2019), exhibited stronger AF activity against diatoms than the nucleosides. Moreover, the oxadiazine nocuolin A, isolated from the cyanobacterial strain *Nodularia* sp. LEGE 06071, showed even higher potency against *Navicula* sp. ($EC_{50} = 0.47 \mu\text{g mL}^{-1}$) (S. Pereira *et al.*, 2024). Despite differences in potency, certain compounds, like nocuolin A, demonstrated activity against *Navicula* sp., but lacked effectiveness against marine biofilm-forming bacteria. This specificity suggests a possible mode of action targeting eukaryotic processes which may explain the differential response observed between bacterial and algal targets in this study. The apparent selectivity towards eukaryotic organisms suggests a more targeted AF profile, a feature typically associated with safer and more environmentally compatible AF agents, especially compared to the broad-spectrum biocides known for their high marine toxicity (Qiu *et al.*, 2024)..

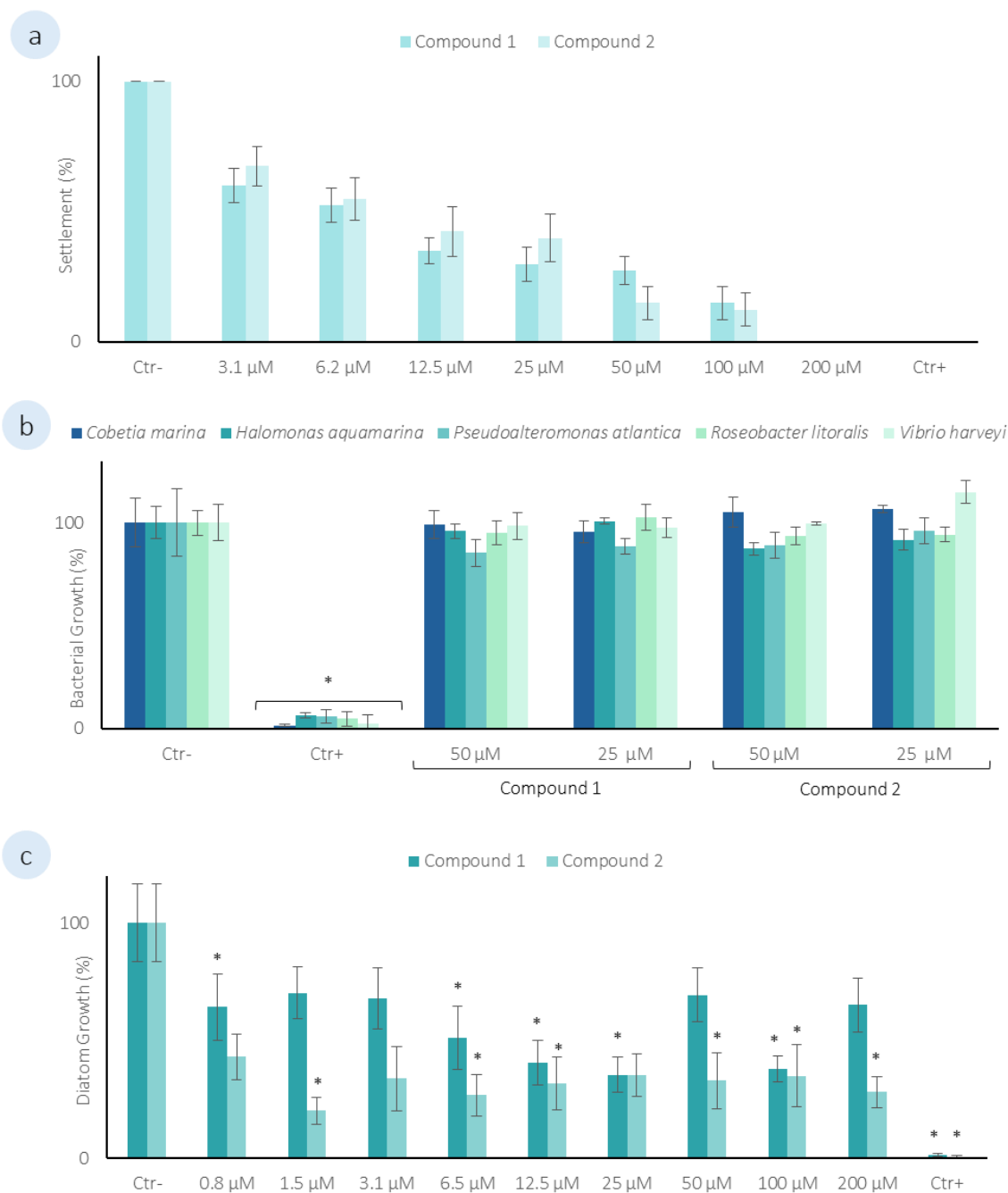


Figure 13. Antifouling activity of **1** and **2**. (a) Dose-response against *M. galloprovincialis* plantigrades. Ctr-: DMSO (0.1%); Ctr+: CuSO₄ (5 μ M); (b) Growth inhibition of five marine biofilm-forming bacteria screening bioassay. Compounds were screened at 25 and 50 μ M. Ctr-: DMSO (1%); Ctr+: 1:100 penicillin-streptomycin-neomycin stabilized solution; (c) Dose-response against the marine biofilm-forming diatom *Navicula* sp. Ctr-: DMSO (1%); Ctr+: Cycloheximide (3.55 μ M). *Significant differences compared to the negative control (Dunnett's, $p < 0.01$).

3.3.3. Molecular targets of the AF activity on *M. galloprovincialis* plantigrades

3.3.3.1. AChE and Tyrosinase activity

Understanding the mechanisms behind the AF action of compounds is essential for the development of new AF agents (L. Chen & Qian, 2017). Furthermore, the environmental

regulation BPR (EU 524/2012) mandates a precise characterization of the mode of action for newly developed AF agents. However, identifying a single molecular target or pathway remains challenging due to the complex biological processes involved in biofouling (Cahill *et al.*, 2024). This study investigated the potential AF protein targets of compounds **1** and **2** in *M. galloprovincialis* planigrades by exploring various pathways. Enzymes such as AChE are known to be affected by AF compounds in fouling organisms, as AChE plays a key role in cholinergic neural signalling during larval settlement (L. Chen & Qian, 2017). Here, we examined the effects of the compounds on this pathway using both docking studies and *in vitro* enzymatic assays. The docking studies, leveraging recent advances in computer-aided drug design (CADD) tools (Arabshahi *et al.*, 2021; Gaudêncio & Pereira, 2022), were conducted to explore the molecular interactions between the compounds and AChE using AutoDock Vina. Previous research has demonstrated the effectiveness of docking approaches in predicting the binding of hydroxyxanthenes to *Electrophorus electricus* AChE (Almeida *et al.*, 2020).

The crystallographic structure of *Electrophorus electricus* AChE is characterized by a long, slender and hydrophobic active-site gorge (Bourne *et al.*, 1999). The catalytic triad (Ser-203, Glu-334, and His-447) of this serine hydrolase is located near the base of the gorge, where acetylcholine (ACh) is converted into choline and acetate (Y. Zhou *et al.*, 2010). The oxyanion hole, formed by Gly-121, Gly-122, and Ala-204 determines the reactive intermediates and transition states that are generated during the process (Silman & Sussman, 2008). The side chains of Trp-86, Glu-202, and Tyr-337 are combined to create the anionic subsite, which is primarily in charge of binding the quaternary trimethylammonium tail group of ACh (Kua *et al.*, 2003). Tyr-72, Asp-74, Tyr-124, Glu-285, Trp-286, and Tyr-341 are among the aromatic residues that make up the peripheral anionic site (PAS) near the gorge's entrance. This PAS forms a hydrophobic region that binds ACh and transfers it to the deep catalytic site (Johnson & Moore, 2006). Studies on AChE from a variety of species indicate that some AChE inhibitors can bind at the catalytic site and act as competitive inhibitors (Colovic *et al.*, 2013). At the active site gorge, AChE inhibitors can also bind to PAS, thus hindering the catalysis by sterically blocking ligands from entering and leaving the active site portal and by allosterically altering the catalytic triad's conformation (Bourne *et al.*, 2003). In the present *in silico* docking study, pulmonarin A and B, two AF natural products isolated from the ascidian *Synoicum*, were used as positive controls. These compounds have been shown to act as competitive AChE inhibitors, without displaying antibacterial or cytotoxic effects (Tadesse *et al.*, 2014; Trepos *et al.*, 2014). The top-ranked poses of pulmonarin A and

B have docking scores of -5.9 and -6.8 kcal.mol $^{-1}$, respectively. Compared to positive controls, **1** and **2** were predicted to have a higher affinity towards the AChE active site (docking score of -7.6 and -7.8 kcal.mol $^{-1}$, respectively) (Figure 14).

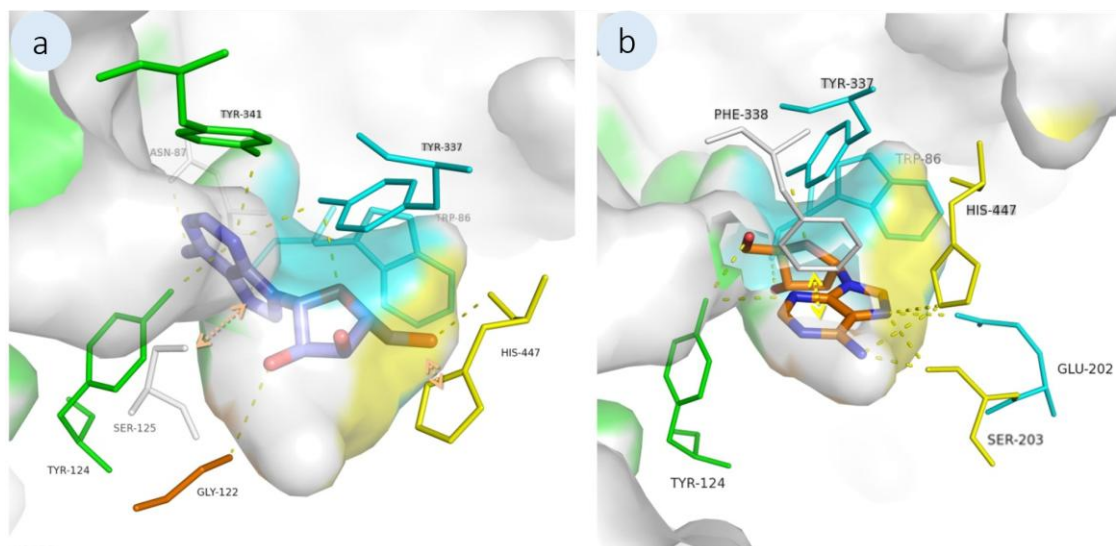


Figure 14. AChE docking. Detailed view of (a) compound **1** (dark blue sticks), and (b) compound **2** (orange sticks) docked in AChE binding site (PDB code: 1C2O). Hydrogen interactions are represented with yellow broken lines. π -Stacking and π -hydrogen donor interactions are represented with a yellow and orange double arrow, respectively. Residues involved in those interactions are represented as lines and labelled. AChE is represented as transparent surface, where the catalytic triad, PAS, anionic sites and oxyanion hole are represented in yellow, green, blue and orange, respectively.

As previously described by our group, the interactions between known inhibitors pulmonarin A and B and the enzyme are characterized by several hydrogen bonds with residues from PAS, oxyanion hole, anionic site and catalytic triad (Almeida *et al.*, 2020). Compound **2** docks inside the groove, with the purinone ring fitting snugly into the catalytic groove and the oxolanyl ring blocking the entrance of the gorge (PAS); oppositely, it is the oxolanyl ring of **1** that binds deeply in the catalytic groove (Figure 14). Compound **1** establishes hydrogen interaction with residues from the catalytic triad (His-447), PAS (Tyr-124 and Tyr-341), anionic sites (Trp-86 and Tyr-337), oxyanionic hole (Gly-122), and with other residues such as Asn-87; it also forms two π -hydrogen donor interactions with Ser-125 and His-447 (Figure 14.a). Compound **2** establishes hydrogen interactions with residues from the catalytic triad (Ser-203 and His-447), PAS (Tyr-124), and anionic sites (Trp-86, Glu-202 and Tyr-337); a T-shaped π -stacking interaction is also formed with Phe-338 (Figure 14.b). Overall, the compounds share most binding patterns, with specific interactions likely driven by the presence or absence of the hydroxyl group in their structures. These binding patterns align with established structural and computational findings on AChE-ligand interactions. Crystallographic studies have

shown the formation of hydrogen bonds between inhibitors and Ser-203, a key catalytic residue (Ortiz *et al.*, 2016), while computational studies indicate that Ser-203 and His-447 are consistently located in proximity to bound inhibitors, contributing to complex stability (Moraga-Nicolás *et al.*, 2018). The involvement of Tyr-337 and Tyr-341 in reversible inhibitor binding has been previously reported (Bourne *et al.*, 2004; Saxena *et al.*, 1994), and Trp-86 has been reported as relevant in stabilizing irreversible inhibitors within the AChE binding pocket (Ranjan *et al.*, 2018). Gly-122 is thought to be involved in the formation of ACh reactive intermediates and transition states that are formed during the reaction (Y. Zhang *et al.*, 2002) and it is predicted as being in contact with most of AChE ligands (Ordentlich *et al.*, 1998). It has previously been reported that Tyr-124, which integrates PAS, plays a crucial role in the binding of *Electrophorus electricus* AChE inhibitors (Bourne *et al.*, 2003; Greenblatt *et al.*, 1999; Loh *et al.*, 2021). Other residues such as Ser-125, Glu-202 and Phe-338 have also been described as important in the interaction of inhibitors with AChE (Gul *et al.*, 2023; Karunakaran *et al.*, 2022; Merzoug *et al.*, 2021; Z. Zhang *et al.*, 2023). Taken together, these findings suggest that compounds **1** and **2** may establish multiple stabilizing interactions within the AChE binding site, which may account for their observed bioactivities.

To further investigate the affinity of the nucleosides to AChE, enzymatic assays were conducted. Initial screenings at 25 and 50 μM were performed in quadruplicate for each nucleoside. Both compounds showed an increase in AChE activity compared to the control (Dunnett's, $p < 0.01$). Therefore, a broader range of concentrations (3.1 – 200 μM) was also tested. The post-hoc Dunnett's test ($p < 0.01$) showed a significant increase in the AChE activity when exposed to lower concentrations of **1** and **2**, with an augment of approximately 30%. However, at 200 μM , a decrease in activity by approximately 20% was observed for both nucleosides (Figure 15.a). According to these results, both compounds seem to have an hormesis effect on AChE (Agathokleous *et al.*, 2024), improving enzyme efficiency by stabilizing an ideal catalytic conformation at low concentrations ($\leq 25 \mu\text{M}$), or competing with acetylcholine for active site interactions, leading to a reduced activity or partial inhibition of AChE, at higher concentrations ($\geq 200 \mu\text{M}$). This biphasic response is not uncommon in natural compounds (Barreiro-sisto *et al.*, 2024) For instance, exposure to concentrations below 5 mg mL^{-1} of lithium can enhance chlorophyll content, mitochondrial activity, and antioxidant capacity on the freshwater microalgae *Chromochloris zofingiensis* (Yaqi Xu *et al.*, 2024). In fact, Noguera *et al.* (2025) observed a possible hormesis effect on the AChE activity of *M. galloprovincialis* exposed to lithium. Furthermore, these *in vitro* results are consistent with the high binding affinity of the compounds for AChE, as demonstrated by docking

studies. The stabilization of the catalytic triad and anionic site, along with π -hydrogen interactions from compound **1** and π -stacking from compound **2**, likely contributes to maintaining the active site conformation and enhancing catalytic efficiency at low concentrations. An increase in AChE activity may enhance the hydrolyzation of acetylcholine and, thereby decreasing its availability and potentially impairing cholinergic signalling. As mentioned before, the cholinergic signalling plays a critical role in regulating byssal thread production. Such disruption could lead to weakened byssal threads and reduced attachment efficiency, which could explain the AF activity of the compounds on mussel plantigrades, since their EC_{50} values are lower than 10 μ M. Nonetheless, information on the effects of increased AChE activity in mussels remains scarce, limiting the interpretation of these preliminary results, as most available studies focus on AChE inhibitors (D. Pereira, Palmeira, *et al.*, 2024; S. Pereira *et al.*, 2024). Therefore, future research should include *in vivo* assays on exposed mussel plantigrades to evaluate AChE activity and validate the significance of the AF compounds' effects on this enzyme.

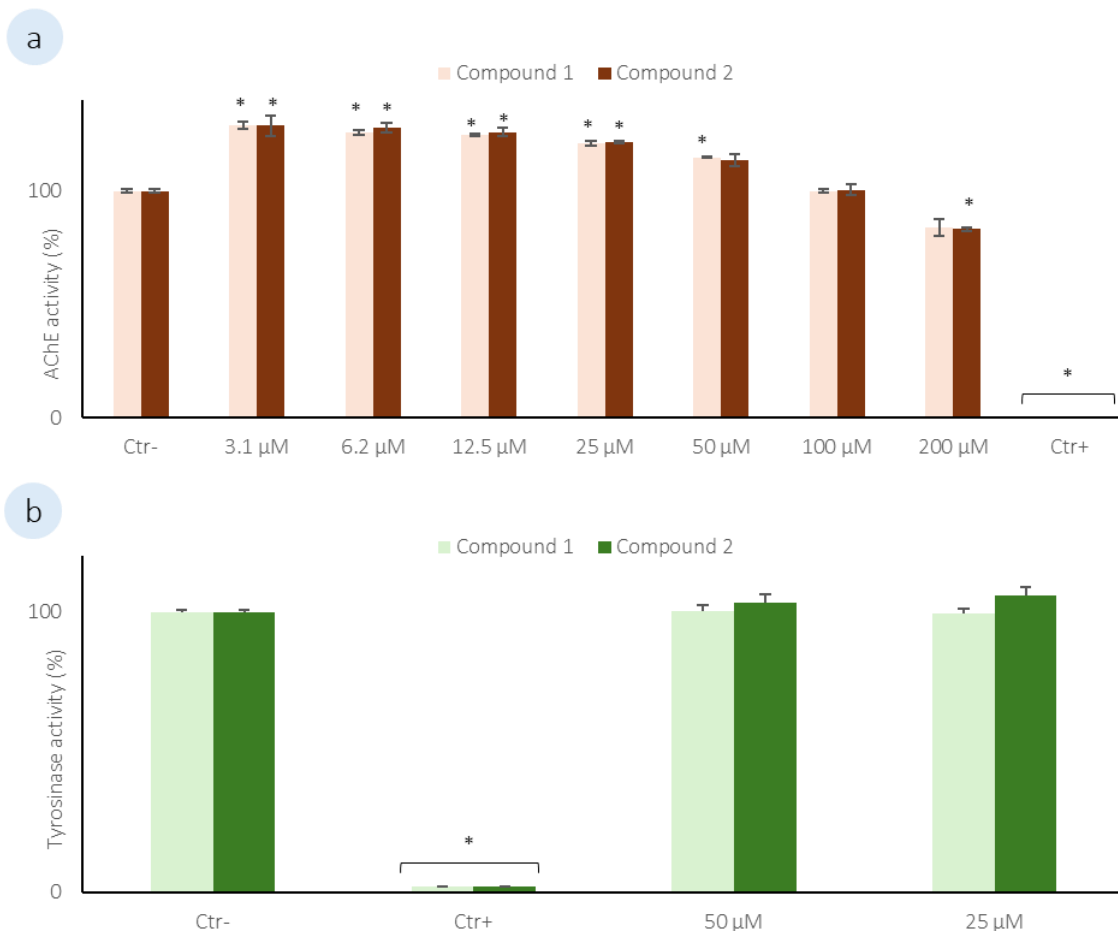


Figure 15. *In vitro* enzymatic activity of **1** and **2**. (a) Dose-response for the AChE activity. Ctr-: DMSO (0.2%); Ctr+: Eserine (200 μ M); (b) Screening for the tyrosinase activity at 25 and 50 μ M. Ctr-: DMSO (0.2%); Ctr+: Kojic acid (200 μ M). * Significant differences compared to the negative control (Dunnett's test, $p < 0.01$).

Another well-known AF target is tyrosinase, an enzyme involved in mussel adhesion (Xinhui Zhang *et al.*, 2019). Tyrosinase facilitates the oxidation of tyrosine residues to reactive DOPA and subsequently to o-quinone in mussel foot proteins within byssal plaques, which is essential for strong adhesion (Lianguo Chen & Qian, 2017; Martín-Rodríguez *et al.*, 2015). An initial screening was performed using concentrations of 25 and 50 μ M for each compound. However, no inhibition was observed for tyrosinase activity when exposed to the compounds at tested concentrations (Dunnett's, $p > 0.01$) (Figure 15.b). Our findings indicate that these compounds' AF activity is not mediated through tyrosinase, as no significant alterations were observed *in vitro*.

3.3.3.2. Untargeted proteomic analysis

To investigate additional molecular targets, we applied an untargeted shotgun proteomics approach on the whole proteome of *M. galloprovincialis* plantigrades. The mussel plantigrades were exposed to 12.5 μ M and 50 μ M for both compounds, alongside

a control group (0.1% DMSO). However, due to experimental limitations that affected the integrity of some treated samples, only the 12.5 μ M treatment for compound **1** and the 50 μ M treatment for compound **2** were included in the final proteomic analysis. Considering that this disparity in concentrations limits the direct comparison between compounds, the datasets were treated individually. For the differentially expressed proteins (DEP) analysis, missing values were filtered, and only proteins identified in all replicates of at least one condition were considered. A fold-change threshold ($FC > 1.5$) was applied to the samples for the proteins to be considered DEPs. A list of all the identified DEPs is described in the Supplementary Table S2. The proteomic data analysis of the exposed *M. galloprovincialis* plantigrades were represented by the principal component analysis (PCA), volcano plot and heatmap (Figure 16). The PCA plot highlights protein expression variations between the control and the exposed groups, as well as variability among replicates. The volcano plots, fold change ($\log_2$) against the adjust p-values ($-\log_{10}$), highlight the differential expression of proteins in the mussel plantigrades exposed to each compound relative to the control. In the heatmap, rows represent DEPs and their relative expression levels across treatments, while columns correspond to the experimental conditions. The colour gradient indicates variations in protein expression across different conditions.

The effect of **1** on the proteome of the mussel plantigrades was evaluated at 12.5 μ M, in comparison to the control group (0.1% DMSO). The LC-MS analysis identified a total of 1562 proteins grouped into 1267 clusters. Out of the 1267 proteins, 750 were present in all replicates of at least one condition and, therefore, were considered for the analysis. After applying a fold change ($FC > 1.5$) threshold to compare modifications in the proteins' expression between the control and the exposed group, no differently expressed proteins were found among them. This result may be due to variability among the replicates, which can hinder the detection of differences in the protein expression.

On the other hand, the effects of **2** were assessed at 50 μ M and compared to the control group (0.1% DMSO). A total of 791 proteins grouped into 480 clusters were identified through LC-MS analysis. Out of the 480 proteins, 223 were present in all replicates of at least one condition and, therefore, were considered for the analysis. Alterations in protein expression were evaluated considering fold change ($FC > 1.5$). The comparison between the exposed and the control group identified a total of 167 DEPs. The PCA in Figure 16.a revealed clear distinction between the tested groups, however some variability was observed among the replicates within the control. The volcano plot indicated that 34 proteins were downregulated, while 133 were upregulated when compared to the control

(Figure 16.b). Additionally, the heatmap (Figure 16.c) confirmed that the identified DEPs were mainly upregulated by the exposed group (50 μ M) of **2**. Among the DEPs identified for **2**, were those containing von Willebrand factor (vWF) and epidermal growth factor-like (EGF) domains.

Exposure to compound **2** at 50 μ M downregulated the expression of vWFA domain-containing proteins (A0A8B6C9L4). In vertebrates, vWF is a glycoprotein involved in haemostasis by promoting platelet adhesion to damaged vessels (Cruz *et al.*, 1995) and contains four domains (A, B, C, and D) (Sun *et al.*, 2002). In mussels, vWF is thought to play a role in underwater adhesion (J. H. Waite, 2017; Yoo *et al.*, 2016), and shell mineralization (Shimizu *et al.*, 2024). Two vWFA domains are present in the structure of proximal thread matrix protein-1 (PTMP1) (Sun *et al.*, 2002), a non-collagenous matrix protein crucial for the structural integrity of byssal threads (Silverman & Roberto, 2007). PTMP contributes to thread stiffening, likely through interactions between vWFA domains and collagen (Suhre *et al.*, 2014; Yoo *et al.*, 2016). Downregulation of these vWF domain-containing proteins in response to **2** may disrupt PTMP production, potentially compromising byssal thread integrity and inhibiting mussel settlement.

Compound **2** upregulated one form of EGF-like domain-containing proteins (A0A8B6CPN1). The EGF polypeptide regulates cell proliferation (Boonstra *et al.*, 1995), and EGF-like domains are found repetitively in mussel foot protein-2 (Mfp-2) across various species (Hwang *et al.*, 2010; K. Inoue, Takeuchi, *et al.*, 1995; S. Li *et al.*, 2018). Mfp-2 is a polyphenolic protein that strengthens byssal plaques, enhancing adhesion resistance to proteases and maintaining byssus stability (Silverman & Roberto, 2007). According to Hwang *et al.* (2010), the EGF-like domains in Mfp-2 may enhance cohesion between the byssal plaque and substratum. The increased expression of EGF-like domain-containing protein after exposure may indicate challenges in the production of Mfp-2, which could affect adhesion, and is potentially compensated by higher expression of this domain.

In summary, no DEPs were detected in samples exposed to compound **1** (12.5 μ M), whereas two domains present in adhesive proteins (EGF-like and vWFA) emerged as potential effectors of the AF response to compound **2** (50 μ M), likely by indirectly modulating adhesion. However, the disparity in compound concentrations prevents a direct comparison of molecular targets. Future work should therefore employ transcriptomic approaches, such as RNA sequencing and real-time PCR, to validate the involvement of EGF-like and vWFA domains at equivalent conditions for both compounds.

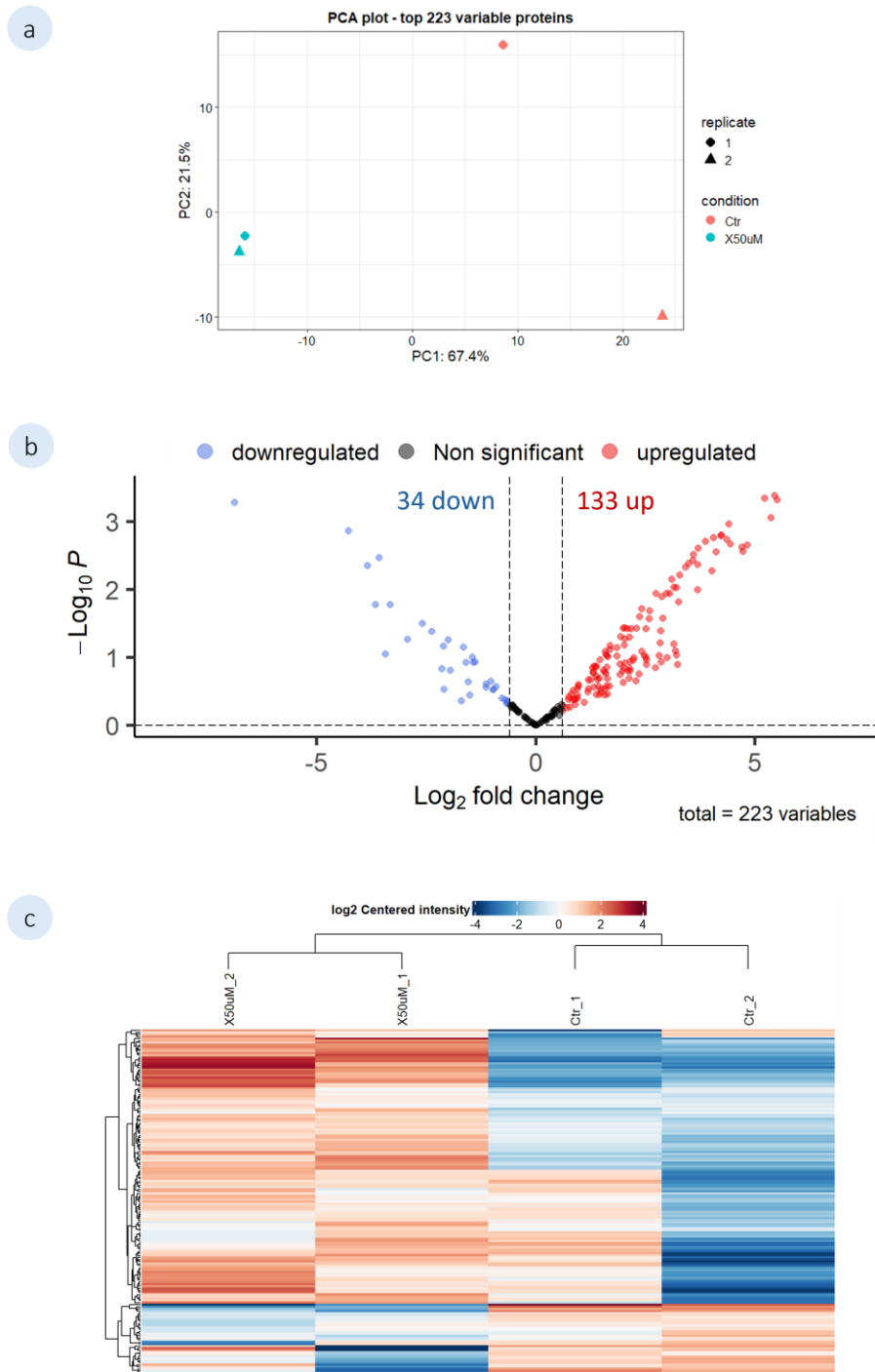


Figure 16. Proteomic analysis of *M. galloprovincialis* planigrades exposed to **2**. Analysis was performed by pairwise comparisons between the exposed group (50 μ M) and the control (0.1% DMSO). **(a)** Principal component analysis (PCA) of the 223 proteins included in the analysis; **(b)** Volcano plot of the DEPs with the p values ($-\log_{10}$) plotted against the fold changes ($\log_2$). Downregulated DEPs are represented by blue dots, and the upregulated by red dots; **(c)** The heatmap illustrates relative expression of DEPs across treatments (red: upregulated; blue: downregulated). The rows represent the DEPs. The columns represent the tested groups.

3.3.3.2.1. Functional enrichment analysis

A functional enrichment analysis was performed to further investigate the response of *M. galloprovincialis* plantigrades to compound 2. Specifically, the DEPs were examined for the presence of known mussel adhesive proteins, including proximal thread matrix proteins, precollagens, foot proteins, and adhesive plaque proteins. However, no proteins directly associated with mussel adhesion were identified.

To gain further insights, the analysis also explored basic cellular functions affected by the compound. This was achieved using homologous sequences from *M. gigas*, a phylogenetically closer species with a genome denoting a higher level of annotation. An assumption was made that protein functions are conserved between these two species, allowing for a more detailed functional characterization. As such, homologous sequences from *M. gigas* were classified using gene ontology (GO) terminology and grouped according to their functions. The results of the BLAST analysis for compound 2 is presented in the Supplementary Table S3. The functional classes were searched using the web tool g:Profiler, and statistically significant enriched biological processes and molecular functions were filtered using a hypergeometric test (Fisher's exact test, $p < 0.05$) with Benjamini-Hochberg's false discovery rate correction. Through this method, we were able to analyse the housekeeping molecular functions and biological processes of the proteins affected by the compounds. Figure 17 illustrates the functional enrichment of the mussel plantigrades exposed to 50 μM of **2**. This analysis identified 167 GO terms associated with the functions of DEPs (Supplementary Table S4).

The analysis showed that several proteins are ribosome constituents or related to ribosome functions (e.g. Small ribosomal subunit protein uS5 (A0A8W8HYQ8), 60S ribosomal protein L7a (A0A8W8L109), 40S ribosomal protein S14 (A0A8W8JKH8), Large ribosomal subunit protein P1 (A0A8W8N7H9), Ubiquitin-ribosomal protein eL40 fusion protein (K1PD36), and Small nuclear ribonucleoprotein Sm D2 (A0A8W8IE99). Proteins related to cytoskeleton organization (e.g. Actin-2 (A0A8W8LZ95), Histidine kinase/HSP90-like ATPase domain-containing protein (A0A8W8HTN3), Histone H2A (A0A8W8NPB5), Alpha-actinin, sarcomeric (K1RH58), Myosin heavy chain, non-muscle (Fragment) (K1QXX7), Paramyosin (K1QTC1), Spectrin alpha chain (K1R401), Alpha-actinin, sarcomeric (K1RH58), F-actin-capping protein subunit alpha (K1PLV6), Plastin-2 (A0A8W8NS22), Myosin heavy chain, striated muscle (A0A8W8KP76), Myosin-Ib (A0A8W8NAI3), Collagen alpha-1(IV) chain (K1RH30), and Histone H2A (A0A8W8NPB5)), and cilia movement (e.g. Radial spoke head protein 9 homolog (A0A8W8ICW5), Tektin (A0A8W8IGH4, A0A8W8JPK9), and Cilia- and flagella-

associated protein 52 (K1RDT2)) were also identified. Several mitochondrial proteins were identified. These are putatively involved in ATP metabolism (e.g. Citrate synthase (A0A8W8MWR9), Fructose-bisphosphate aldolase (A0A8W8MQD3), Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial (A0A8W8I7C9), Malate dehydrogenase, mitochondrial (A0A8W8K8K1), 2-oxoglutarate dehydrogenase, mitochondrial (A0A8W8HLX2), and ATP-dependent 6-phosphofructokinase (A0A8W8NW87)). The analysis also found proteins involved in ATP binding (e.g. 78 kDa glucose-regulated protein (K1QIR8), Histidine kinase/HSP90-like ATPase domain-containing protein (A0A8W8HTN3), Glucose-regulated protein 94 (A5LGG7), Stress-70 protein, mitochondrial (A0A8W8J1A5), and T-complex protein 1 subunit alpha (A0A8W8LAX1)), calcium binding (e.g. Annexin (A0A8W8MW14), Calmodulin (K1Q3D9), Calreticulin (A0A8W8KPL5), and EF-hand domain-containing protein (A0A8W8MAQ3)), and oxidoreductase activity (Protein disulphide-isomerase (K1Q6X5), and Thioredoxin domain-containing protein (A0A8W8MXV3)).

This analysis suggests a stress response to **2**, with altered expression of proteins involved in metabolism, cell structure, and cellular stress response. Notably, biological processes affected included cilium movement and ATP metabolism. In terms of cilium movement, compound **2** upregulated tektins and radial spoke head proteins. Radial spoke proteins play a role in regulating ciliary motility (Grossman-haham *et al.*, 2021), while tektins are structural components essential for the ciliary microtubule cytoskeleton. In mussels, cilia direct water flow for respiration and feeding (Cannuel *et al.*, 2009; DRAL, 1966), control the biointerface strength and stem release of the byssus (Sivasundarampillai *et al.*, 2023), and, on the foot of *Limnoperna fortunei*, cilia have been suggested to assist in temporary adhesion (Andrade *et al.*, 2015). Therefore, the upregulation of the expression of cilia-related proteins could imply a defensive response of the mussels to the compound, affecting the overall health, aligning with previous findings on AF compounds affecting ciliary motility in mussel plantigrades (Antunes *et al.*, 2019). Regarding the ATP metabolism, compound **2** upregulated ATP synthase, citrate synthase, 2-oxoglutarate dehydrogenase and malate dehydrogenase. Enzymes like citrate synthase, 2-oxoglutarate dehydrogenase and malate dehydrogenase are key to the citric acid cycle (Krebs cycle), producing electron carriers required for the mitochondrial electron transport chain (Osellame *et al.*, 2012). ATP synthase is part of the electron transport chain on the inner mitochondrial membrane, facilitating ATP synthesis through oxidative phosphorylation (Osellame *et al.*, 2012). Upregulation of these proteins by the compound may suggest a potential compensation for mitochondrial

dysfunction, increased oxidative stress, and metabolic dysregulation. These findings align with previous observations of bioenergetic modulation in response to other AF compounds (Antunes *et al.*, 2019; Dash *et al.*, 2012; Han *et al.*, 2013; S. Pereira *et al.*, 2024; Qian, Wong, *et al.*, 2010; Y. F. Zhang *et al.*, 2012). Overall, mussel plantigrades exhibited a global stress response to **2**. Specifically, the compound influenced proteins critical for metabolism, cell structure maintenance, and stress response, potentially impairing physiological functions essential for mussel adhesion and survival.

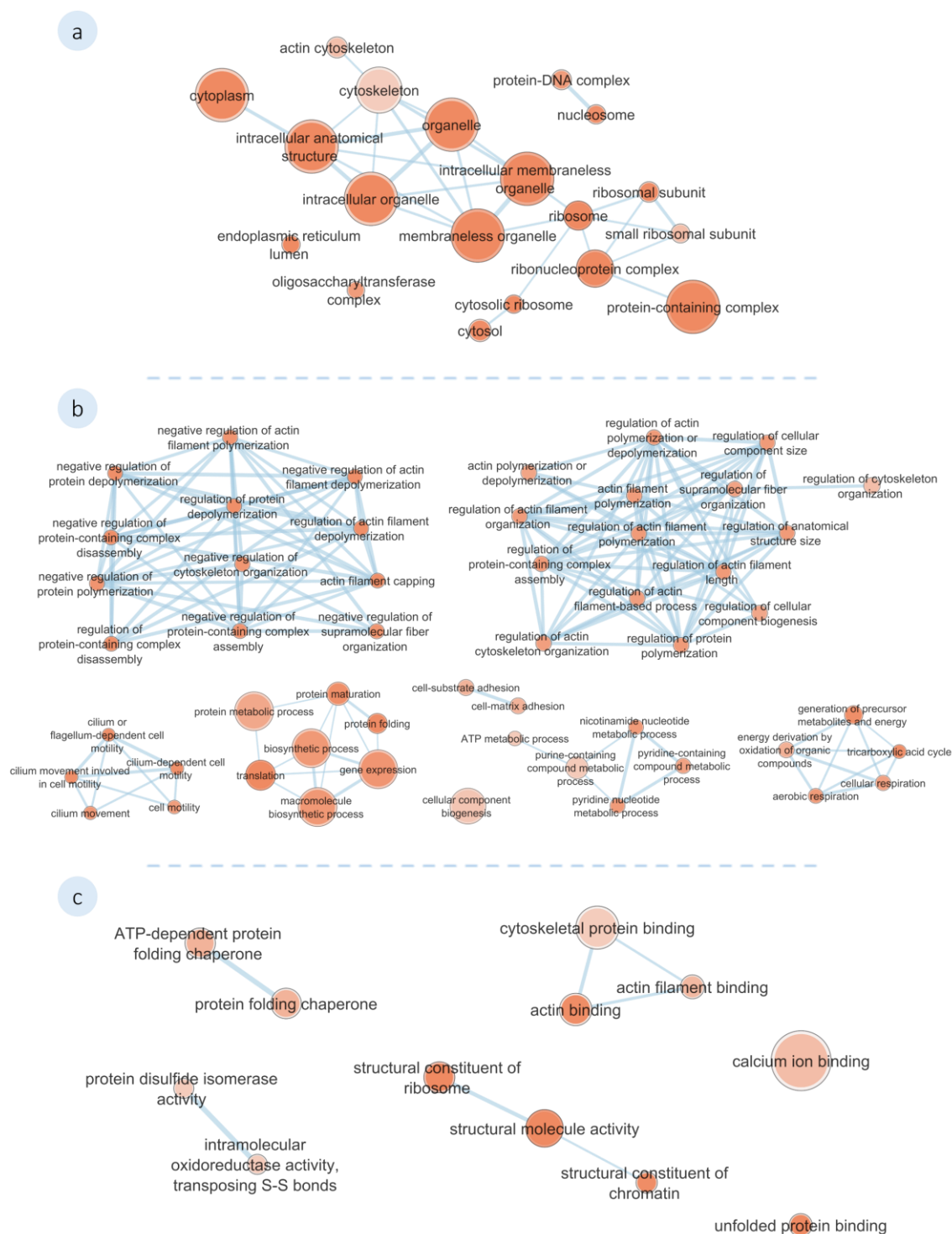


Figure 17. Functional network of differentially expressed proteins in the *M. galloprovincialis* plantigrades exposed to 50 μ M of **2**. Represented gene ontology (GO) categories are (a) Cellular Components, (b) Biological Processes, and (c) Molecular Functions. Node sizes increase with the number of proteins associated with the GO term and colour intensifies with significance (Node cut-off = 0.03). Blue edges connections between the nodes.

3.3.4. Environmental toxicity

3.3.4.1. *In vivo* effects

In addition to effectively preventing the attachment and growth of fouling organisms, through a more specific, rather than broadly toxic, mode of action, new AF agents must also be environmentally compatible. To address this, we evaluated compound's potential toxicity in both target (Table 9). and non-target marine organisms. For target species, a therapeutic ratio (LC_{50}/EC_{50}) higher than 15 is considered indicative of efficacy relative to toxicity, although these thresholds are not absolute (Dan Rittschof *et al.*, 2003). For the tested target species - marine biofilm-forming bacteria, microalgae and mussel plantigrades - no lethal effects were observed for either compound **1** or **2** at any tested concentrations. Notably, both compounds were active against *Navicula* sp., without causing cellular disruption ($LC_{50} > 53.45 \mu\text{g mL}^{-1}$; $> 200 \mu\text{M}$ for **1**; $LC_{50} > 50.25 \mu\text{g mL}^{-1}$; $> 200 \mu\text{M}$ for **2**). Comparable LC_{50} values were observed for mussel plantigrades, yielding therapeutic ratios higher than 15 ($LC_{50}/EC_{50} > 30.17$ for **1**; > 22.89 for **2**). The toxicity of other nucleosides, including kipukasin D, E, H, and diacetylkipukasin D and E, was evaluated against barnacle cyprids, yielding LC_{50} values ranging from > 59.2 to $> 123 \mu\text{M}$ (C. Y. Wang *et al.*, 2016). This similar low toxicity reinforces the non-toxic profile of nucleoside derivatives such as compounds **1** and **2**.

Complementary ecotoxicological evaluation using model species further confirmed the low environmental risk of the compounds. For *A. amphitrite* nauplii, no mortality was observed within the test range ($0.125\text{--}8 \mu\text{g mL}^{-1}$; $0.5\text{--}32 \mu\text{M}$), supporting an $LC_{50} > 8 \mu\text{g mL}^{-1}$ ($32 \mu\text{M}$), well above the EC_{50} values for mussel plantigrades. Similarly, in *A. salina* nauplii, neither compound induced mortality at 25 and 50 μM (Dunnett's, $p > 0.01$), reinforcing the suitability of both compounds as AF agents due to their low environmental toxicity. A comparable safety profile has been reported for furanone derivatives omF1 and omF2 from *Streptomyces violaceoruber* SCH-09, which were active yet non-toxic against *Navicula annexa* ($LC_{50}/EC_{50} = 173\text{--}290$), *Mytilus edulis* ($LC_{50}/EC_{50} = 92\text{--}94$), and *A. salina* ($LC_{50} = 118.9\text{--}81.9 \mu\text{g mL}^{-1}$) (Hong & Cho, 2013).

3.3.4.2. *In silico* predictions

As a complementary information to the ecotoxicity of the compounds, their physicochemical properties and environmental fate were estimated through EPI Suite™ (Table 10). In ecotoxicology, the octanol-water partition coefficient (known as $\text{Log } K_{ow}$) is an indicator of the bioaccumulation potential of a substance. $\text{Log } K_{ow}$ values greater than 4.5 suggest a high affinity for lipids in organisms, which can pose a risk to marine organisms due to potential bioaccumulation (ECHA, 2017). Compounds **1** and **2** in this

study exhibit Log K_{ow} values below the threshold of 4.5, indicating a low potential for bioaccumulation in organisms and retention in organic matter. In fact, the negative values estimated for the Log K_{ow} are frequently related to high water solubility of molecules, which can be correlated with the compounds' chemical structure. The organic carbon-water partition coefficient, (Log K_{oc}) estimates the distribution of a chemical between a soil or sediment and the aqueous phase that it is in contact with. The adsorption predicted values for the compounds were also negative (Log K_{oc} = -1.11 and -0.55, for **1** and **2** respectively), indicating a low environmental persistence in soils and sediments. Given their negative Log K_{oc} and Log K_{ow} values, the compounds are highly water-soluble and mobile in aquatic systems. The bioconcentration factor (BCF) predicts the tendency of a substance to bioconcentrate in tissues of aquatic organisms, by relating rate of chemical uptake from water and its elimination from the organism. The obtained BCF values for the compounds (BCF = 3.16 L Kg⁻¹) were much lower than the threshold of BCF > 2000 L Kg⁻¹ (ECHA, 2017) defined in Annex XIII of the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) for classifying substances as PBT (persistent, bioaccumulative and toxic), which indicates that they have low tendency to bioconcentrate in the tissues of aquatic organisms. The probability of rapid aerobic and anaerobic biodegradation of an organic compound in the presence of mixed populations of environmental microorganisms (BIOWIN) was also estimated for the compounds. Both compounds are predicted to be not readily biodegradable in anaerobic conditions but would degrade within days to weeks in aerobic conditions. Vilas-Boas *et al.* (2021) studied the *in silico* environmental parameters of 3rd generation biocides: ECONEA®, Medetomidine, and Triphenylborane pyridine (TPBP). All biocides showed strong sediment binding (Log K_{oc} > 3.5), high bioaccumulation potential (Log K_{ow} > 3), and limited biodegradability (Vilas-Boas *et al.*, 2021). In contrast, the nucleosides showed high water solubility, mobility, and low bioaccumulation potential, making them environmentally low risk in terms of persistence and accumulation in food chains. Overall, these preliminary *in vivo* and *in silico* findings highlight the biodegradability, low toxicity, and low bioaccumulation of the compounds, in alignment with regulatory criteria (namely, BPR and REACH). This supports their potential as environmentally compatible candidates for practical application in AF coatings, offering a safer alternative to conventional agents currently in use. However, further *in vivo* ecotoxicological studies on additional target and non-target species, along with *in silico* predictions of environmental concentrations in harbour areas (Oliveira *et al.*, 2017; S. Pereira *et al.*, 2024; Wissner *et al.*, 2024), are recommended to support their environmental safety profile.

Table 10. Predicted data of **1** and **2** from EPI Suite™. Includes biodegradability, sediment-water partition (Log K_{oc}), octanol-water partition (Log K_{ow}), and bioconcentration factor (BCF) of **1** (adenosine) and **2** (2'-Deoxyadenosine). BIOWIN™ criteria: (3) predicted time for ultimate biodegradation; (4) primary biodegradation in aerobic conditions; (5) MITI linear model prediction; and (7) predicted probability for fast biodegradation in anaerobic conditions. Results for (3) and (4): values 5.00 = hours, 4.00 = days, 3.00 = weeks, 2.00 = months, 1.00 = recalcitrant. Results for (5) and (7): values < 0.5 = does not biodegrade fast. ^aNRB: Not Readily Biodegradable; ^bBF: Biodegrades Fast; ^cDNBF: Does not biodegrade fast.

Compound	BIOWIN™				KOCWIN™	KOWWIN™	BCF™
	Ultimate biodegradation (3)	Aerobic conditions (4)	MITI Linear Model (5)	Anaerobic conditions (7)	Log K_{oc}	Log K_{ow}	BCF (LKg/wet-wt)
1	Weeks (2.94)	Days-Weeks (3.73)	NRB ^a (0.36)	BF ^b (0.69)	0.19	-1.38	3.16
2	Weeks (2.82)	Days-Weeks (3.63)	NRB ^a (0.25)	DNBF ^c (0.24)	0.50	-0.71	3.16

3.4. Conclusions

This study investigated the cyanobacterial strain *Leptothoe* sp. LEGE 181152 as a potential biotechnological source of AF agents resulting in the isolation and structure identification of two nucleosides: adenosine (**1**) and 2'-deoxyadenosine (**2**) with novel AF activity. Although these primary metabolites are ubiquitous across organisms, their consistent detection in *Leptothoe* sp. LEGE 181152 active fractions, together with their absence in controls and other cyanobacterial extracts (data not shown), strongly suggests their assignment as metabolites associated with this strain, particularly considering that cultures were unialgal but not axenic. These nucleosides were further evaluated for AF efficacy across different levels of biological organization, with insights into their mode of action, alongside assessments of their environmental toxicity and degradation potential, in order to meet regulatory criteria for new AF agents. Compounds **1** and **2** effectively inhibited the growth of *Navicula* sp. and prevented mussel plantigrade settlement without causing toxicity to either species. Further analysis revealed that compounds **1** and **2** modulated AChE in a hormesis effect. In mussel plantigrades exposed to compound **2**, alterations in the expression of vWFA and EGF-like domain-containing proteins were observed, indicating these proteins as candidate effectors in the AF response to **2**. Functional enrichment analysis also revealed effects on ATP metabolism and ciliary movement. Both compounds demonstrated AF activity against micro- and macrofouling organisms, with no observed lethality toward target or non-target organisms. The observed selectivity towards eukaryotic organisms seem to indicate a more specific AF profile, a characteristic commonly linked to safer and more

environmentally sustainable AF agents. Their predicted low bioaccumulation potential and low environmental persistence further support their minimal ecological risk, positioning them as promising AF agents with fair environmental compatibility. This study identifies adenosine and 2'-deoxyadenosine as novel, low-risk natural AF agents, offering promising alternatives to conventional biocides in preventive AF coatings. Future research should focus on expanding ecotoxicological testing across other key species, evaluating the compounds' incorporation into marine coating matrices, and conducting *in situ* proof-of-concept studies.

Author contributions

S.P.: Formal analysis, investigation, methodology, validation, writing – original draft. L.F.: Investigation, writing – review & editing. I.B.O.: Investigation, writing – review & editing. C.G.: Investigation, writing – review & editing. A.P.: Methodology, validation, writing – review & editing. M.T.: Methodology, validation, writing – review & editing. V.V.: Supervision, resources, writing – review & editing. A.C.: Supervision, validation, resources, writing – review & editing. M.R.: Funding acquisition, project administration, validation, resources, writing – review & editing. J.R.A.: Conceptualization, funding acquisition, project administration, validation, resources, supervision, writing – review & editing.

Conflicts of interest

The authors declare no conflicts of interest.

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(<https://doi.org/10.54499/CEECIND/01368/2018/CP1574/CT0005> and
<https://doi.org/10.54499/2022.03876.CEECIND/CP1728/CT0005>, respectively).

Declaration of competing interest

The authors declare no conflicts of interest.

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Data availability

Data will be made available on request.

4. Structure-Antifouling Activity Relationships, Molecular Mechanisms, and Ecotoxicological Evaluation Underlying Nucleoside-Mediated Antifouling Activity

The content of this chapter corresponds to a published original research article:

Pereira, S., Oliveira, I. B., Palmeira, A., Turkina, M., Vasconcelos, V., Campos, A., & Almeida, J. R. (2026). Structure-Antifouling Activity Relationships, Molecular Mechanisms, and Ecotoxicological Evaluation Underlying Nucleoside-Mediated Antifouling Activity. *Biomolecules*, 16 (4), 584. <https://doi.org/10.3390/biom16040584>

Sandra Pereira performed all the bioassays (*M. galloprovincialis*, *Navicula* sp., marine biofilm-forming bacterial strains, and *Artemia salina*), enzymatic assays, FASP, protein identification, quantification and functional classification as well as *in silico* environmental predictions. All was conducted at CIIMAR.

The *Amphibalanus amphitrite* bioassay was performed by Isabel Oliveira at CIIMAR.

Acetylcholinesterase docking studies were conducted by Andreia Palmeira at LQOF/FFUP.

Peptides LC-MS/MS analysis were conducted by Maria Turkina at BKV/MEDFAK.LiU.

Structure-Antifouling Activity Relationship, Molecular Mechanisms, and Ecotoxicological Assessment of Nucleosides Hypoxanthine arabinoside and 2'-Deoxyinosine

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Abstract

Marine biofouling remains a major challenge for maritime industries, affecting submerged structures and vessels worldwide. The long-standing reliance on biocidal coatings, together with their well-documented environmental impacts, has led to increasingly restrictive regulations and an urgent demand for environmentally compatible antifouling (AF) solutions. This study evaluates the AF potential and toxicological profile of two nucleoside analogues, hypoxanthine arabinoside (**1'**) and 2'-deoxyinosine (**2'**), selected based on the previously reported non-lethal AF activity of the naturally occurring nucleosides adenosine and 2'-deoxyadenosine from cyanobacteria. Both analogues inhibited the growth of *Navicula* sp. by approximately 60% without inducing mortality and significantly reduced settlement of *Mytilus galloprovincialis* plantigrades, with EC₅₀ values of 5.50 µM (**1'**) and 8.54 µM (**2'**), and no lethality detected (LC₅₀ > 200 µM). At near-EC₅₀ values, both compounds increased acetylcholinesterase and tyrosinase activities, supported by molecular docking results, suggesting involvement of neurotransmission- and byssal formation-related pathways. Proteomic analysis revealed compound-specific molecular responses. No lethal effects were detected in non-target organisms (LC₅₀ > 32 µM for *A. amphitrite* and LC₅₀ > 50 µM for *A. salina*), and environmental fate modelling predicted low bioaccumulation potential and rapid

degradation, indicating minimal ecological risk. Overall, substitution of the amino group by a carbonyl group preserved AF efficacy without increasing toxicity, highlighting nucleosides as promising low-toxicity AF agents.

Keywords

Antifouling chemical alternatives, Bio-based solutions, Nucleosides, Hypoxanthine arabinoside, 2'-Deoxyinosine

4.1. Introduction

Marine biofouling – the accumulation of diverse micro- and macrofouling organisms such as bacteria, fungi, diatoms, barnacles, mussels, tubeworms, and algae on submerged surfaces (Qiu *et al.*, 2024) – is a naturally occurring process that has evolved into a significant global issue, particularly affecting maritime industries. It contributes to increased drag on vessels, elevated fuel consumption, and the degradation of marine infrastructure. Although biocidal coatings remain the predominant strategy for biofouling control (Weber & Esmaeili, 2023), their effectiveness is coupled with environmental drawbacks due to the release of toxic substances into marine ecosystems, raising concerns for both environmental and human health (Paz-Villarraga *et al.*, 2022; Vilas-Boas *et al.*, 2023). In response to the European Union's shift towards a sustainable blue economy (European Commission: Deltares *et al.*, 2021), and the implementation of the Biocidal Products Regulation (BPR, EU 528/2012), there is growing pressure to replace persistent toxic biocides with environmentally safer alternatives. Significant recent efforts have been directed toward the discovery of novel antifouling (AF) compounds, with several comprehensive reviews highlighting advances in marine natural product-based AF agents (Cahill *et al.*, 2024; D. Pereira, Almeida, *et al.*, 2024; K. L. Wang *et al.*, 2017). However, new AF agents must meet stricter criteria, demonstrating target-specific efficacy, a well-defined mode of action, minimal toxicity to non-target species, and low potential for bioaccumulation and environmental persistence. Within this context, our investigation of the biotechnological potential of the cyanobacterium *Leptothoe* sp. LEGE 181152 led to the identification of two naturally occurring nucleosides, adenosine (**1**) and 2'-deoxyadenosine (**2**), as novel AF agents. These compounds inhibited the growth of *Navicula* sp. by approximately 40–60% and prevented the settlement of *Mytilus galloprovincialis* plantigrades ($EC_{50} = 6.63\text{--}8.74\text{ }\mu\text{M}$), while displaying no lethality toward fouling organisms ($LC_{50} > 200\text{ }\mu\text{M}$) or non-target species such as *Artemia salina* and *Amphibalanus amphitrite* nauplii. Mechanistic insights further suggested a modulation of acetylcholinesterase (AChE) activity by both compounds, characterized by a hormetic response. In addition, proteomic analysis of mussel plantigrades exposed to compound

2 revealed altered expression of von Willebrand factor (vWF) and epidermal growth factor (EGF)-like proteins, alongside effects on proteins associated with ciliary function and ATP metabolism (S. Pereira et al., 2025). Despite these promising results, nucleosides and their derivatives remain largely unexplored as AF agents. To date, only the nucleoside derivatives kipukasins – isolated from *Aspergillus versicolor* and *Ganoderma australe* – have been evaluated for AF activity against *Amphibalanus amphitrite* cyprids (C. Y. Wang et al., 2016) and *Bugula neritina* larvae (Wu et al., 2020). Among nature-inspired compounds, AF activity has been reported only for benzimidazole C-nucleosides, where selected acyclic gulo- and gluco-analogues reduced bacterial biofilm formation when incorporated into marine coatings (Sallam et al., 2020). Consequently, the structural elements regulating AF activity in nucleosides remain essentially unknown. Given that AF activity is a recently described property of nucleosides **1** and **2**, a critical next step is to identify the structural features responsible for their efficacy and selectivity. As reviewed in (Seley-Radtke & Yates, 2018), simple modifications on nucleoside scaffold purine base can influence hydrogen bonding interactions, electronic distribution, and enzyme recognition, and therefore significantly impact the biological activity. To test the contribution of amino functional group (-NH₂) at the 6-position of the purine base, two closely related nucleoside analogues – hypoxanthine arabinoside (**1'**) and 2'-deoxyinosine (**2'**) – were selected. These analogues preserve the ribose or deoxyribose moiety and overall molecular scaffold of **1** and **2**, differing only by the substitution of the amino group with a carbonyl group (C=O) (Figure 18). This conservative modification allows the evaluation of structure–AF activity relationships while minimizing confounding effects arising from major structural changes. Hypoxanthine arabinoside (**1'**) was originally developed as an antiviral agent (Bryson & Connor, 1976) and is commonly encountered as a degradation product of vidarabine, whereas 2'-deoxyinosine (**2'**) is a naturally occurring product of 2'-deoxyadenosine (**2**) deamination, catalysed by adenosine deaminase during nucleotide metabolism (Bhagavan & Ha, 2011). Notably, 2'-deoxyinosine (**2'**) has also been associated with biotechnological potential, particularly in the context of anti-cancer and anti-inflammatory activities (P. Liu et al., 2024; Zhao et al., 2024).

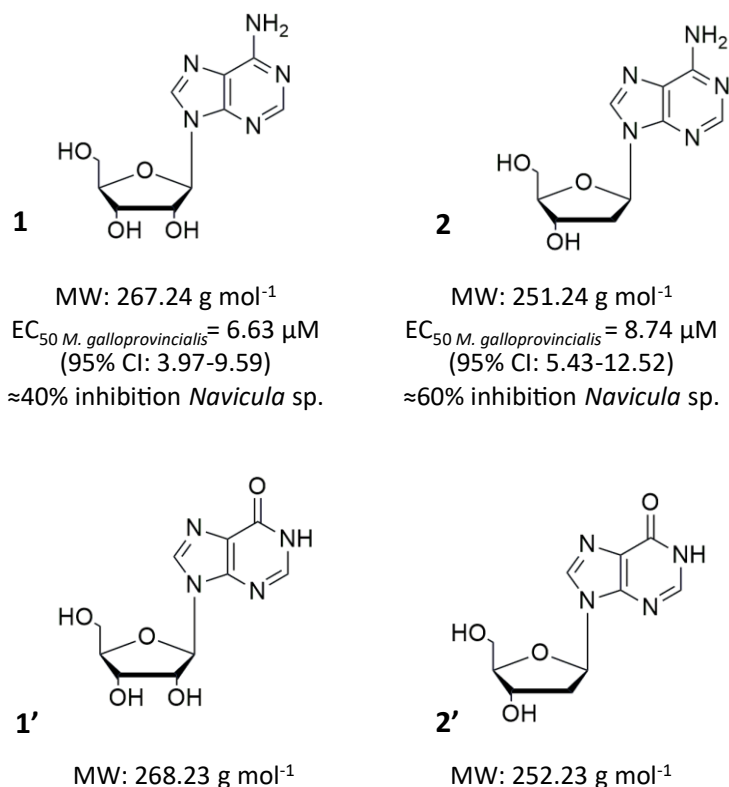


Figure 18. Chemical structure of the nucleosides. **1**: Adenosine; **1'**: Hypoxanthine arabinoside; **2**: 2'-Deoxyadenoside; **2'**: 2'-Deoxyinosine. MW: molecular weight. EC₅₀ values obtained for *M. galloprovincialis* plantigrades. Growth inhibition against the marine biofilm-forming diatom *Navicula* sp (S. Pereira *et al.*, 2025).

In this study, we investigated the structure–AF activity relationship of **1'** and **2'** by systematically evaluating their AF efficacy, molecular mechanisms, and predicted environmental impact. AF performance was assessed against representative micro- and macrofouling organisms. Molecular mechanisms were explored through *in vitro* enzymatic assays focusing on tyrosinase (TYR) and AChE, complemented by untargeted whole-organism shotgun proteomics and AChE-target docking simulations. Finally, the environmental safety profile of the compounds was evaluated using toxicity assays on non-target species and *in silico* predictions of bioaccumulation and degradation potential.

4.2. Methodology

4.2.1. General

The compound hypoxanthine arabinoside (**1'**) (purity ≥98%, CAS: 7013-16-3) was procured from Biosynth Ltd, UK, and 2'-deoxyinosine (**2'**) (purity ≥98%, CAS: 890-38-0) was acquired from Merck, Germany.

4.2.2. Antifouling activity

4.2.2.1. Settlement inhibition of *M. galloprovincialis* plantigrades

The settlement inhibition assay followed the methodology previously reported by Almeida *et al.* (2017). Briefly, samples were obtained from intertidal rock pools at Memória Beach, Matosinhos, Portugal (41° 13' 59" N; 8° 43' 28" W), during periods of low neap tides. Active plantigrades of *M. galloprovincialis* were selected and distributed into 24-well microplates, using four replicates per condition (five individuals per well). Organisms were exposed to the test solutions for 15 h at 18 ± 1 °C under dark conditions. Test compounds were evaluated at 25 and 50 µM. Each assay included a negative control (0.1% DMSO) and a positive control (5 µM CuSO₄), with all the solutions prepared in filtered seawater. Anti-settlement effects were determined by assessing the formation of byssal threads and their attachment to the substrate. Additionally, the half-maximal effective concentration (EC₅₀), corresponding to 50% inhibition of larval settlement, and the median lethal dose (LC₅₀) were calculated.

4.2.2.1.1. *M. galloprovincialis* settlement recovery

The reversibility of the AF effects on mussel plantigrades was assessed following short-term exposure (15 h), according to previously described procedures (Antunes *et al.*, 2019). Initially, a settlement inhibition assay was conducted as outlined above, in which plantigrades were exposed to the compounds for 15 h, at 3.1 and 12.5 µM for **1'** and at 6.2 and 25 µM for **2'**. These concentration ranges were selected to include the respective EC₅₀ values. Assays included both negative (0.1% DMSO) and positive (5 µM CuSO₄) controls. The inhibitory effect on settlement was confirmed immediately after the exposure period. Subsequently, each individual was carefully transferred to a clean microplate containing only filtered natural seawater, with appropriate controls maintained. After an additional 15 h recovery period, settlement re-evaluated under all conditions to determine the reversibility of the observed effects.

4.2.2.2. Growth inhibition of marine bacteria and diatom

To further investigate the activity of the compounds against microfouling organisms, a panel of five marine biofilm-forming bacterial strains - *C. marina* CECT 4278, *V. harveyi* CECT 525, *H. aquamarina* CECT 5000, *P. atlantica* CECT 570, and *R. littoralis* CECT 5395 - was acquired from the Spanish Type Culture Collection (CECT). Antibacterial activity was evaluated following previously reported procedures (Almeida *et al.*, 2017). Briefly, bacterial strains were cultured in Marine Broth (Condalab, Madrid, Spain) and distributed into 96-well flat-bottom microplates, ensuring an initial optical density of 0.1 at 600 nm (OD₆₀₀). Cells were exposed to compounds at 25 and 50 µM and incubated

at 26 °C for 24 h, except for *R. littoralis*, which required 72 h. Each condition was tested in quadruplicate. A solution of Marine broth containing 1% DMSO served as the negative control, while a 1:100 penicillin-streptomycin-neomycin stabilized solution (Sigma P4083, Merck, Darmstadt, Germany) was used as the positive control. Bacterial growth inhibition was determined by measuring absorbance at 600 nm using a microplate reader (Biotek Synergy HT, Winooski VT, USA).

For the evaluation of anti-microalgal activity, a benthic marine diatom (*Navicula* sp.) was obtained from the Spanish Collection of Algae and tested according to Antunes *et al.*, 2019. Diatom cultures were prepared in Guillard's (F/2) medium supplemented with silica (Sigma G9903), at an initial concentration of $2 - 4 \times 10^4$ cells mL⁻¹. The assays were conducted in 96 well flat-bottom microplates, where cells were exposed to the compounds at 25 and 50 µM over a 10-day period at 20 °C. All treatments were performed in quadruplicate. Guillard's (F/2) medium containing 1% DMSO was used as negative control, while cycloheximide (3.55 µM) served as the positive control. Inhibition of diatom growth was assessed by comparing cell densities across different conditions.

4.2.3. Molecular Targets on *M. galloprovincialis*

4.2.3.1. *In Vitro* TYR Activity

TYR inhibitory activity was evaluated using *Agaricus bisporus* tyrosinase (EC 1.14.18.1), following the method described by Adhikari *et al.*, (2008) with minor modifications (Almeida *et al.*, 2017). For each assay condition, 50 µL of TYR (25 U mL⁻¹) prepared in phosphate buffer (50 mM, pH 6.5) was added. Test compounds were assessed at 25 and 50 µM. The reaction was initiated by the addition of L-DOPA (25 mM), prepared in the same phosphate buffer (50 mM, pH 6.5). DMSO (0.2%) was used as negative control, while kojic acid (100 µM) was used as positive control. All experiments were performed in quadruplicate. Enzymatic activity was monitored by measuring absorbance at 475 nm over 5 min at 25 °C.

4.2.3.2. *In Vitro* AChE Activity

AChE inhibition activity of the compounds was evaluated using AChE Type V-S from *Electrophorus electric* (SIGMA C2888, E.C. 3.1.1.7), following the method originally described by Ellman *et al.* (1961) with modifications reported by Almeida *et al.* (2015). Succinctly, the reaction solution consisted of 30 mL of phosphate buffer (0.1 M, pH = 7.2), supplemented with 1 mL of 5,5'-Dithiobis(2-nitrobenzoic acid (DTNB, 10 nM), prepared in phosphate buffer (0.1 M, pH = 7.2) containing sodium hydrogen carbonate, and 200 µL of acetylthiocholine iodide (0.075 M). For each condition, 50 µL of pure AChE solution (0.25 U mL⁻¹) was combined with 250 µL of the reaction solution. The tested

compounds were evaluated at 25 μ M and 50 μ M. DMSO (0.2%) and eserine (200 μ M) were used as negative and positive controls, respectively, while a blank was prepared using 50 μ L of phosphate buffer instead of the enzyme. All conditions were conducted in quadruplicates. Enzymatic activity was monitored by measuring absorbance at 412 nm over 5 min at 25 °C.

4.2.3.2.1. Docking Study of AChE

The crystal structure of *Electrophorus electric* AChE (PDB code: 1C2O) (Bourne *et al.*, 1999) was retrieved from the protein databank (PDB) and used as the receptor for the *in silico* molecular docking analyses. The chemical structures of the test compounds, along with two references AF agents exhibiting AChE inhibitory activity - pulmonarins A and B (Trepas *et al.*, 2014) - were drawn and minimized. Geometry optimization was carried out using the semi-empirical Austin Model 1 (AM1) method, applying the Hyperchem 7.5 software (Hypercube, Florida, USA) (Froimowitz, 1993), until the energy gradient between consecutive steps fell below 10⁻¹ kcal mol⁻¹ Å⁻². Docking simulations were performed with Autodock Vina v1.2x (Molecular Graphics Lab, The Scripps Research Institute, San Diego, CA, USA) (Trott & Olson, 2010), using an exhaustiveness of 8. The search space was defined by a grid box of 15.0 × 20.0 × 15.0 Å, encircling the acetylcholine active site. For each ligand, nine binding conformations were generated. The poses exhibiting the lowest docking scores were selected for further evaluation of interaction patterns and visual inspection using PyMol 1.3 (Schrödinger, New York, NY, USA) (Lill & Danielson, 2011).

4.2.3.3. Untargeted proteomic analysis and functional enrichment of mussel plantigrades

M. galloprovincialis plantigrades were exposed to the tested compounds at 12.5 μ M, a concentration close to the EC₅₀ previously determined in plantigrade settlement assays. Following exposure, plantigrades from both negative control (0.1% DMSO) and treated groups were collected for proteomic analysis, using four replicates per condition (ten plantigrades per replicate), in accordance with the approach described by S. Pereira *et al.*, (2025). Protein extraction and peptide preparation for liquid chromatography-mass spectrometry (LC-MS) were carried out as previously reported by Campos *et al.*, (2016). Succinctly, defrosted samples were solubilized in lysis buffer (composed of 2% (w/v) SDS, 100 mM Tris-HCl at pH 7.6, 0.1 M DTT, and 1:50 protease inhibitor) and filter-aided sample preparation (FASP) was used for protein digestion. After reconstitution in formic acid (0.1%, v/v), peptide separation was performed on an EASY nLC 1200 chromatographic system (Thermo Scientific, Waltham, MA, USA) equipped with C18

Acclaim PepMap 100, 75µm × 2 cm pre-column (Thermo Scientific, Waltham, MA, USA) and EASY-Spray C18 analytical column (PepMap RSLC C18, 2 µm, 100A 75 µm × 25 cm, Thermo Scientific, Waltham, MA, USA). Elution used was achieved at a flow rate of 0.3 µL/min using a gradient of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in 80% acetonitrile), progressing from 6% to 30% B over 65 min, followed by an increase to 100% B in 20 min and a final hold at 100% B for 5 min. The eluting peptides were analyzed online by a QExactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA), operating in positive ion mode under data-dependent acquisition mode. Full MS scans were acquired at a resolution of 120 000 across an m/z range of 380–1400. The 15 most intense multiple charged ions (isolation window of 1.2 m/z) were selected for fragmentation at a resolution of 30 000, with a dynamic exclusion of 30 s. Protein identification was performed using Sequest HT algorithm implemented in Proteome Discoverer (v. 2.5.0.400, Thermo Scientific, Waltham, MA, USA), searching against the Mollusca UniProt Knowledgebase (The UniProt Consortium, 2023) (Taxon ID: 6447, March 2023, 753,986 entries). Search parameters included trypsin specificity, a parent ion tolerance of 10.0 ppm, and a fragment ion mass tolerance of 0.080 Da. Carbamidomethylation of cysteine residues was set as a fixed modification, while methionine oxidation was allowed as a variable modification. Peptide and protein identifications were further validated using Scaffold (v5.3.2). Acceptance criteria required peptide identifications with a probability greater than 95.0% (based on Percolator posterior error probability) (Käll *et al.*, 2008), and protein identifications exceeding 99.0% probability with at least two identified peptides, as determined by the Protein Prophet algorithm (Nesvizhskii *et al.*, 2003). Proteins sharing significant peptide evidence were grouped according to parsimony principles.

4.2.3.4. Bioinformatic analysis

The set of identified proteins was examined for entries previously described in the literature as being associated with mussel adhesion mechanisms. In parallel, broader functional insights were obtained through sequence homology analysis using BLASTp against *Magallana gigas* protein databases (UniProt KB 2025_01, Ensembl Metazoa 2024_08), implemented in Blast2GO (v6.0.3) (Conesa *et al.*, 2005; Conesa & Götz, 2008; Götz *et al.*, 2008), applying an e-value cutoff of 1×10^{-3} . Given the more extensive annotation available for *M. gigas* compared to *M. galloprovincialis*, functional enrichment and classification analyses were conducted using g:Profiler (e112_eg59_p19_25aa4782, updated 3 February 2025) (Kolberg *et al.*, 2023). The resulting functional interaction networks were then constructed and visualized in Cytoscape (v3.10.3) (Shannon *et al.*, 2003).

4.2.4. Environmental behaviour

4.2.4.1. *Artemia salina* bioassay

General marine ecotoxicity of the tested compounds was evaluated using the brine shrimp (*A. salina*) nauplii lethality test assay, as previously described (Almeida *et al.*, 2017). Cysts were hatched in nutrient-enriched seawater for approximately 24 h at 26 °C to obtain active nauplii. The compounds were tested at 25 and 50 µM. Assays were carried out in 96-well microplates, with 15-20 nauplii per well and eight replicates per condition. Exposure was conducted in the dark at 25 °C over a period of 48 h. DMSO (1%) was used as negative control, while potassium dichromate (13.6 µM) was used as positive control.

4.2.4.2. *Amphibalanus amphitrite* bioassay

The toxicity of the compounds was further evaluated using *A. amphitrite* nauplii, following the procedure described by Pereira *et al.* (2024). Newly released and actively swimming nauplii (< 3 h post-release) were exposed to the test solutions at concentrations ranging from 0.125–8 µg mL⁻¹ (0.5–32 µM). Exposures were conducted in glass tubes containing 3 mL of solution, with approximately 30–60 larvae per tube, in triplicate. The assay was carried out for 24 h at 28 °C under dark conditions, without feeding. Mortality was determined based on the absence of movement in the nauplii. A negative control consisting of DMSO (0.1%) was included. The assay was performed twice, and the results were combined for subsequent analysis.

4.2.4.3. *In silico* environmental fate predictions

A set of physicochemical properties and environmental fate of the compounds was predicted using computational models available within the Estimation Programs Interface (EPI) Suite™, developed by the US Environmental Protection Agency's Office of Pollution Prevention and Toxics and Syracuse Research Corporation (SRC) (US EPA, 2024). The analysis included bioconcentration potential (BCFBAF™ v3.01), partitioning behaviour (KOWWIN™ v1.68), biodegradability (BIOWIN™ v4.10), and soil adsorption capacity (KOCWIN™ v2.00).

4.2.5. Data analysis

Prior to statistical analysis, all datasets were evaluated for normality using the Shapiro-Wilk test and for homogeneity of variances with Levene's test. When required, data transformations were applied to meet test assumptions. The screening results from the different bioassays were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's test ($p < 0.01$). In cases where assumptions were not met, Welch's ANOVA was applied, followed by Dunnett's T3 test ($p < 0.01$). For the diatom growth inhibition

assay, differences between concentrations were assessed using Tukey HSD test ($p < 0.01$) was used to assess differences between compounds' concentrations. EC_{50} for *M. galloprovincialis* plantigrade settlement inhibition was estimated through Probit regression analysis. Model fit was evaluated using Pearson Goodness-of-Fit Chi-Square test ($p < 0.01$), and 95% lower and upper confidence limits (LCL; UCL) were calculated. All ANOVA and Probit analyses were conducted using IBM SPSS Statistics (version 30). For mussel plantigrades, the compounds' efficacy *versus* toxicity was expressed as the therapeutic ratio (LC_{50}/EC_{50}) (Almeida *et al.*, 2017; Dan Rittschof *et al.*, 2003). Proteomic data analysis was performed in R: programming language (version 4.4.1) (R Core Team, 2021) using the "DEP" package (Xiaofei Zhang *et al.*, 2018). Proteins with missing values were initially filtered, retaining only those consistently identified in all replicates of at least one experimental condition. Remaining missing values were subsequently imputed. Differently expressed proteins (DEPs) between treatment and control groups were defined based solely on a $\log_2$ fold change ($\log_2FC = \log_2(1.5)$).

4.3. Results and Discussion

4.3.1. AF activity and toxicity against micro and macrofouling organisms

In this work, the nucleoside analogues hypoxanthine arabinoside (**1'**) and 2'-deoxyinosine (**2'**) were evaluated for their AF potential, following the previously reported AF activity of their structural counterparts **1** and **2**. As this represents the first study exploring the biotechnological AF potential of **1'** and **2'**, their activity was assessed against a panel of microfouling organisms, including five strains of marine biofilm-forming bacteria and a marine biofilm-forming diatom, as well as against a widely distributed macrofouling species. To evaluate microfouling inhibition, marine biofilm-forming bacterial strains and the diatom *Navicula* sp. were exposed to **1'** and **2'**. A growth inhibition threshold of $\geq 30\%$ was established as the criterion for AF activity. In the initial screening against the five bacterial strains, neither compound induced significant growth inhibition at concentrations of 25 and 50 μM (Dunnett's test, $p > 0.01$) (Figure 19.a). In contrast, both nucleosides displayed significant inhibitory effects against the marine diatom *Navicula* sp., with growth inhibition $\geq 30\%$ at the same concentrations (Dunnett's test, $p < 0.01$), prompting further dose-response evaluation. Across the entire concentration range tested (0.8–200 μM), both **1'** and **2'** consistently inhibited diatom growth by approximately 60% compared to the control (Dunnett's test, $p < 0.01$) (Figure 19.b). This response contrasts with the differential inhibitory effect previously observed for **1** ($\approx 40\%$) and **2** ($\approx 60\%$), which were proposed to be associated with an increased lipophilicity resulting from the absence of hydroxyl group (-OH) at the 2' position of the ribose moiety (Novotny *et al.*, 2000; S. Pereira *et al.*, 2025). Since **2'** also lack this

hydroxyl group, a plausible explanation for their comparable inhibitory profile is that the increased lipophilicity conferred by the carbonyl group (=O) at the position 6 of the purine system, substituting the amino group (-NH₂), may outweigh the structural differences between **1'** and **2'**, resulting in a similar inhibitory response (Novotny *et al.*, 2000). Nonetheless, no clear dose-response relationship could be established for either compound, suggesting that a threshold-like mechanism may underlie their AF activity against *Navicula* sp., consistent with observations reported for **1** and **2** (S. Pereira *et al.*, 2025).

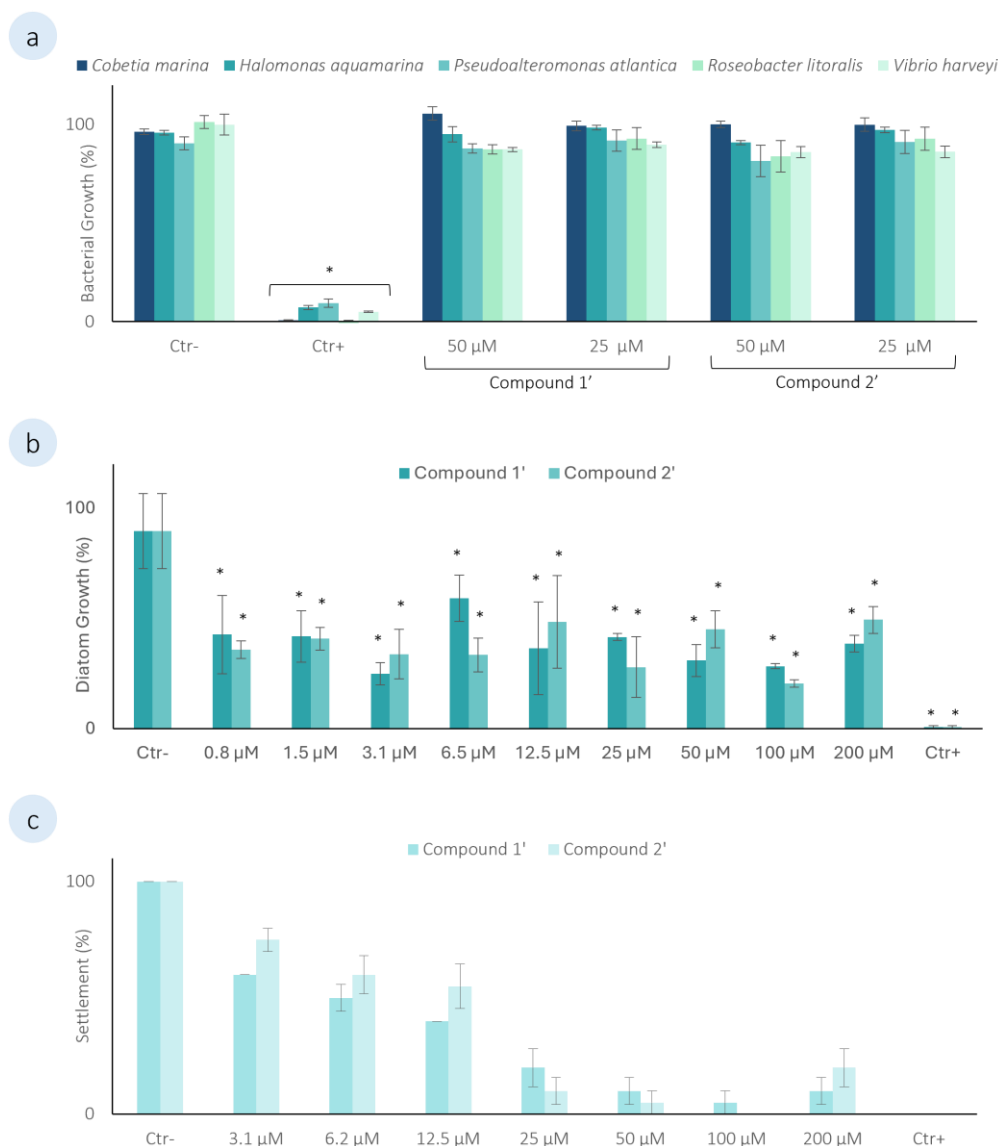


Figure 19. Antifouling activity of **1'** and **2'**. (a) Growth inhibition of five marine biofilm-forming bacteria screening bioassay. Compounds were screened at 25 and 50 μ M. Ctr-: DMSO (1%); Ctr+: 1:100 penicillin-streptomycin-neomycin stabilized solution; (b) Dose-response against the marine biofilm-forming diatom *Navicula* sp. Ctr-: DMSO (1%); Ctr+: Cycloheximide (3.55 μ M); (c) Dose-response against *M. galloprovincialis* plantigrades. Ctr-: DMSO (0.1%); Ctr+: CuSO₄ (5 μ M). *Significant differences compared to the negative control (Dunnett's test, $p < 0.01$).

The AF activity of the compounds was further evaluated against the macrofouling species *M. galloprovincialis*, one of the most widespread macrofoulers globally and a well-established model organism for settlement inhibition bioassays (O. Ferreira et al., 2020; Vilas-Boas et al., 2021). Mussel plantigrades were exposed to the compounds for 15 h, and, after the initial screening at concentrations of 25 and 50 μM , both compounds showed significant settlement inhibition (Dunnett's test, $p < 0.01$). A dose-response bioassay was performed (Figure 19.c), and the half-maximal effective concentration that inhibited 50% of mussel settlement was assessed for **1'** ($\text{EC}_{50} = 5.50 \mu\text{M}$) and **2'** ($\text{EC}_{50} = 8.54 \mu\text{M}$) (Table 11). Results showed EC_{50} values for **1'** and **2'** below the U.S. Navy's program reference threshold ($\text{EC}_{50} < 25 \mu\text{g mL}^{-1}$). The values were comparable to those reported for **1** and **2**, suggesting that the AF activity may not be linked to the amino group. Other nucleosides, such as kipukasins (D, E, H, K and L) and their diacetylated derivatives (diacetylkipukasin D and E), have previously been evaluated for AF activity using different model organisms. Kipukasins D, E and H as well as diacetylkipukasin D and E exhibited low inhibitory effects on the settlement of *A. amphitrite* cyprids, with reported EC_{50} values ranging from 44.50 to 61.30 μM (C. Y. Wang et al., 2016). In contrast, kipukasins K and L were shown to reduce larval settlement of the bryozoan *Bugula neritina* at a concentration of 25.00 $\mu\text{g mL}^{-1}$ (Wu et al., 2020). In comparison, the inhibitory activity of compounds **1'** and **2'** against mussel plantigrades was considerably stronger. Additionally, the efficacy of compound **1'** can be comparable to the commercial AF agent ECONEA® ($\text{EC}_{50} = 1.40 \mu\text{g mL}^{-1}$) efficacy in preventing the settlement of mussel plantigrades (Almeida et al., 2017).

Table 11. Antifouling effectiveness *versus* toxicity of compounds **1'** and **2'** against biofouling species. The half maximum effective concentration (EC_{50}), the median lethal concentration (LC_{50}), and the therapeutic ratio ($\text{LC}_{50}/\text{EC}_{50}$) are represented. The EC_{50} , EC_{30} and LC_{50} are provided in both μM and $\mu\text{g mL}^{-1}$ for comparative purposes. Confidence intervals for EC_{50} and EC_{30} values are in μM (in parentheses). **1'**: hypoxanthine arabinoside; **2'**: 2'-deoxyinosine. n.a. – not assessed.

	Target species	EC_{50} (μM ; $\mu\text{g mL}^{-1}$)	LC_{50} (μM ; $\mu\text{g mL}^{-1}$)	$\text{LC}_{50}/\text{EC}_{50}$
1'	<i>M. galloprovincialis</i>	5.50 (95% CI: 2.86 - 8.50); 1.47	> 200; > 53.6	> 36.38
	<i>Navicula</i> sp.	n.a.		n.a.
2'	<i>M. galloprovincialis</i>	8.54 (95% CI: 3.54 – 14.95); 2.15	> 200; > 50.4	> 23.41
	<i>Navicula</i> sp.	n.a.		n.a.

Compounds exhibiting AF activity with EC_{50} values around $\sim 2 \mu\text{g mL}^{-1}$, such as the nucleoside derivatives described in this study, fall within the range commonly reported

for many effective marine AF natural products. Indeed, a wide variety of bioactive compounds—including terpenoids, alkaloids, and polyketides—typically display EC_{50} values in the range of 1–10 $\mu\text{g mL}^{-1}$ against relevant fouling organisms (Almeida *et al.*, 2017; Qian *et al.*, 2015; K. L. Wang *et al.*, 2017). Although some recently described metabolites, such as madeirone and related compounds (Wissner *et al.*, 2024), exhibit higher potency at sub-microgram levels, AF performance cannot be evaluated solely based on EC_{50} values. Other factors, including environmental compatibility, mode of action, chemical stability, and synthetic accessibility, are critical for practical applications. Therefore, despite not always reaching the lowest EC_{50} values reported for certain marine natural products, nucleosides constitute a valuable and competitive addition to the current AF chemical toolbox.

Overall, the AF profile **1'** and **2'** showed activity against *Navicula* sp. and *M. galloprovincialis* plantigrades, but no inhibitory effect on the growth of marine biofilm-forming bacteria, indicating a degree of biological specificity, with putative molecular mechanisms preferentially targeting eukaryotic processes rather than prokaryotes. This aligns with that observed for **1** and **2** and supports a more selective AF mode of action. Such selectivity is typically associated with safer and more environmentally compatible AF agents, particularly when compared to broad-spectrum biocides known for their high marine toxicity (Cahill *et al.*, 2024). Future works should focus on testing other eukaryotic biofouling organisms to confirm this apparent selectivity.

4.3.2. Putative molecular mechanisms of the AF activity on *M. galloprovincialis* plantigrades

Understanding the mechanisms underlying the AF action of compounds is becoming increasingly important for the development of new AF agents, particularly considering the regulatory demands set by the Biocidal Products Regulation (BPR, EU 528/2012). However, pinpointing a single molecular target or pathway remains a considerable challenge due to the complexity of the biological processes involved in biofouling (Cahill *et al.*, 2024). In this study, the potential protein targets underlying the AF effects of compounds **1'** and **2'** were investigated in *M. galloprovincialis* plantigrades, with a focus on exploring multiple pathways potentially involved in settlement inhibition.

4.3.2.1. AChE and TYR pathways

Enzymes such as AChE, are affected by AF compound exposure in fouling organisms, as AChE is implicated in cholinergic neural signalling during larval settlement (L. Chen & Qian, 2017). In here, we explore the effects of the compounds on this pathway through docking studies and *in vitro* enzymatic assays. Recently, ligand- and structure-based

computer-aided drug design (CADD) tools have been used to anticipate the AF activities of compounds as well as their molecular mechanism of action (Arabshahi *et al.*, 2021; Gaudêncio & Pereira, 2022). Furthermore, docking studies have already been employed in our research to anticipate how two hydroxyxanthenes will bind to *Electrophorus electric* AChE (Almeida *et al.*, 2020). As such, the molecular binding mode of the **1'** and **2'** to AChE was conducted by docking studies using AutoDock Vina. A long, tight, and hydrophobic cavity is visible in the crystallographic structure of the serine hydrolase *Electrophorus electric* AChE (Bourne *et al.*, 1999). The catalytic triad (Ser-203, Glu-334, and His-447) that makes up the AChE active site is situated near the base of the gorge. Here, acetylcholine is converted into choline and acetate (Y. Zhou *et al.*, 2010). The oxyanion hole formed by Gly-121, Gly-122, and Ala-204 determines the reactive intermediates and transition states that are generated during the process (Silman & Sussman, 2008). The side chains of Trp-86, Glu-202, and Tyr-337 are combined to create the anionic subsite, which is primarily in charge of binding the quaternary trimethylammonium tail group of Ach (Kua *et al.*, 2003). Tyr-72, Asp-74, Tyr-124, Glu-285, Trp-286, and Tyr-341 are among the aromatic residues that make up the peripheral anionic site (PAS) near the gorge's entrance. This PAS forms a hydrophobic region that binds acetylcholine and transfers it to the deep catalytic site (Johnson & Moore, 2006). Studies on AChE from a variety of species indicate that some AChE inhibitors can bind at the catalytic site and act as competitive inhibitors (Colovic *et al.*, 2013). At the active site gorge, AChE inhibitors can also bind to PAS, thus hindering the catalysis by sterically blocking ligands from entering and leaving the active site portal and by allosterically altering the catalytic triad's conformation (Bourne *et al.*, 2003). As positive controls in the *in silico* docking study, pulmonarin A and B, two *Ascidian synoicum* AF compounds, were found to exhibit competitive AChE inhibition without any antibacterial or cytotoxic effect (Tadesse *et al.*, 2014; Trepos *et al.*, 2014). The top-ranked poses of pulmonarin A and B have docking scores of -5.9 and -6.8 kcal mol⁻¹, respectively. Compared to positive controls, compounds **1'** and **2'** were predicted to have a higher affinity towards the AChE active site (docking score of -7.3 and -7.1 kcal mol⁻¹, respectively) (Figure 20).

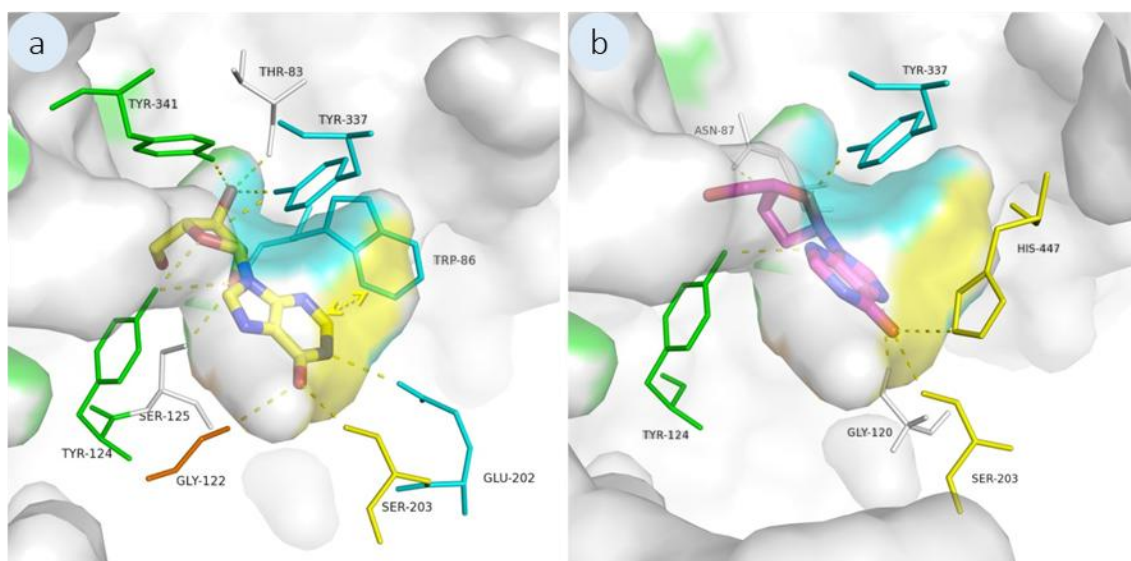


Figure 20. AChE docking study for **1'** and **2'**. Detailed view of (a) **1'** (yellow sticks) and (b) **2'** (pink sticks) docked in AChE binding site (PDB code: 1C2O). Hydrogen interactions are represented with yellow broken lines. π -Stacking and π -hydrogen donor interactions are represented with a yellow and orange double arrow, respectively. Residues involved in those interactions are represented as lines and labelled. AChE is represented as transparent surface, where the catalytic triad, PAS, anionic sites and oxyanion hole are represented in yellow, green, blue and orange, respectively.

As previously described by our group, the interactions between known inhibitors pulmonarin A and B and the enzyme are characterized by several hydrogen bonds with residues from PAS, oxyanion hole, anionic site, and catalytic triad (Almeida *et al.*, 2020). Both compounds dock inside the groove with the purinone ring fitting deeply into the catalytic groove and the oxolanyl ring blocking the entrance of the gorge (PAS) (Figure 20). Compound **1'** establishes hydrogen a bond network that encloses it securely in the target cavity through residues from the catalytic triad (Ser-203), PAS (Tyr-124), anionic site (Glu-202 and Tyr-337), oxyanion hole (Gly-122) and other residues such as Thr-83, Ser-125 and Tyr-341 (Figure 20.a); while **2'** establishes hydrogen interactions with residues from the catalytic triad (His-447 and Ser-203), PAS (Tyr-124), anionic site (Tyr-337), and also with other residues such as Asn-87 and Gly-120 (Figure 20.b). Most binding patterns are shared among **1'** and **2'**, differing mainly in the number and distribution of hydrogen bonds, particularly the interaction with the oxyanion hole, which was exclusively observed for **1'**. These differences are likely influenced by subtle structural variations between the two nucleosides, including the presence or absence of hydroxyl groups in their sugar moieties. When compared with previously reported docking studies for **1** and **2**, additional distinctions emerge. Specifically, π -hydrogen donor interactions were observed for **1**, whereas π -stacking interactions characterized the binding of **2**, interactions that were not prominent for **1'** and **2'**. These differences

may be associated with the substitution of the amino group in the purine ring by a carbonyl group in the hypoxanthine derivatives, highlighting how small modifications in the nucleobase can influence interaction patterns and, ultimately, structure–activity relationships. Crystallographic structures of AChE have shown that hydrogen bonds are established between the inhibitor and residue Ser-203 (Ortiz *et al.*, 2016). Moreover, computational studies have revealed that the most stable conformations of AChE: inhibitor complexes show Ser-203 and His-447 residues close to the inhibitor (Moraga-Nicolás *et al.*, 2018). Amino acid residues Tyr-337 and Tyr-341 are involved in the binding of reversible known AChE inhibitors (Bourne *et al.*, 2004; Saxena *et al.*, 1994). Moreover, Trp-86 has been reported as relevant in stabilizing irreversible inhibitors in the AChE binding pocket (Ranjan *et al.*, 2018). Gly-122 is thought to be involved in the formation of acetylcholine reactive intermediates and transition states that are formed during the reaction (Y. Zhang *et al.*, 2002) and it is predicted as being in contact with most AChE ligands (Ordentlich *et al.*, 1998). It has previously been reported that Tyr-124, which integrates PAS, plays a crucial role in the binding of *Electrophorus electricus* AChE inhibitors (Bourne *et al.*, 2003; Greenblatt *et al.*, 1999; Loh *et al.*, 2021). Other residues such as Thr-83, Glu-202 and Ser-125 have also been described as important in the interaction of inhibitors with AChE (Gul *et al.*, 2023; Karunakaran *et al.*, 2022; Merzoug *et al.*, 2021; Z. Zhang *et al.*, 2023). Taken together, the docking analyses suggest that **1'** and **2'** establish multiple interactions within the AChE catalytic gorge, with predicted binding affinities surpassing those of known inhibitors, reinforcing the relevance of this target and reflecting the trends previously observed for **1** and **2**.

The effects of compounds **1'** and **2'** on AChE were further evaluated through in vitro enzymatic assays. An initial screening was performed at concentrations of 25 μ M and 50 μ M (four replicates per treatment) revealed that both nucleosides significantly increased AChE activity following exposure (Dunnett's test, $p < 0.01$). To better characterize this response, a wider concentration range (3.1–200 μ M) was subsequently tested (Figure 21.a), revealing a biphasic effect. At lower concentrations, both nucleosides significantly enhanced AChE activity by approximately 23% (Dunnett's test, $p < 0.01$), whereas at higher concentrations a significant inhibition of enzymatic activity ($\approx 20\%$) was observed (Dunnett's test, $p < 0.01$), consistent with a hormetic response (Agathokleous *et al.*, 2024). This concentration-dependent modulation of AChE activity is in line with the high binding affinities predicted by the docking studies. At low concentrations, the stabilization of residues within the catalytic triad and anionic site may promote a more favourable active-site conformation, enhancing catalytic efficiency. Conversely, at higher concentrations, excessive target occupancy or subtle

conformational constraints may impair enzyme turnover, resulting in reduced activity. Functionally, enhanced AChE activity at low concentrations may accelerate acetylcholine hydrolysis, reducing neurotransmitter availability and potentially disrupting cholinergic signalling pathways. Given the known role of cholinergic regulation in byssal thread production in mussels, such modulation could impair attachment processes and settlement efficiency, providing a plausible mechanistic link to the observed AF activity at concentrations close to the EC_{50} values. From a structure–activity relationship perspective, the similar enzymatic responses produced by **1'** and **2'** suggest that their effects on AChE activity are not primarily driven by the presence or absence of the amino group in the purine moiety. This interpretation is further supported by comparable biphasic responses previously reported for **1** and **2**, reinforcing the notion that nucleoside-based structures can act as modulators rather than classical inhibitors of AChE. However, there is limited information about the effects of AChE activity modulation in mussels, since most existing studies focus on enzyme inhibition rather than activation (D. Pereira, Palmeira, *et al.*, 2024; S. Pereira *et al.*, 2024), restricting the interpretation of these early findings. Considering this, future studies should focus on validating the significance of the effect of these nucleosides on the AChE activity, through *in vivo* assays on exposed mussel plantigrades.

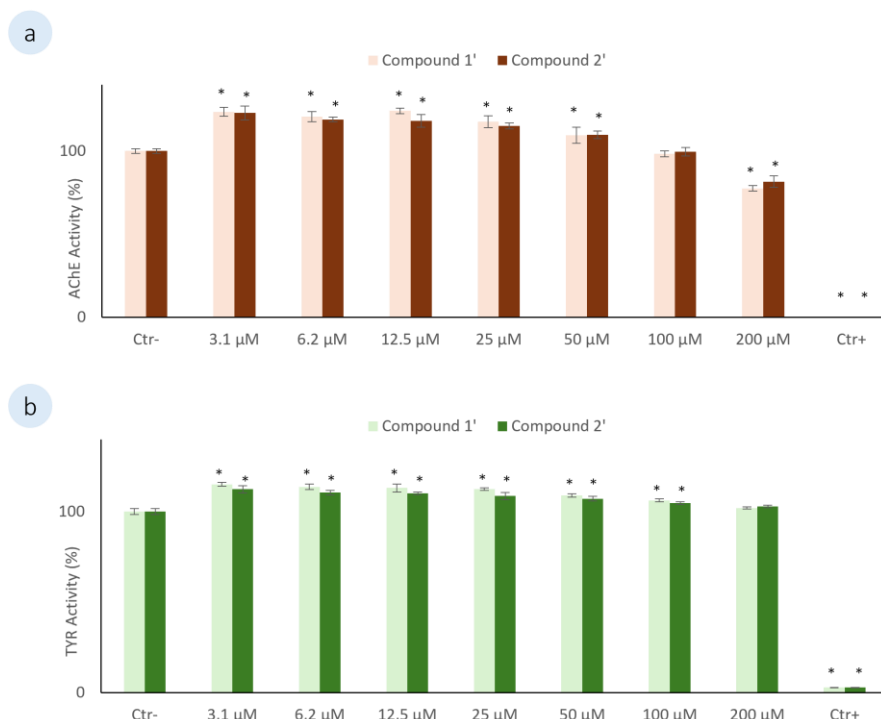


Figure 21. In vitro enzymatic activity assays of **1'** and **2'**. (a) AChE activity. Ctr-: DMSO (0.2%); Ctr+: Eserine (200 μ M). (b) TYR activity. Ctr-: DMSO (0.2%); Ctr+: Kojic acid (200 μ M). *Significant differences compared to the negative control (Dunnett's test, $p < 0.01$).

TYR is another well-known molecular target for investigating the effects of AF agents. This enzyme plays a pivotal role in mussel adhesion by catalysing two key reactions: the oxidation of tyrosine residues to reactive DOPA residues in mussel foot protein-1 within the byssal plaques, and the conversion of DOPA to o-quinone, which promotes the curing and hardening of byssal threads. Both processes are essential for strong attachment (Silverman & Roberto, 2007). TYR is also involved in various physiological processes, including immune responses, cuticle sclerotization, wound healing, and shell biomineralization (Miglioli *et al.*, 2019). To evaluate the effects of compounds **1'** and **2'** on TYR, *in vitro* assays were conducted at concentrations of 25 μ M and 50 μ M, with four replicates per treatment. An initial screening revealed a significant increase in TYR activity following exposure to both compounds (Dunnett's test, $p < 0.01$). A broader concentration range (3.1–200 μ M) was then tested (Figure 21.b), showing a significant 15% increase in TYR activity at lower concentrations (Dunnett's test, $p < 0.01$). However, no significant effects were observed at higher concentrations (Dunnett's test, $p > 0.01$). These findings align with previous observations by Almeida *et al.*, 2020, in which a nature-inspired xanthone also enhanced TYR activity *in vitro*. The authors suggested that this may be linked to the upregulation of immune responses upon exposure or to increased demand for proteins involved in cytoskeletal dynamics and adhesive structure formation. Supporting this hypothesis, the proteomic analysis in the present study revealed changes in the expression of cytoskeleton-associated and TYR-related proteins, particularly in response to compound **1'**. Interestingly, this outcome contrasts with previous results for compounds **1** and **2**, which did not induce any changes in TYR activity, suggesting that minor structural modifications in nucleosides may differentially influence enzymatic pathways involved in mussel adhesion.

4.3.2.2. Mussel plantigrades' untargeted proteome analysis

The action of **1'** and **2'** on mussel plantigrades was investigated using shotgun proteomics to ascertain the effects on the AF mechanisms. The mussel plantigrades were exposed to 12.5 μ M of each compound, and a control group (0.1% DMSO) also included in the analysis. During the DEP analysis, proteomic data missing values were filtered and only proteins identified in all replicates of at least one condition were considered. A list of all the DEPs identified for both compounds is described in the Supplementary Table S5 and Supplementary Table S6. The proteomic data analysis of *M. galloprovincialis* plantigrades exposed to both compounds were represented in the principal component analysis (PCA), Venn diagrams, volcano plots and heatmaps (Figure 22 e Figure 23). The PCA shows the variations of the proteins between the control and the exposed groups, as well as the variations among replicates. Furthermore,

volcano plots highlight the differential expression of proteins in the mussel plantigrades in response to the compounds, when compared to the control ($\log_2FC = \log_2(1.5)$). In the heatmaps, the rows represent the altered proteins and their relative expression levels under different conditions (control and 12.5 μ M for **1'** and **2'**). The heatmap columns correspond to the three experimental conditions, and the colour gradient indicates variations in protein expression across different conditions.

For compound **1'**, proteomic analysis identified a total of 1562 proteins grouped into 1267 clusters. Out of the 1267 proteins, 622 were present in all replicates of at least one condition and, therefore, were considered for the analysis. A comparison between the exposed groups and the control group identified a total of 512 differentially expressed proteins (DEPs). The PCA (Figure 22.a) of the 622 proteins included in the analysis, revealed a distinct separation between the control and the exposed group of compound **1'**. Although some variability could be observed among the replicates within the exposed group. The expression profile of the mussel plantigrades proteins altered after exposure to **1'**, showed a prevalence of downregulated DEPs, as illustrated in the volcano plot (Figure 22.b). Furthermore, the volcano plot also highlighted two proteins with most altered expression, enolase (A0A8S3VIN2) with a $FC = -6.64$ and von Willbrand factor D (vWFD) domain-containing protein (A0A8B6G6F1) with a $FC = 14.2$. Among the DEPs were also identified other von Willebrand factors (vWF), epidermal growth factor-like (EGF) domains and TYR. Exposure to compound **1'** lead to the downregulation of three EGF-like forms (A0A8B6CPN1, A0A8B6DVI4, A0A8S3RV51), and TYR (EC 1.14.18.1) (A0A8B6FHC2). As mentioned before, **1'** upregulated one form of vWFD (A0A8B6G6F1), and altered the expression of two forms of vWFA (A0A8B6BU43, A0A8B6C9L4). Furthermore, a manual search was conducted on the DEPs for proteins associated with mussel adhesion, specifically mussel foot proteins, precollagens, and proximal thread matrix proteins. Nonetheless, none of the described proteins directly involved in the adhesion process were found among the DEPs.

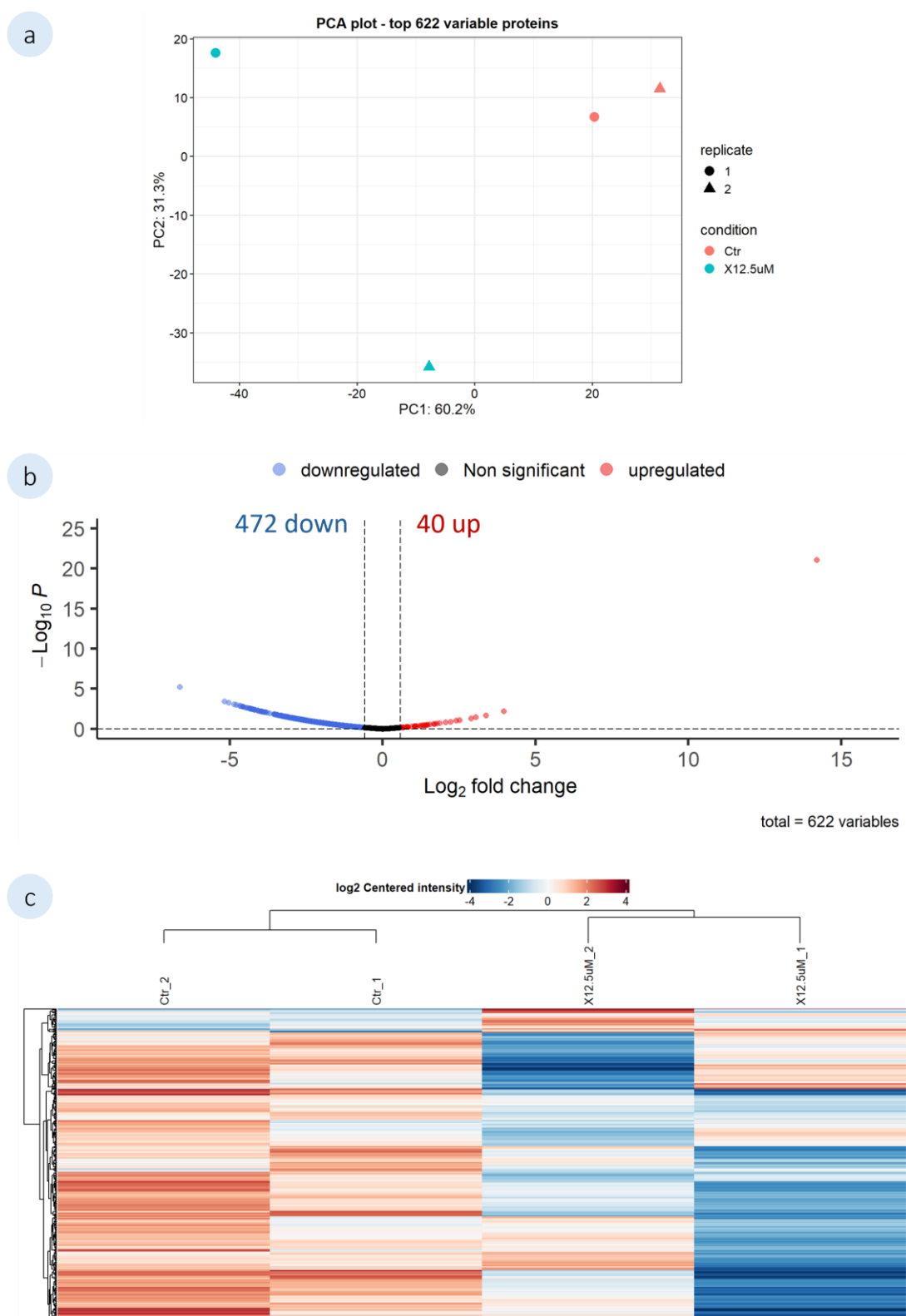


Figure 22. Proteomic analysis of *M. galloprovincialis* planigrades exposed to **1**[†]. Analysis was performed by pairwise comparisons between the exposed group (12.5 μ M) and the control (0.1% DMSO). (a) Principal component analysis (PCA) of the 622 proteins included in the procedure; (b) Volcano plot of the DEPs in response to the compounds, when compared to the control ($\log_2 FC = \log_2(1.5)$). Downregulated DEPs are represented by blue dots, and the upregulated by red dots. Non significant proteins are displayed as grey dots; (c) The heatmap illustrates relative expression DEPs, across

treatments (red: upregulated; blue: downregulated). The rows represent the DEPs. The columns represent the tested groups.

A total of 791 proteins grouped into 480 clusters were identified through proteomic analysis of mussel plantigrades exposed to compound **2'**. Out of the 480 proteins, 119 were present in all replicates of at least one condition and, therefore, were considered for the analysis. The comparison between the exposed group and the control identified a total of 68 DEPs. A high variability was observed among the samples, particularly on 12.5 μ M replicates, as seen in PCA which can be further verified by the proximity of replicate 1 from the exposed group to the control replicates on the heatmap (Figure 23.a and Figure 23.c). The volcano plot showed a similar number of proteins upregulated and downregulated by compound **2'** (Figure 23.b). The DEPs identified for **2'** included several proteins, among which were vWF proteins. Results showed the upregulation of one form of vWFA domain-containing protein (A0A8B6BV40). However, as observed for **1'**, no proteins directly involved in the adhesion process were found among the DEPs.

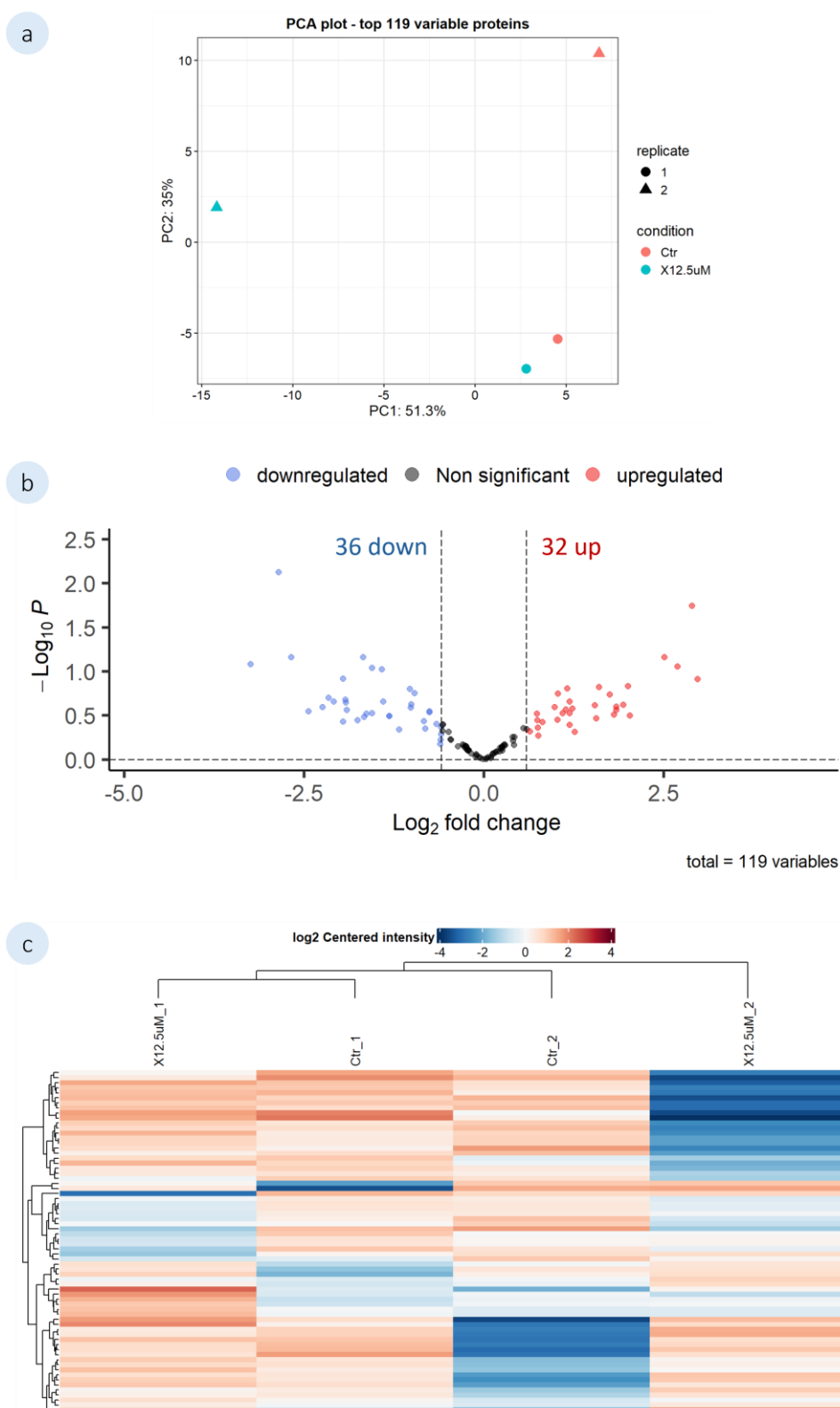


Figure 23. Proteomic analysis of *M. galloprovincialis* plantigrades exposed to **2'**. Analysis was performed by pairwise comparisons between the exposed group (12.5 μ M) and the control (0.1% DMSO). (a) Principal component analysis (PCA) of the 119 proteins included in the procedure; (b) Volcano plot of the DEPs in response to the compounds, when compared to the control ($\log_2 FC = \log_2(1.5)$). Downregulated DEPs are represented by blue dots, and the upregulated by red dots. Non-significant proteins are displayed as grey dots; (c) The heatmap illustrates relative expression of DEPs,

across treatments (red: upregulated; blue: downregulated). The rows represent the DEPs. The columns represent the tested groups.

Among the identified DEPs several were altered following exposure to both compounds. Notably, von Willebrand factor (vWF) domain-containing proteins were modulated by both **1'** and **2'**. Specifically, compound **1'** affected the expression of vWFD and vWFA proteins, while **2'** upregulated vWFA proteins. In vertebrates, vWF is a glycoprotein involved in haemostasis, promoting platelet adhesion at sites of vascular injury (Cruz *et al.*, 1995). It contains four conserved domains: A, B, C, and D (Sun *et al.*, 2002). In mussels, however, vWF domain-containing proteins are believed to contribute to underwater adhesion (J. H. Waite, 2017; Yoo *et al.*, 2016), and shell mineralization (Shimizu *et al.*, 2024). Of particular interest are the two vWFA domains present in proximal thread matrix protein-1 (PTMP1), a non-collagenous matrix protein that plays a central role in the structural integrity of byssal threads (Silverman & Roberto, 2007; Sun *et al.*, 2002). PTMP likely contributes to thread stiffening through interactions between its vWFA domains and collagen (Suhre *et al.*, 2014; Sun *et al.*, 2002; Yoo *et al.*, 2016). Dysregulation of vWF domain-containing proteins upon exposure to the compounds may impair PTMP production, thereby compromising thread strength and potentially inhibiting mussel settlement.

Additionally, certain proteins were downregulated only by compound **1'**, such as EGF-like proteins and TYR. EGF-like domains are known components of mussel foot protein-2 (Mfp-2), a polyphenolic protein found across several mussel species (Hwang *et al.*, 2010; Hwang & Waite, 2012; K. Inoue, Waite, *et al.*, 1995; S. Li *et al.*, 2018; J. H. Waite, 2017). Mfp-2 enhances adhesion resistance to enzymatic degradation and contributes to the stability of byssal plaques (Silverman & Roberto, 2007). According to Hwang *et al.*, 2010, the EGF-like domains in Mfp-2 may also promote cohesion between the plaque and substratum. Therefore, the downregulation of these EGF-like proteins by **1'** could interfere with Mfp-2 production, potentially weakening plaque adhesion. TYR, as previously discussed, is a key enzyme involved in multiple physiological processes, including mussel adhesion. Its downregulation may reduce DOPA availability, thus impairing byssal thread curing and settlement. However, given that TYR activity was found to increase *in vitro* at the same concentrations, this may reflect a compensatory response by mussel plantigrades to elevated enzymatic activity. It is important to note that the enzymatic and proteomic approaches are complementary but not directly comparable. Interestingly, although both compounds induced similar increases in the *in vitro* TYR activity, TYR was not differentially expressed in the proteome of mussels

exposed to compound **2'**. This discrepancy may suggest compound-specific regulatory effects at the transcriptional or post-translational level.

In summary, modulation of vWF domain-containing proteins was shared between **1'** and **2'**, whereas **1'** additionally affected EGF-like proteins. These proteins can be considered potential effectors of their AF activity on mussel plantigrades, as both are key constituents of adhesive proteins (PTMP and Mfp-2, respectively). Their modulation may therefore indirectly interfere with byssal thread formation and adhesion, supporting a putative mechanistic basis for the AF activity of **1'** and **2'**. In addition, TYR was identified as a DEP exclusively in samples exposed to **1'**. Collectively, these findings suggest that the structural modifications in **1'** and **2'** enhance their biological potency, enabling measurable proteomic disruption at lower concentrations. The results reinforce a structure–activity relationship in which subtle chemical substitutions modulate the specificity of AF mechanisms. However, a direct comparison of the molecular AF effects between carbonyl-substituted nucleosides (**1'** and **2'**) and their amino-substituted counterparts (**1** and **2**) is constrained by limitations in the proteomic datasets of the latter compounds. Specifically, compound **1** did not yield detectable DEPs at 12.5 µM, likely due to high biological variability, while proteomic analysis of compound **2** was conducted at a substantially higher concentration (50 µM versus 12.5 µM for **1'** and **2'**), limiting direct quantitative comparisons.

4.3.2.3. Functional enrichment analysis

A functional enrichment analysis was performed on the identified DEPs in *M. galloprovincialis* plantigrades exposed to **1'** and **2'**, using homologous sequences from *Magallana gigas* (formerly *Crassostrea gigas*) due to the absence of an annotated genome for this non-model organism in current g:Profiler databases. This approach enabled exploration of common molecular functions and biological processes impacted by compound exposure. The results of the BLAST analysis for both compounds are presented in the Supplementary Table S7 and Supplementary Table S8. Homologous proteins were classified using gene ontology (GO) terminology and grouped according to their functions (see Supplementary Table S9 and Supplementary Table S10), using the g:OST function.

The resulting functional network for **1'** is illustrated in Figure 24. The analysis yielded 257 GO terms that describe the functions of the differentially expressed proteins. Several proteins were found associated to ribosome constituents (e.g. Small ribosomal subunit protein uS15 (A0A8W8N791), Large ribosomal subunit protein uL13 (A0A8W8J403), 60S ribosomal protein L7a (A0A8W8L109), RRM domain-containing protein

(A0A8W8J5R5), 40S ribosomal protein S3 (A0A8W8LEA1), Ribosomal protein L37 (A0A8W8IQU1), Small nuclear ribonucleoprotein Sm D2 (A0A8W8IE99), and 60 kDa SS-A/Ro ribonucleoprotein (K1QRQ0)), whereas other proteins were associated to cytoskeleton (e.g. Villin-1 (K1RGK4), Alpha-actinin, sarcomeric (K1RH58), Filamin-A (A0A8W8J8A2), Actin-depolymerizing factor 6 (D7EZG8), Fascin (K1QEZ3), Myosin-VI (K1Q1S3), Vinculin (A0A8W8MX28), and Collagen alpha-1(IV) chain (K1R1A5). Among these, were proteins involved in cilium movement (e.g. Tektin (A0A8W8IGH4), Radial spoke head protein 4-like protein A (A0A8W8IBB4), and Cilia- and flagella-associated protein 52 (K1RDT2)). Proteins putatively involved in ATP metabolism (e.g. ATP synthase subunit beta (A0A8W8MB85), 2-oxoglutarate dehydrogenase, mitochondrial (A0A8W8HLX2), Citrate synthase (A0A8W8MWR9), Cytochrome b-c1 complex subunit 6, mitochondrial (K1QKV1), Cytochrome c oxidase subunit 5B, mitochondrial (K1PZT2), Isocitrate dehydrogenase (A0A8W8HL81), Malate dehydrogenase (Fragment) (K1PU26), and Succinate dehydrogenase (A0A8W8N2K5)) and oxidoreductase activity (e.g. Acyl-coenzyme A oxidase (A0A8W8KG37), glutamate dehydrogenase (K1QRQ2), Glutathione S-transferase omega (A0A8W8K6J4), Heat shock 70 kDa protein 4L (A0A8W8KS31), Peroxidase (A0A8W8J0I5), Sulfide:quinone oxidoreductase, mitochondrial (K1QTY5), and Thioredoxin (A0A8W8L209)) were also identified.

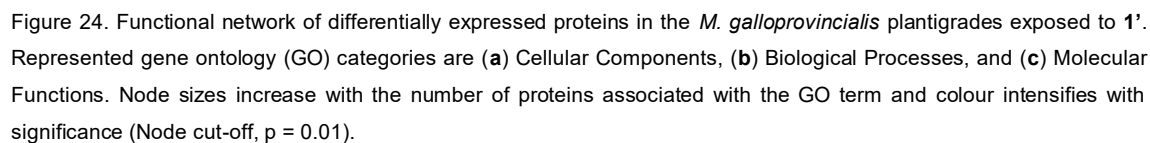


Figure 25 illustrates the functional enrichment of the mussel plantigrades exposed to compound **2'**. This analysis identified 140 GO terms associated with the functions of DEPs. Most of the identified proteins affected by **2'** have shown to be related to ribosome constituents (e.g. Histone H2B (K1R2L4), Large ribosomal subunit protein P1 (A0A8W8N7H9), Lamin Dm0 (K1RFA3), Ubiquitin-ribosomal protein eL40 fusion protein (K1PD36), 40S ribosomal protein S26 (A0A8W8LH25), Small ribosomal subunit protein uS2 (A0A8W8MQU1), and 60S acidic ribosomal protein P0 (K1QWX2)) and ATP metabolism (e.g. ATP synthase subunit gamma, mitochondrial (A0A8W8LM86), Nucleoside diphosphate kinase (K1RHA5), ATP-dependent 6-phosphofructokinase (A0A8W8NW87), Citrate synthase (A0A8W8MWR9), Cytochrome b-c1 complex subunit 6, mitochondrial (K1QKV1), Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial (A0A8W8I7C9), Glucose-6-phosphate isomerase (K1PTI6), and Malate dehydrogenase, mitochondrial (A0A8W8K8K1)).



Figure 25. Functional network of differentially expressed proteins in the *M. galloprovincialis* plantigrades exposed to **2'**. Represented gene ontology (GO) categories are (a) Cellular Components, (b) Biological Processes, and (c) Molecular Functions. Node sizes increase with the number of proteins associated with the GO term and colour intensifies with significance (Node cut-off $p = 0.01$).

The analysis revealed a shared stress response to both compounds, with altered expression of proteins involved in metabolism, cell structure, and cellular stress responses. ATP metabolism was notably affected by both compounds. Compound **1'** downregulated key enzymes involved in ATP production, such as ATP synthase, citrate synthase, 2-oxoglutarate dehydrogenase, and malate dehydrogenase. In contrast, compound **2'** modulated their expression. Enzymes like citrate synthase, 2-oxoglutarate dehydrogenase, and malate dehydrogenase are essential for the citric acid cycle (Krebs cycle), generating electron carriers necessary for the mitochondrial electron transport chain (Osellame *et al.*, 2012). ATP synthase, a critical component of the electron transport chain on the inner mitochondrial membrane, facilitates ATP synthesis through oxidative phosphorylation (Osellame *et al.*, 2012). The downregulation of these proteins by **1'**, and the dysregulation observed with **2'**, suggests potential compensation mechanisms in response to mitochondrial dysfunction, increased oxidative stress, and metabolic dysregulation. Additionally, ciliary motility appeared to be affected by compound **1'**, which downregulated cilia-related proteins such as tektins and radial spoke proteins. Radial spoke proteins are involved in regulating ciliary motility (Grossman-haham *et al.*, 2021), while tektins are structural components vital for the ciliary microtubule cytoskeleton. In mussels, cilia are essential for directing water flow for respiration and feeding (Cannuel *et al.*, 2009; DRAL, 1966), control the biointerface strength and stem release of the byssus (Sivasundarampillai *et al.*, 2023). Furthermore, in *Limnoperna fortune* foot, cilia have been suggested to assist in temporary adhesion (Andrade *et al.*, 2015). Therefore, the downregulation of cilia-related proteins may indicate a defensive response of mussels to the compound, potentially affecting their overall health and physiological processes.

Taken together, these findings indicate that the AF activity of the tested compounds likely arises from a multifaceted mode of action involving both metabolic disruption and impairment of structural and adhesion-related functions. The observed interference with key components of energy metabolism suggests a reduction in ATP availability, which may compromise essential cellular processes and limit the energetic capacity required for active behaviours such as attachment, byssus production, and maintenance of cellular homeostasis. Concurrently, the downregulation of proteins associated with ciliary structure and function points to a direct impact on motility and surface interaction mechanisms, which are critical for effective substrate colonization and stable adhesion in fouling organisms. Importantly, these combined effects may not necessarily induce acute toxicity but rather weaken the organism's physiological performance and its ability to successfully attach and persist on surfaces. This mode of action is particularly relevant

in the context of environmentally friendly AF strategies, as it suggests a non-lethal interference with key biological processes rather than broad-spectrum toxicity. Overall, the proteomic alterations provide valuable mechanistic insight, highlighting how targeted disruption of energy metabolism and adhesion-related pathways can synergistically contribute to AF efficacy at the molecular level.

4.3.3. Predicted environmental behaviour of the compounds

4.3.3.1. Toxicity assessment on target and non-target organisms

In addition to effectively preventing the attachment and growth of fouling organisms, candidate AF agents must display a favourable environmental safety profile. Accordingly, the toxicity of compounds **1'** and **2'** was evaluated in both target and non-target species (Table 11). A therapeutic ratio (LC_{50}/EC_{50}) greater than 15 was used as a benchmark to assess efficacy relative to toxicity in mussel plantigrades (Dan Rittschof *et al.*, 2003). For target organisms, including marine biofilm-forming bacteria, microalgae, and mussel plantigrades, no lethal effects were observed following exposure to either compound across the tested concentration ranges. In *Navicula* sp., although significant growth inhibition was detected, no evidence of cellular disruption or mortality was observed at any concentration. Consequently, LC_{50} values exceeding 200 μM were obtained for both compounds (**1'**: $LC_{50} > 200 \mu M$; $> 53.6 \mu g mL^{-1}$; **2'**: $LC_{50} > 200 \mu M$; $> 50.4 \mu g mL^{-1}$), confirming their low toxicity towards this diatom. Similarly, mussel plantigrades exhibited no lethal effects upon exposure to either nucleoside, with LC_{50} values above 200 μM and therapeutic ratios well above the established threshold ($LC_{50}/EC_{50} > 33.99$ for **1'** and > 23.59 for **2'**). From a structure–activity relationship perspective, this non-toxic profile mirrors that previously reported for **1** and **2** (Table 12), indicating that substitution of the amino group by a carbonyl group within the purine moiety does not compromise environmental safety. This suggests that the conserved nucleoside scaffold, rather than specific substituents on the purine ring, is the dominant determinant of low toxicity.

Table 12. Comparative toxicity of compounds **1'** and **2'** with **1** and **2** against target and non-target organisms. The median lethal concentration (LC₅₀) is represented in both μM and $\mu\text{g mL}^{-1}$ for comparative purposes. **1'**: hypoxanthine arabinoside; **1**: adenosine; **2'**: 2'-deoxyinosine; **2**: 2'-deoxyadenosine. *Results previously described (S. Pereira *et al.*, 2025).

Compounds	LC ₅₀ (μM ; $\mu\text{g mL}^{-1}$)				
	Target species			Non-target species	
	<i>Marine biofilm-forming bacteria</i>	<i>Navicula</i> sp.	<i>M. galloprovincialis</i>	<i>A. salina</i>	<i>A. amphithrite</i>
1'	> 50; 13.40	> 200; > 53.60	> 200; > 53.60	> 50; 13.40	> 32; > 8
2'	> 50; 12.60	> 200; > 50.40	> 200; > 50.40	> 50; 12.60	> 32; > 8
1*	> 50; 13.36	> 200; 53.45	> 200; 53.45	> 50; 13.36	> 32; > 8
2*	> 50; 12.56	> 200; 50.25	> 200; 50.25	> 50; 12.56	> 32; > 8

To further investigate whether the observed settlement inhibition reflected acute toxicity or reversible biological effects, settlement recovery assays were performed using concentrations spanning the EC₅₀ values (3.1–12.5 μM for **1'** and 6.2–25 μM for **2'**). Initial exposure resulted in significant settlement inhibition at the higher concentrations for both compounds (Dunnett's test, $p < 0.01$; Figure 26). However, following transfer to clean filtered natural seawater, settlement levels were fully restored, with no significant differences relative to the control (Dunnett's test, $p < 0.01$). Notably, settlement increased by approximately 30% for **1'** and 40% for **2'** at the higher concentrations. These results indicate the absence of acute toxicity and suggest that settlement inhibition is mediated by a reversible mechanism, consistent with previous observations for cyanobacterial portoamides (Antunes *et al.*, 2019). Such reversibility further supports a mode of action based on temporary physiological modulation rather than irreversible cellular damage.

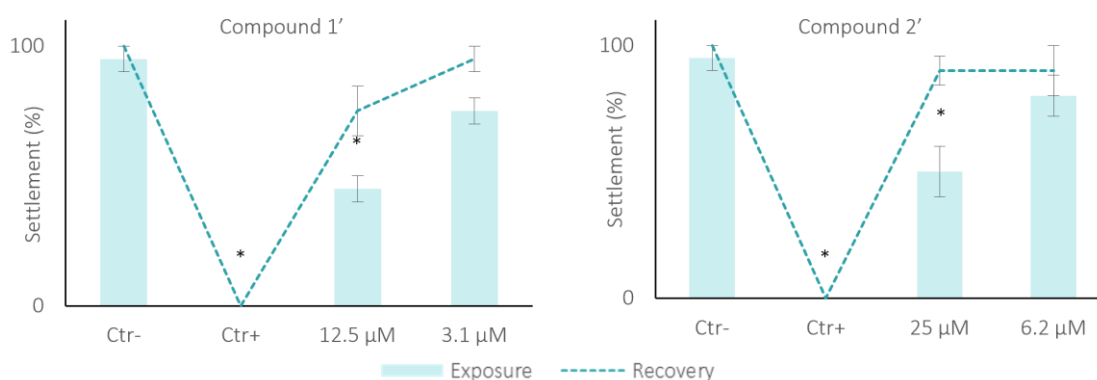


Figure 26. Settlement recovery bioassay of **1'** and **2'**. Compound **1'** was assessed at concentrations of 3.1 and 12.5 μM . Compound **2'** was tested at concentrations of 6.2 and 25 μM . Ctr-: DMSO (0.1%). Ctr+: CuSO₄ (5 μM). *Significant differences compared to the negative control (Dunnett's test, $p < 0.01$).

The effects of **1'** and **2'** on non-target organisms were also assessed using nauplii of the marine brine shrimp *A. salina* and the barnacle *A. amphitrite*. No mortality was observed in *A. amphitrite* nauplii across the tested concentration range (0.5–32 μM ; 0.125–8 $\mu\text{g mL}^{-1}$) or in *A. salina* nauplii at 25 and 50 μM (Dunnett's test, $p > 0.01$) for either compound. These results support LC_{50} values greater than 32 μM for *A. amphitrite* and greater than 50 μM for *A. salina*, both well above the EC_{50} values determined for mussel plantigrades. Comparable toxicity thresholds were previously reported for compounds **1** and **2** in the same non-target species, further reinforcing the conclusion that nucleoside-based AF agents combine biological efficacy with a high degree of environmental compatibility.

4.3.3.2. Persistence, Bioaccumulation and Adsorption Predictions

As complementary information to the ecotoxicological assessment, the physicochemical properties and predicted environmental fate of the four nucleosides (**1**, **2**, **1'**, and **2'**) were estimated using EPI Suite™ (Table 13). The predicted octanol–water partition coefficients ($\text{Log } K_{ow}$) for **1'** and **2'** were well below the threshold of 4.5 established by (ECHA, 2017), indicating a low potential for bioaccumulation and sorption to organic matter. Notably, both compounds exhibited negative $\text{Log } K_{ow}$, reflecting high water solubility, which is consistent with their overall polar nucleoside structures. Predicted soil adsorption coefficients ($\text{Log } K_{oc}$) were also negative for all compounds, with values of -1.00 and -0.79 for **1'** and **2'**, respectively, further supporting low affinity for soils and sediments and, consequently, low environmental persistence in benthic compartments. Similar trends were observed for **1** and **2**, indicating that modifications at the purine 6-position (amino *versus* carbonyl group) do not substantially alter sorption behaviour, reinforcing a consistent SAR across the nucleoside series. Bioconcentration factors (BCF) were low for **1'** and **2'** ($\text{BCF} = 3.16 \text{ L kg}^{-1}$), remaining several orders of magnitude below the REACH Annex XIII threshold for bioaccumulative substances ($\text{BCF} \geq 2000 \text{ L kg}^{-1}$; (ECHA, 2017), which was in accordance with the observed for **1** and **2**. These values confirm the negligible potential for bioconcentration in aquatic organisms. Biodegradation potential was assessed using BOWIN models, revealing distinct SAR trends linked to functional group substitutions. Compounds **1'** and **2'** were predicted to undergo rapid aerobic biodegradation (within days), whereas **1** and **2** were predicted to degrade over slightly longer timescales (days to weeks). This enhanced aerobic biodegradability of **1'** and **2'** may be associated with the presence of a carbonyl group at the 6-position of the purine ring, which could facilitate enzymatic attack. Under anaerobic conditions, compounds **1'** and **1** were predicted to degrade rapidly, while **2'** and **2** were not, suggesting that the presence of the 2'-hydroxyl group may play a role in degradation

pathways under oxygen-limited conditions. Collectively, these predictions highlight how subtle structural differences within the nucleoside framework can modulate degradation behaviour without compromising overall environmental safety. Taken together, the negative Log K_{ow} and Log K_{oc} values indicate high mobility in aquatic environments; however, the rapid predicted biodegradation suggests a short environmental residence time, particularly in biological systems. Consequently, **1'** and **2'** present a low concern in terms of persistence, bioaccumulation, and long-term ecological impact. While expanding ecotoxicological testing to additional non-target species would further strengthen risk assessment, the predicted PBT profiles strongly support the suitability of these nucleosides as environmentally compatible antifouling agents for marine coating applications.

Table 13. Predicted data of **1'** and **2'** from EPI Suite™. Includes biodegradability, sediment-water partition (Log K_{oc}), octanol-water partition (Log K_{ow}), and bioconcentration factor (BCF) of **1'** (hypoxanthine arabinoside) and **2'** (2'-deoxyinosine). BIOWIN™ criteria: (3) predicted time for ultimate biodegradation; (4) primary biodegradation in aerobic conditions; (5) MITI linear model prediction; and (7) predicted probability for fast biodegradation in anaerobic conditions. Results for (3) and (4): values 5.00 = hours, 4.00 = days, 3.00 = weeks, 2.00 = months, 1.00 = recalcitrant. Results for (5) and (7): values < 0.5 = does not biodegrade fast. ^aRB: readily biodegradable; ^bBF: Biodegrades Fast; ^cDNBF: Does not biodegrade fast.

Compound	BIOWIN™				KOCWIN™	KOWWIN™	BCF™
	Ultimate biodegradation (3)	Aerobic conditions (4)	MITI Linear Model (5)	Anaerobic conditions (7)	Log K_{oc}	Log K_{ow}	BCF (LKg/wet- wt)
1'	Weeks (3.02)	Days (4.04)	RB ^a (0.64)	BF ^b (0.57)	-1.00	-1.84	3.16
2'	Weeks (2.90)	Days (3.94)	RB ^a (0.59)	DNBF ^c (0.24)	-0.79	-1.18	3.16

4.4. Conclusion

Two nucleoside analogues, hypoxanthine arabinoside (**1'**) and 2'-deoxyinosine (**2'**), derived from the AF-active nucleosides adenosine (**1**) and 2'-deoxyadenosine (**2**), were investigated to elucidate the influence of substituting the amino group on the purine base with a carbonyl group on AF performance and environmental behaviour. The analogues were assessed for AF efficacy against representative micro- and macrofouling organisms, molecular mechanisms of action in *M. galloprovincialis* plantigrades, and ecotoxicological effects and environmental fate.

Both compounds retained AF efficacy, significantly inhibiting diatom growth and mussel plantigrade settlement, without inducing lethality in target fouling organisms. Neither

compound affected marine biofilm-forming bacteria, indicating that the structural modification preserved the selectivity of nucleosides toward eukaryotic fouling taxa. Mechanistic studies revealed modulation of AChE and TYR activity *in vitro*, suggesting involvement of neurotransmission and byssal thread formation-related pathways in the observed AF effects. Molecular docking studies confirmed high binding affinity to AChE, while TYR modulation – absent for compounds **1** and **2** – appears to be associated with the carbonyl substitution on the purine base.

Proteomic analysis of exposed mussel plantigrades revealed modulation of vWF domain-containing proteins for both analogues, together with compound-specific molecular responses. Compound **1'** additionally induced downregulation of EGF-like proteins and TYR, whereas **2'** selectively upregulated vWFA proteins. Functional enrichment analysis further indicated shared effects on ATP metabolism, while **1'** exerting additional influence on proteins associated with ciliary function and oxidoreductase activity. These results demonstrate that while overall AF efficacy is preserved, subtle chemical modifications can alter the magnitude and specificity of molecular responses. From an ecotoxicological perspective, both compounds exhibited a favourable environmental profile. No lethal effects were observed in non-target species (*A. salina* and *A. amphitrite* nauplii), and environmental fate modelling predicted low persistence, bioaccumulation, and bioconcentration potential, combined with high water solubility and rapid degradation. This benign profile closely mirrors that of the parent nucleosides, indicating that carbonyl substitution does not compromise environmental safety.

Overall, this study demonstrates that nucleoside-mediated AF activity is not dependent on the presence of an amino functional group on the purine base, although structural modifications can influence the underlying molecular mechanisms. By integrating chemical structure–activity relationships with mechanistic and ecotoxicological endpoints, these findings support nucleosides as a promising class of environmentally compatible AF agents and provide a framework for their further development as low-impact alternatives to conventional biocidal coatings.

Author contributions

S.P.: Formal analysis, investigation, methodology, writing – original draft. I.B.O.: Investigation, methodology, writing – review & editing. A.P.: Investigation, methodology, validation, writing – review & editing. M.T.: Investigation, methodology, validation, writing – review & editing. V.V.: Supervision, resources, writing – review & editing. A.C.: Supervision, validation, resources, writing – review & editing. J.R.A.: Conceptualization,

funding acquisition, project administration, validation, resources, supervision, writing – review & editing.

Conflicts of interest

The authors declare no conflicts of interest.

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Declaration of competing interest

The authors declare no conflicts of interest.

5. Antifouling activity and ecotoxicological profile of the cyanobacterial oxadiazine Nocuolin A

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Sandra Pereira performed *M. galloprovincialis*, *Navicula* sp., marine biofilm-forming bacterial strains, and *Artemia salina* bioassays, enzymatic assays, FASP, protein identification, quantification and functional classification as well as *in silico* environmental predictions, all conducted at CIIMAR.

The *Amphibalanus Amphitrite* bioassay and zebrafish embryo acute toxicity test (ZFET), as well as the prediction of environmental concentrations were performed by Isabel Oliveira at CIIMAR.

Isolation of the nocuolin A was performed by Maria Lígia Sousa and Marco Preto at CIIMAR.

Initial screening was conducted by Catarina Gonçalves at CIIMAR.

Acetylcholinesterase docking studies were conducted by Andreia Palmeira at LQOF/FFUP.

Peptides LC-MS/MS analysis was conducted by Maria Turkina at BKV/MEDFAK.LiU.

Antifouling activity and ecotoxicological profile of the cyanobacterial oxadiazine Nocuolin A

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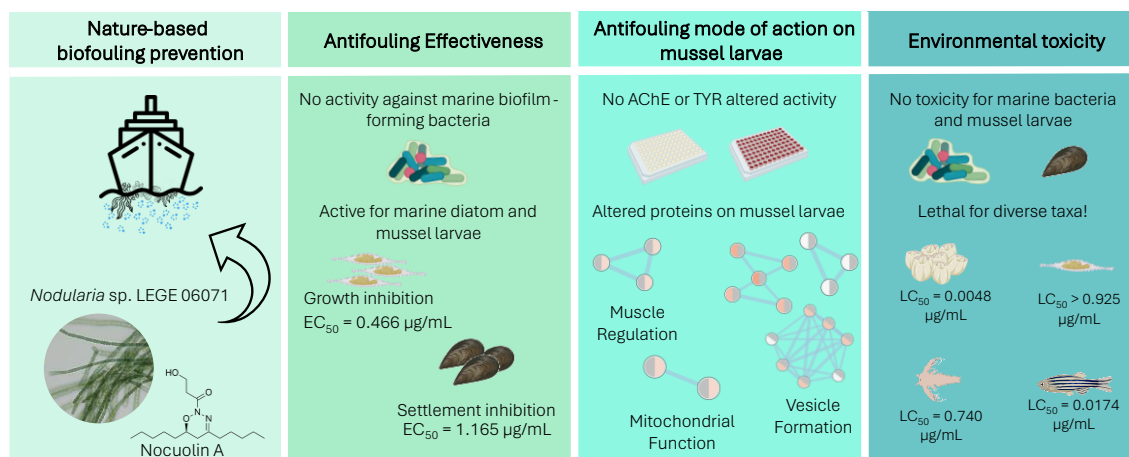
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Abstract

Pursuing effective and biocompatible natural compounds to supplant current biocidal AF technologies remains crucial and challenging. Among natural product hosts, cyanobacteria are recognized as producers of bioactive secondary metabolites that are underexplored in terms of anti-biofilm and AF potential. Nocuolin A, a natural oxadiazine previously isolated and known to be produced by different cyanobacterial strains, has demonstrated bioactive potential, particularly against tumour cell lines. Considering this potential and its exquisite chemical structure, here nocuolin A was investigated as a potential natural AF agent through an integrative approach including AF bioactivity testing across distinct levels of biological organization, mode of action assessment, ecotoxicity evaluation, and ecological risk predictions. Nocuolin A was found to inhibit the settlement of mussel plantigrades of *Mytilus galloprovincialis* ($EC_{50} = 3.905 \mu M$) while showing no toxicity to this biofouling species ($LC_{50} > 100 \mu M$). Additionally, while exhibiting no inhibitory activity against the growth of five marine biofilm-forming bacterial strains, it significantly suppressed the growth of the marine biofilm-forming diatom *Navicula* sp. ($EC_{50} = 1.561 \mu M$) and had a lethal effect on this diatom species ($> 3.1 \mu M$). The AF targets of nocuolin A on mussel plantigrades revealed no correlation with acetylcholinesterase and tyrosinase metabolic processes; however, proteins involved in oxidative stress, muscle regulation, and energy production were highlighted. The results also provide insights into the ecological risk of nocuolin A, including its ecotoxicity against *Artemia salina* nauplii ($LC_{50} = 2.480 \mu M$), *Amphibalanus amphitrite* nauplii ($LC_{50} = 0.0162 \mu M$), and *Danio rerio* embryos ($LC_{50} = 0.0584 \mu M$). When matching these results with

simulated environmental values, nocuolin A was deemed a considerable threat to the ecosystems. While this research highlights the AF activity of nocuolin A, it also emphasizes the potential adverse environmental impact when applied in preventive coatings.

Graphical Abstract



Keywords

Biofouling, Antifouling, Natural products, Cyanobacteria, Nocuolin A, Environmental Compatibility

5.1. Introduction

Marine biofouling is the natural process that occurs on all submerged surfaces, characterized by the rapid colonization of organic molecules, and successively a micro and macro biological community. This biological colonization leads to serious problems and requires large investments for the maritime industries, as well as raising environmental concerns due to the AF biocidal agents currently in use as components of marine coatings (Paz-Villarraga *et al.*, 2022; Schultz *et al.*, 2011; Yebra *et al.*, 2004). In compliance with recent EU strategies for sustainability, new effective and environmentally compatible alternatives to biofouling control are needed (European Commission: Deltares *et al.*, 2021). Natural products are believed to have the potential to provide solutions for a wide range of biotechnological applications, including AF, which could be effective and ecologically compatible. Following this premise, several natural antifoulants have been described so far from both terrestrial and marine sources (D. Pereira, Almeida, *et al.*, 2024), however, their environmental impact is often overlooked based on the assumption that they are derived from natural sources.

Among the natural sources, cyanobacteria- a biodiverse group of photosynthetic

prokaryotes- are known to produce a variety of chemically diverse secondary metabolites, encouraging further investment in the bioprospection of this particular group for AF bioactive compounds (Antunes *et al.*, 2019; Brown *et al.*, 2004; Dahms *et al.*, 2006; L. T. Tan *et al.*, 2010). In addition, a better understanding of natural products' AF mode of action and ecotoxicity profile remains necessary, keeping some promising compounds far from being considered for commercial purposes. Among the cyanobacterial-derived compounds discovered so far, nocuolin A, a natural oxadiazine, was originally isolated from the cyanobacterium *Nostoc* sp. CCAP1453/38 and is notable for its unique structure with a six-membered 1,2,3-oxadiazine heterocycle (Figure 27), exhibiting pro-apoptotic properties and a potent antiproliferative activity (0.7 - 4.5 μ M) against several human cancer lines (Voráčková *et al.*, 2017). The compound was later found in three independent cyanobacterial strains of genera *Nostoc*, *Nodularia*, and *Anabaena*, all of which have the enzymes of the *noc* biosynthetic gene cluster for nocuolin A biosynthesis in their genomes (Voracova *et al.*, 2017; Martins *et al.*, 2022). The antiproliferative effect of the compound was further investigated and associated with the weakening of mitochondrial oxidative phosphorylation followed by cell autophagy, after nocuolin A isolation from a cyanobacterial strain *Nodularia* sp. LEGE 06071 (M. L. Sousa *et al.*, 2019).

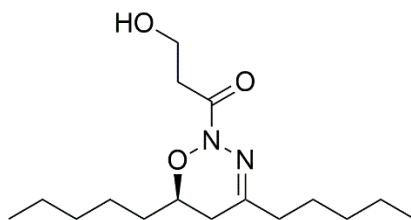


Figure 27. Cyanobacteria-derived compound nocuolin A.

Considering its uniqueness and biotechnological potential, recent studies have been conducted on nocuolin A, including optimization of production parameters (Chmelík *et al.*, 2019), pursuing its natural biosynthetic pathways (Martins *et al.*, 2022), the discovery of new nocuolin A hybrids (Figueiredo *et al.*, 2021), and the synthesis of new nocuolin A semisynthetic analogues (V. T. Pérez *et al.*, 2023). However, the investigation of nocuolin A as a promising biotechnological tool for applications targeting unrelated molecular pathways, such as AF activity, remains unexplored. In light of this, here we aim to unveil nocuolin A's overall potential for use in environmentally compatible biofouling preventive systems, through an integrative approach testing AF efficiency, putative AF molecular targets, and predicted marine ecotoxicity. AF efficiency was evaluated against representative micro and macrofouling organisms. In addition, the

elucidation of molecular targets underlying the AF activity observed in mussel plantigrades was performed, as the understanding of the mechanisms of action associated with AF compounds remains a research challenge. Since biofouling is a complex phenomenon involving a wide range of different organisms, AF compounds are believed to affect colonization/settlement through distinct pathways (Almeida & Vasconcelos, 2015; L. Chen & Qian, 2017). Considering this, *in vitro* tyrosinase and acetylcholinesterase (AChE) enzymatic assays, were performed in the presence of nocuolin A, and a whole organism-untargeted shotgun proteomic approach was used with mussel larvae after exposure to nocuolin A, to assess the most prominent proteins affected. Finally, the environmental toxicity of nocuolin A was investigated in different organisms using lethal and sub-lethal endpoints and *in silico* predictions. The observed multi-organism interactions provided insights into the potential ecological function of nocuolin A within its native cyanobacterial hosts. As seen in other studies on AF compounds (Oliveira *et al.*, 2014, 2017; Wang *et al.*, 2014), the information generated in this study was also used, together with predicted environmental concentration, as a proxy for nocuolin A's putative ecological risk.

5.2. Material and Methods

5.2.1. Nocuolin A Isolation

Nocuolin A was obtained following the method described by Sousa *et al.*, 2019, with minor adjustments. Briefly, the cyanobacterial strain *Nodularia* sp. LEGE 06071, from the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) (<https://lege.ciimar.up.pt/>) (Ramos *et al.*, 2018) was cultured and upscaled under standard conditions. A crude organic methanolic extract with a final dichloromethane extraction step was obtained from the lyophilized biomass of *Nodularia* sp. LEGE 06071 (40 g). The fractionation process was initiated by Vacuum-Liquid Chromatography. The presence of the compound on the fractions was confirmed by LC/MS analysis, and fractions with nocuolin A were pooled together and submitted to flash chromatography with increasing polarity solvents. The chemical profile of the subsequent fractions was compared through thin-layer chromatography, and fractions were pooled according to their profile similarities. After the isolation of nocuolin A (16.4 mg) by high-pressure liquid chromatography, its abundance and purity (>99%, ¹H NMR) were obtained by LC-MS and ¹H nuclear magnetic resonance.

5.2.2. Antifouling bioactivity

5.2.2.1. Settlement inhibition of *M. galloprovincialis* plantigrades bioassay

Small aggregates of juvenile mussels (*M. galloprovincialis*) were collected during low neap tides in intertidal pools, at Memória beach, Matosinhos, Portugal (41° 13' 59" N; 8° 43' 28" W). At the laboratory, samples were screened in a binocular magnifier (Olympus SZX2-ILLT) for mussel post-larvae - plantigrades (0.5 – 2 mm) (Alfaro *et al.*, 2004; Carl *et al.*, 2011). Mussel plantigrades displaying foot exploratory behavior were transferred to a petri dish with filtered seawater and gently cleansed of adhered organic particles before being used in the bioassays. The settlement inhibition of *M. galloprovincialis* plantigrades bioassay followed the description in Almeida *et al.*, 2017. Briefly, active post-larvae mussel plantigrades were exposed to nocuolin A in 24-well microplates with 4 replicates (5 larvae per well) for 15 h, at 18 ± 1 °C, in the darkness. Nocuolin A was screened at 12.5 and 25 µM by diluting the compound stock solution (50 mM in DMSO). Negative (0.1% DMSO) and positive controls (5 µM CuSO₄) were included in bioassays. All tested solutions were prepared in filtered natural seawater. After exposure, the presence/absence of efficiently attached byssal threads produced by each post-larva for all the tested conditions was used to assess the settlement inhibition.

5.2.2.2. Antibacterial and anti-diatom bioassays

The antibacterial activity of nocuolin A was assessed against five strains of marine biofilm-forming bacteria *C. marina* CECT 4278, *V. harveyi* CECT 525, *H. aquamarina* CECT 5000, *P. atlantica* CECT 570, and *R. litoralis* CECT 5395 selected from the Spanish Type Culture Collection (CECT) as described in previous studies (Almeida *et al.*, 2017; Antunes *et al.*, 2019; Resende *et al.*, 2021). After inoculation in Marine Broth (Condalab), bacteria were exposed to nocuolin A in 96 well flatbottom microtiter plates and incubated for 24 h (72 h for *R. litoralis*), at 26 °C. Nocuolin A was screened at 12.5 and 25 µM. Negative (1% DMSO) and positive controls (1:100 penicillin-streptomycin-neomycin stabilized solution (Sigma P4083)) were also included. Four replicates per condition were used. Initial optical density was ensured to be at 0.1 (OD600). Bacterial growth inhibition was determined at 600 nm with a microplate reader (Biotek Synergy HT, Vermont, USA).

Navicula sp. from the Spanish Collection of Algae was selected as a representative of the benthic marine diatoms, for the anti-microalgal screening, performed according to Antunes *et al.*, 2019. Briefly, diatoms, inoculated in F/2 medium (Sigma) with silica (Sigma G9903) at an initial concentration of $2-4 \times 10^4$ cells mL⁻¹, were exposed to nocuolin A, at 12.5 and 25 µM, in 96 well flat-bottom microtiter plates for 10 days, at 20

°C. Negative (1% DMSO) and positive controls (3.55 μ M cycloheximide) were included. Each condition was assessed in quadruplicate. Growth inhibition of diatoms was quantified by the difference in cell densities among conditions.

5.2.3. Antifouling Mechanism of Action

5.2.3.1. *In vitro* AChE inhibition assay

The *in vitro* AChE inhibition assay used Electrophorus electric AChE Type V-S (SIGMA C2888, E.C. 3.1.1.7), as described by Ellman *et al.*, (1961) and modified by Almeida *et al.*, (2015). The reaction solution included 30 mL of phosphate buffer (0.1 M, pH = 7.2), 1 mL of the reagent dithiobisnitrobenzoate (DTNB) at 10 nM (5,5'-Dithiobis(2-nitrobenzoic acid) and sodium hydrogen carbonate dissolved in phosphate buffer at 0.1 M, pH = 7.2) and 200 μ L of acetylthiocholine iodide at 0.075 M. During the assay, 50 μ L of pure AChE (0.25 U mL⁻¹) was added to 250 μ L of the reaction solution, for each condition. Nocuolin A was screened at 25 and 50 μ M. Negative (0.2% DMSO) and positive controls (100 μ M eserine) were included. All conditions were assessed in quadruplicate. The activity of the AChE was detected at 412 nm for 5 minutes at 25 °C.

5.2.3.2. *In vitro* tyrosinase inhibition assay

Tyrosinase inhibition assay was conducted using *Agaricus bisporus* tyrosinase (EC 1.14.18.1) according to Adhikari *et al.*, (2008) with appropriate adaptations (Almeida *et al.*, 2017). Briefly, 50 μ L of tyrosinase (25 U/mL) in phosphate buffer (50 mM, pH 6.5) was added to each condition. Nocuolin A was screened at 12.5 and 25 μ M. The enzymatic activity was initiated by L-dopa (25 mM) also prepared in phosphate buffer (50 mM, pH 6.5). Negative (0.2% DMSO) and positive controls (100 μ M kojic acid) were included. All conditions were assessed in quadruplicate. Tyrosinase activity was detected at 475 nm for 5 minutes at 25 °C.

5.2.3.3. Proteomic profiling of the *M. galloprovincialis* plantigrades

5.2.3.3.1. Sample preparation and protein extraction

M. galloprovincialis plantigrades were exposed to nocuolin A at 5 μ M and 10 μ M as described previously for the settlement inhibition assay. The concentration of 5 μ M was selected to represent a closer concentration to the EC₅₀ value observed for the mussel larvae while the 10 μ M concentration purpose was to explore the differences in the proteomic expression of the larvae when exposed to higher concentrations of nocuolin A. After the exposure, mussel plantigrades from negative control (0.1% DMSO) and tested nocuolin A concentrations (5 μ M and 10 μ M) were collected (10 larvae/replicate, 3 replicates/condition). Protein extraction and subsequent proteome analysis were performed as described by Campos *et al.*, (2016). After defrosting in ice, samples were

solubilized in lysis buffer (composed of 2% (w/v) SDS, 100 mM Tris-HCl at pH 7.6, 0.1 M DTT, and 1:50 protease inhibitor) and incubated at 25 °C in the dark at 500 rpm overnight. After confirming in the stereoscope that all shells were open and empty, protein denaturation was performed for 3 min at 95 °C. The supernatant was collected, and the total protein concentration was estimated using the Bradford method, with diluted samples (1:10).

5.2.3.3.2. Filter-Aided Sample Preparation (FASP)

Proteins were subsequently digested through FASP (Wiśniewski *et al.*, 2009). Entire protein extracts were diluted in 200 µL of UA buffer (8 M urea, in 0.1 M Tris/HCl, pH 8.5) and transferred to filter units (Microcon 10 kDa NMWL, Merck Millipore Ltd., Tullagreen, Ireland) previously washed in water and UA buffer. After centrifugation at 14,000 G for 20 min, 100 µL of 0.05 M iodoacetamide was added to the filter units, mixed at 600 rpm in the dark, for 1 min (Thermomixer compact, Eppendorf, Hamburg, Germany), and incubated for 20 min at room temperature. Filter units were centrifuged at 14,000 G for 20 min, at room temperature, followed by two successive washings with 100 µL UA buffer, with centrifugations, and 100 µL of 0.05 M ammonium bicarbonate. Peptide digestion was initiated by adding recombinant proteomics-grade trypsin (Roche, Mannheim, Germany) at a 1:100 enzyme-to-protein ratio in 0.05 M ammonium bicarbonate to the samples. After mixing for 1 min at room temperature, samples were incubated in a wet chamber at 37 °C for 18 h. Digested peptides were eluted in new tubes by adding 50 µL of 0.5 M NaCl between centrifugations. Before desalting, the peptide concentrations were measured at 280 nm and acidified with 5 µL of formic acid (10% v/v). For peptide desalting were used C18 columns (UptiTip™ C18 Packed C18 10-200 µL, Interchim Innovations, Montluçon, France) and conditioned with acetonitrile (60% v/v) and formic acid (0.1%, v/v), as described in the manufacturer's protocol. The samples were then added to the columns and washed with formic acid (0.1% v/v). The final elution of peptides was performed with acetonitrile (60% v/v) and formic acid (0.1%, v/v) in new tubes. The peptide concentration was measured at a wavelength of 280 nm. The samples were dried in a vacuum concentrator (Concentrator Plus™, Eppendorf).

5.2.3.3.3. LC-MS Analysis

Obtained peptides were analysed by nano-LC coupled to a hybrid Ion trap-Orbitrap mass spectrometer (LTQ Orbitrap Velos Pro, Thermo Scientific, Waltham, EUA). Full scans were performed at 30,000 resolution with scan ranges of 380–2000 m/z and the top 20 most intense ions isolated and fragmented. Collision-induced fragmentation (CID) was used to fragment precursor ions. Charge state deconvolution and deisotoping were not

performed. All MS/MS spectra were analysed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version IseNode in Proteome Discoverer 2.4.0.305). Sequest was set up to search Mollusca 20201001.fasta (unknown version, 365732 entries, UniProtKB database) assuming the digestion enzyme trypsin. Sequest was searched with a fragment ion mass tolerance of 0.020 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine was specified in Sequest as a fixed modification. Oxidation of methionine was specified in Sequest as a variable modification.

5.2.3.3.4. Protein identification and quantification

Scaffold program (version Scaffold_4.11.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identification. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Percolator posterior error probability calculation (Käll *et al.*, 2008). Protein identification was accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides. Protein probabilities were assigned using the Protein Prophet algorithm (Nesvizhskii *et al.*, 2003). Proteins that contained similar peptides and could not be differentiated by MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins that shared significant peptide evidence were grouped into clusters.

5.2.3.3.5. Functional classification of protein and pathways analysis

Obtained protein sequences were compared (blast) against *the Crassostrea gigas* sequences (91.786 sequences, downloaded from UniProt Knowledgebase release 2023_05 (The UniProt Consortium, 2023), and cgigas_uk_roslin_v1 peptide sequences 2023_11, downloaded from Ensembl Metazoa), using the local BLASTp function from Blast2GO version 6.0.3 (basic) (Conesa *et al.*, 2005; Conesa & Götz, 2008; Götz *et al.*, 2008), with a cut-off e-value of 1×10^{-3} . This analysis was required due to the insufficient annotation of the *M. galloprovincialis* protein sequence. A multi-query analysis compared homologous *C. gigas* sequences from both concentrations, grouping them into functional classes (Gene Ontology classes) through the g:OSt function in g:Profiler (version e111_eg58_p18_30541362). Statistically significant enriched biological processes, molecular functions, and cellular components were detected using a hypergeometric test. Results were validated with Benjamini-Hochberg's false discovery rate correction (p-value < 0.05) (Kolberg *et al.*, 2023). A functional network was constructed on Cytoscape version 3.10.2 (Shannon *et al.*, 2003).

5.2.4. Ecotoxicity assessment

5.2.4.1. *Artemia salina* bioassay

The brine shrimp (*A. salina*) nauplii lethality test was used to assess the general marine ecotoxicity of the nocuolin A (Almeida *et al.*, 2017). Nauplii hatched in nutrient-enriched seawater after approximately 24 h at 26 °C. Initially, nocuolin A concentrations of 12.5 and 25 µM were evaluated. The lethality tests were performed with 15-20 nauplii per well, eight replicates per condition, in 96-well microplates in darkness, at 25 °C, for 48 h. A negative (1% DMSO) and a positive control (13.6 µM K₂Cr₂O₇) were included in the assays.

5.2.4.2. *Amphibalanus amphitrite* bioassay

The barnacle *A. amphitrite* was used to perform a nauplii lethality test (Piazza *et al.*, 2012; Rittschof *et al.*, 1992). Adult barnacle broods were brought from the field, cleaned from epibionts, and maintained in the lab, in filtered natural seawater (FNSW) at room temperature (22 ± 5 °C). The broodstock was fed a mixture of microalgae (*T-iso lutea*, *Tetraselmis suecica*, and *Dunaliella tertiolecta*, every day) and newly hatched brine shrimp (*A. salina* every other day) *ad libitum*. Whenever nauplii are needed, adult barnacles are left dried overnight and, the next morning, FNSW at 28 °C was added to the beaker and placed in the dark near a pointed light source to stimulate the release of the nauplii. Nauplii were collected using a Pasteur pipette and transferred to another beaker with FNSW to release them from debris and ensure they were actively swimming. Toxicity tests were conducted in glass tubes containing 3 mL of test solutions (3 replicates) using newly hatched nauplii collected less than 3 h after release from adult barnacles. The assay consisted of adding a drop of these larvae (50 µL, 30–60 larvae) to each test tube and incubating at 28 °C in darkness without feeding for 24 h. Mortality was determined as the proportion of immotile nauplii. Controls and solvent controls (0.1% DMSO) were included in the assays which were repeated twice. Data was combined for statistical analysis.

5.2.4.3. Zebrafish Embryo Acute Toxicity Test (ZFET)

Zebrafish (*Danio rerio*) embryos were used to assess the lethal and sub-lethal effects of nocuolin A. Breeding maintenance and embryo collection was conducted in our certified Bioterium of Aquatic Organisms (BOGA, CIIMAR, Porto). All experiments were conducted following ethical guidelines of the European Union Council (Directive 2010/63/EU) and the Portuguese Ministry of Agriculture, Sea, Environment, and Spatial Planning (Decree-Law nr. 113/2013, 7 August) for the protection of animals used for experimental and other scientific purposes.

Fish embryo exposures were conducted in an E3 medium (pH 8.6) following an adaptation of the OECD guideline (OECD, 2013) using 96-well plates. Briefly, newly fertilized eggs were exposed to a range of nocuolin A concentrations (ten embryos per concentration) from approximately 1 h post-fertilization (hpf) until 96 hpf at 28 °C. Embryos were inspected under a microscope every 24 h for lethal and sub-lethal effects, with solution renewal at each inspection. Indicators of lethality included coagulation of fertilized eggs, absence of heartbeat or somite formation, and failure of tail detachment from the yolk sac. Recorded sub-lethal endpoints included hatching time, tail deformities, presence of heart or yolk sac oedema, and locomotion difficulties. Controls and solvent controls (DMSO 0.1%) were included in the assays. The assay was repeated three times.

5.2.4.4. *In silico* predictions of nocuolin A

Based on the molecular structure of a given substance, it is possible to predict important parameters related to its physicochemical properties and PBT (persistence, bioaccumulation, and toxicity) criteria. This is particularly relevant when little or no information exists. In this study, we used EPISUITE™, a Windows-based suite of programs developed by the U.S. Environmental Protection Agency (EPA's Office of Pollution Prevention Toxics) and Syracuse Research Corporation (SRC), to estimate the physicochemical properties and environmental fate of nocuolin A. Input values included the compound name, molecular weight, chemical formula, and SMILES notation. To distinguish nocuolin A between a baseline toxicant or a specifically toxic substance, we used the Toxic Ratio, which is the ratio between an estimated LC₅₀ (using a QSAR - quantitative structure-activity relationship - a model for baseline toxicity) and the experimental LC₅₀ value for the given species (Maeder *et al.*, 2004). To assess the potential ecological risk of nocuolin A, we used MAMPEC (Marine Antifoulant Model to Predict Environmental Concentrations, Version 3.0; Deltares, Netherlands) to calculate the predicted environmental concentrations (PEC). MAMPEC can predict environmental concentrations by considering different environments (e.g., harbours, marinas), emission scenarios (including leaching rates, underwater surface area, and shipping characteristics), and the antifoulant's physicochemical properties (Van Hattum *et al.*, 2006). For input values, we followed the approach established by the OECD-EU working group for the risk assessment of antifoulants in an OECD commercial harbour environment and emission scenario descriptors (OECD, 2004) without modifications. The compound descriptor input values included the molecular formula and mass, and values estimated with EPISUITE™ (US EPA, 2024), such as solubility, octanol-water partition coefficient (Log K_{OW}), and partition coefficient (Log K_{OC}).

5.2.5. Data analysis

All data were first checked for normality (Kolmogorov–Smirnov test) and homogeneity of variances (Levene’s test), and appropriate transformations were made when necessary. Data from the nocuolin A screening activities across all bioassays were analysed using one-way analysis of variance (ANOVA) followed by Dunnett’s test ($p < 0.01$). ANOVA analysis was also applied to determine the values for no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) in the ecotoxicity assessment assays. The half-maximal effective concentrations (EC_{50}) were determined for the settlement inhibition of *M. galloprovincialis* plantigrades and the growth inhibition of *Navicula* sp. The median lethal dose (LC_{50}) was also assessed for those assays and for the *A. salina* nauplii, *A. amphitrite* nauplii, and *D. rerio* embryo toxicity assays. Probit regression analysis was employed to calculate EC_{50} and LC_{50} values. The Pearson Goodness-of-Fit Chi-Square test ($p < 0.01$) was applied to the Probit regression analysis, with 95% lower and upper confidence limits (95% LCL; UCL). ANOVA and Probit analyses were conducted using IBM SPSS Statistics 28. The therapeutic ratio (LC_{50}/EC_{50}) was used to evaluate the compound's effectiveness versus toxicity (Almeida *et al.*, 2017; Qian, Xu, *et al.*, 2010; Dan Rittschof *et al.*, 2003). For proteomic data, differentially expressed proteins (DEPs) were assessed using the “DEP” R package, with R: a language and environment for statistical computing version 4.3.3 (R Core Team, 2021; Zhang *et al.*, 2018). Significant differences between nocuolin A and control conditions were identified by applying a $\log_2$ fold change ($lfc = \log_2(1.5)$) as a threshold to classify proteins as DEPs.

5.3. Results

5.3.1. Antifouling activity of nocuolin A

The AF activity of nocuolin A was characterized using both micro and macroorganisms representative of marine biofouling communities. *M. galloprovincialis* was selected for the settlement inhibition assay due to its prominence as a marine macrofouling organism and model species in AF bioassays (Neves, Vilas Boas, *et al.*, 2022). Screening of nocuolin A at 12.5 and 25 μ M against mussel post-larvae (plantigrades) was performed to assess their ability to resettle (anti-settlement activity). Significant settlement inhibition ($p < 0.01$) was observed at both concentrations ($15 \pm 9.6\%$ and $0 \pm 0\%$ of settlement, respectively). Further bioassays were performed to determine the half-maximal effective concentration (EC_{50}) that inhibited 50% of the mussel plantigrades settlement, which was found to be 3.905 μ M (Figure 28.a). For the assessment of microfouling organisms, five marine biofilm-forming marine bacteria and microalgae (the diatom *Navicula* sp.) were used for growth inhibition assays. Diatoms were exposed to nocuolin A for 10 days in an

initial screening at 12.5 and 25 μM , resulting in high mortality ($100 \pm 0\%$) at these concentrations. Lower concentrations were tested (25, 12.5, 6.2, 3.1, 1.5, and 0.8 μM) and an EC_{50} value of 1.561 μM was determined in the dose-response assay for nocuolin A (Figure 28. b). In addition, five marine biofilm-forming bacterial strains (*C. marina*, *V. harveyi*, *H. aquamarina*, *P. atlantica*, and *R. litoralis*) were exposed to nocuolin A, at 12.5 and 25 μM , for 24 h, except for *R. litoralis*, which was exposed for 72 h. Promising antibacterial activity was considered if a threshold of $\geq 30\%$ of growth inhibition was achieved. However, nocuolin A showed no significant inhibition of bacterial growth for any of the tested marine bacterial strains (Figure 28. c).

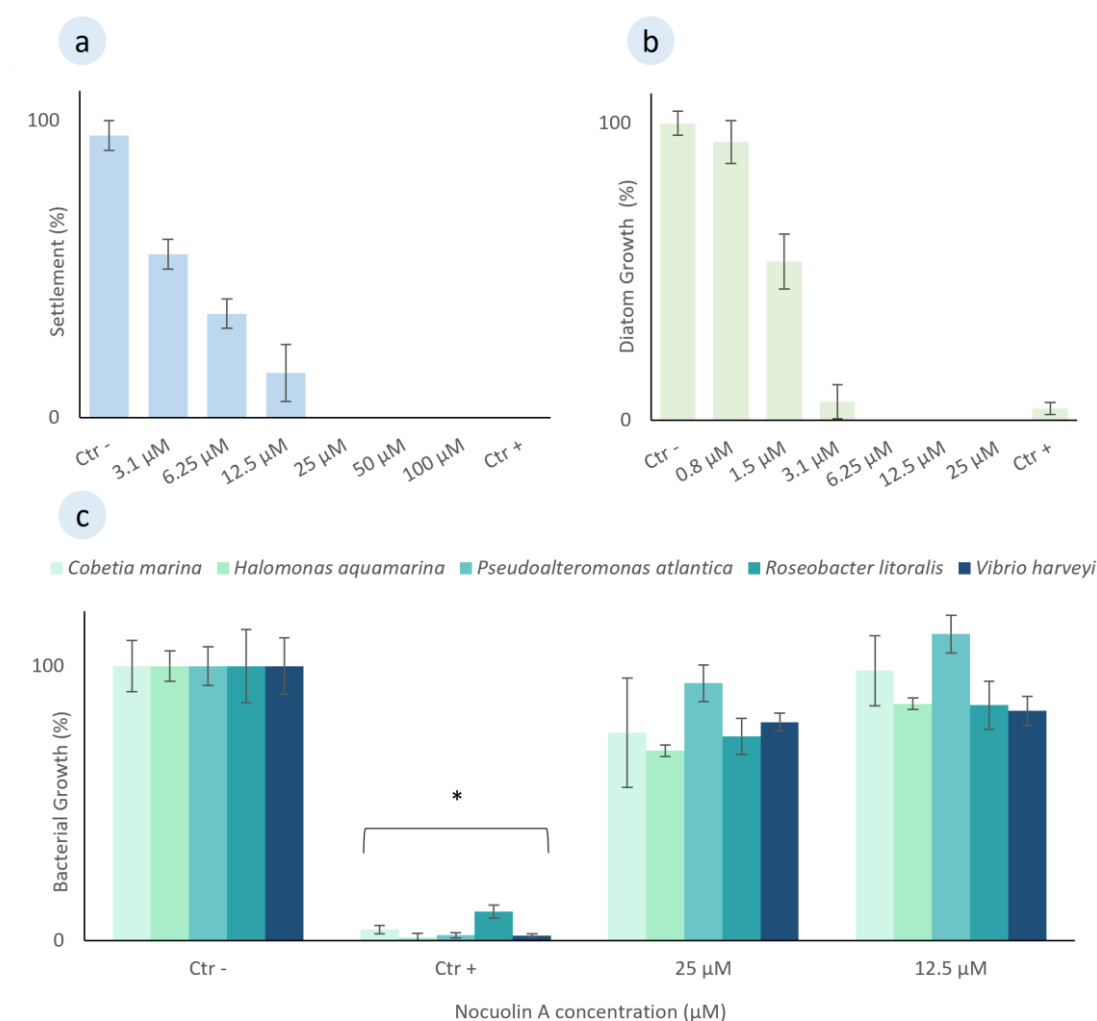


Figure 28. Antifouling activity of nocuolin A against target organisms. (a) Dose-response curve of the settlement inhibition activity in *M. galloprovincialis* plantigrades. Ctr-: DMSO (0.1%), Ctr+: CuSO_4 (5 μM); (b) Dose-response curve of the growth inhibition of *Navicula* sp. Ctr-: DMSO (1%), Ctr+: Cycloheximide (3.55 μM); (c) Growth inhibition of five marine biofilm-forming bacteria screening bioassay, where the compound was tested at 12.5 and 25 μM . Ctr-: DMSO (1%), Ctr+: 1:100 penicillin-streptomycin-neomycin stabilized solution. *Significant differences compared to the negative control ($p < 0.01$, Dunnett's test).

5.3.2. *In vitro* enzymatic inhibition assays for AChE and Tyrosinase

AChE and tyrosinase assays were conducted to evaluate a possible molecular target that could be implicated in the AF activity of nocuolin A, as these pathways are involved in mussel adhesion mechanisms. The enzymatic assays revealed no inhibition of AChE or tyrosinase activities compared to the negative control, excluding these pathways as possible molecular targets for nocuolin A (Figure 29).

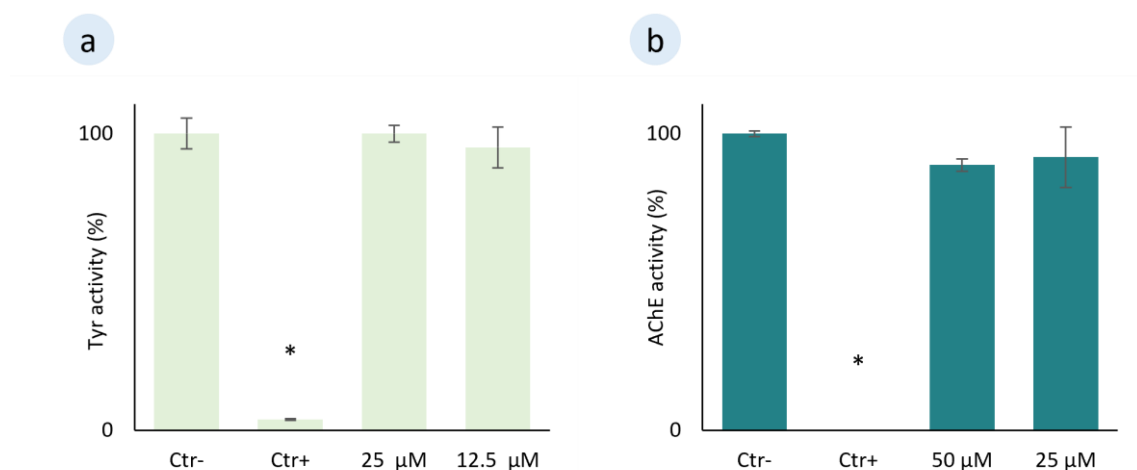


Figure 29. *In vitro* enzymatic inhibition assays of nocuolin A. (a) Tyrosinase inhibition. Ctr-: DMSO (0.2%); Ctr+: Kojic acid (100 µM). (b) AChE inhibition. Ctr-: DMSO (0.2%); Ctr+: Eserine (100 µM). *Significant differences compared to the negative control ($p < 0.01$, Dunnett's test).

5.3.3. Proteomic profiling of the *M. galloprovincialis* plantigrades

The putative effect of nocuolin A on mussel plantigrades at a molecular level was further investigated by shotgun proteomic analysis. Larvae were exposed to two concentrations of nocuolin A (5 and 10 µM) and compared to a control group (0.1% DMSO). The analysis identified 1718 proteins. Since a low number of proteins was identified in one of the three replicates representing the 5 µM nocuolin A group, this sample was excluded, and the analysis continued with only two replicates for this concentration. Therefore, changes in protein expression were analysed using fold change ($\log_2$ fold change of 1.5). In total, 222 proteins were differentially expressed across all conditions (Supplementary Table S11). Figure 30.a presents the proteomic profile in a heatmap, where rows represent the differentially expressed proteins and their relative expression levels under different conditions (control, 5 µM nocuolin A, and 10 µM nocuolin A). Proteins were clustered with distances between clusters indicating differences in expression levels. The heatmap columns correspond to the three experimental conditions. The colour gradient in the heatmap shows variations in protein expression across different conditions. The Venn diagram in Figure 30.b shows that out of the 222 DEPs, 18 were common to nocuolin A

groups when compared to the control. In contrast, 61 and 48 DEPs were unique to the 5 μ M and 10 μ M groups, respectively. Notably, the presence of precollagen NG (Q8MW55) and precollagen D (O44367) among the identified proteins was observed, as these are constituents of the mussel byssal threads. However, none of these proteins were classified as differentially expressed when comparing the nocuolin A conditions with the negative control after applying the $\log_2$ fold change of 1.5 threshold.

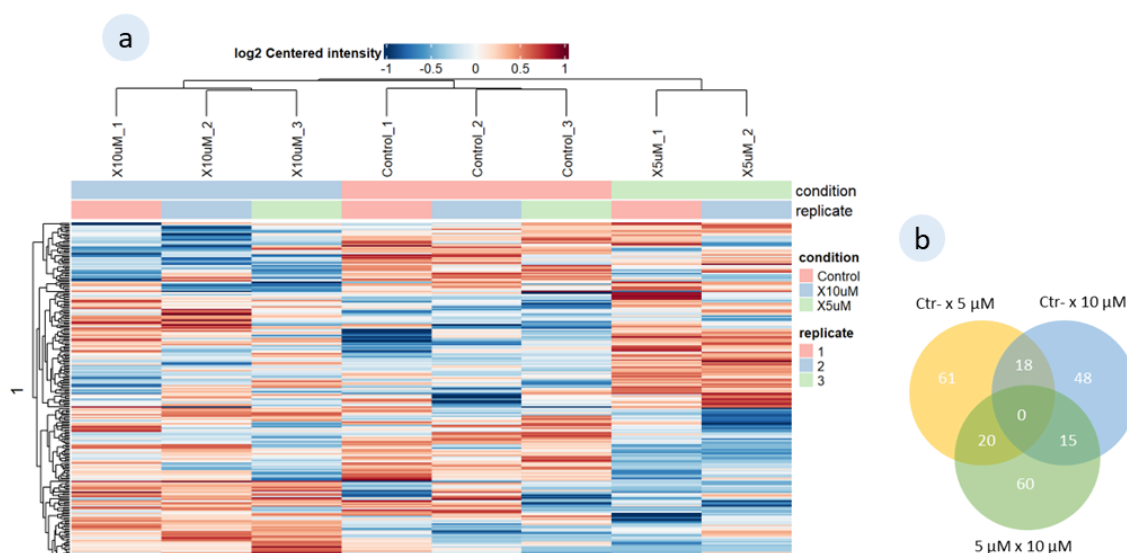


Figure 30. Heatmap and Venn's diagram showing differentially expressed proteins (DEPs) in mussel plantigrades when exposed to nocuolin A at 5 and 10 μ M and control. (a) The heatmap illustrates DEPs for each condition, showing the differences as a $\log_2$ fold change of 1.5, depicted in a colour map (red: upregulated; blue: downregulated). The rows represent DEPs clustered by k-means clustering (1). The columns represent the tested conditions: DMSO 0.1% (control), nocuolin A concentrations at 5 μ M (x5uM), and 10 μ M (x10uM). (b) The Venn diagram highlights the number of unique and shared DEPs identified by pairwise comparisons between each condition.

Further insights into the molecular processes of the differentially expressed proteins affected by nocuolin A were investigated through functional enrichment analysis. Homologous *Crassostrea gigas* sequences (phylogenetically closer species available) were classified using gene ontology (GO) terminology and grouped according to their functions. Blast results are displayed in Supplementary Table S12. Functional classes were searched using the web tool g:Profiler, and statistically significant enriched biological processes, molecular functions, and cellular components were filtered using a hypergeometric test (Fisher's exact test, p-value < 0.05) with Benjamini-Hochberg's false discovery rate correction. The resulting functional network is illustrated in Figure 31. This analysis retrieved 49 GO terms describing the functions of differentially expressed proteins (Supplementary Table S13). Most GO terms belonged to the cellular components category and were identified for both concentrations. Clusters in cellular

components, common to both concentrations, included protein and ribonucleoprotein complexes (AP-2 complex subunit alpha (K1QAB1), eukaryotic translation initiation factor 3 subunit A (A0A8W8INP3), eukaryotic translation initiation factor 3 subunit B (A0A8W8HZ31), RRM domain-containing protein (A0A8W8IE27), Arp2/3 complex 34 kDa subunit (A0A8W8M9C6), 26S proteasome non-ATPase regulatory subunit 6 (K1QUE6), proteasome subunit beta (A0A8W8KC84), collagen alpha-2(I) chain (K1PDS7), dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2 (K1QHM2), proteasome subunit alpha type (A0A8W8JYP9), 40S ribosomal protein S25 (A0A8W8LCY1), 60S ribosomal protein L21 (A0A8W8L8N9), large ribosomal subunit protein eL30 (A0A8W8NG46), 60S ribosomal protein L13 (A0A8W8JYD4), cyclic nucleotide-binding domain-containing protein (A0A8W8MFI8), 60 kDa SS-A/Ro ribonucleoprotein (K1QRQ0), KOW domain-containing protein (A0A8W8IPA8), splicing factor 3B subunit 1 domain-containing protein (A0A8W8M508), collagen alpha-2(I) chain (A0A8W8NCS6), fibrillar collagen NC1 domain-containing protein (A0A8W8KPZ6), myosin-XV (K1RD93), proteasome subunit alpha type (A0A8W8J5Q6), proteasome subunit beta (K1QBT2), protein transport protein SEC23 (A0A8W8IF30), and succinate dehydrogenase (A0A8W8N2K5)); and peptidase/proteasome complex (26S proteasome non-ATPase regulatory subunit 6 (K1QUE6), proteasome subunit alpha type (A0A8W8JYP9), proteasome subunit beta (K1QBT2)). Regarding the clusters from cellular components only present for 5 μ M of nocuolin A, these include the vesicle components group (coatamer subunit delta (A0A8W8KS23), and AP-2 complex subunit alpha (K1QAB1) and the eukaryotic translation complex (eukaryotic translation initiation factor 3 subunit A (A0A8W8INP3), eukaryotic translation initiation factor 3 subunit B (A0A8W8HZ31)). In contrast, the cellular components exclusively found at 10 μ M nocuolin A, were related to the muscle constituent group, which included striated muscle thin filament, troponin complex, and myofilament, which all shared the association to the same protein, troponin T (A0A8W8NMI9), the mitochondrial membrane group (cytochrome c oxidase subunit 5A, mitochondrial (K1PVD7), calcium-binding mitochondrial carrier protein SCaMC-2 (K1QR48), succinate dehydrogenase (A0A8W8N2K5)), the actin cytoskeleton (Arp2/3 complex 34 kDa subunit (A0A8W8M9C6), myosin-XV (K1RD93), and troponin T (A0A8W8NMI9)) and ribosome constituents (60S ribosomal protein L13 (A0A8W8JYD4), KOW domain-containing protein (A0A8W8IPA8), 40S ribosomal protein S25 (A0A8W8LCY1), large ribosomal subunit protein uL1 (A0A8W8MU73), and 60S ribosomal protein L21 (A0A8W8L8N9)). For other functional categories, such as molecular functions and biological processes, the GO terms were only found for the 10 μ M concentration. Clusters related to biological

processes included protein metabolism and organonitrogen compounds (calpain-5 (K1R1C6), 60S ribosomal protein L13 (A0A8W8JYD4), calcium/calmodulin-dependent protein kinase (A0A8W8ID93), cyclic nucleotide-binding domain-containing protein (A0A8W8MFI8), inter-alpha-trypsin inhibitor heavy chain H3 (A0A8W8LTT6), KOW domain-containing protein (A0A8W8IPA8), mitogen-activated protein kinase (A0A8W8KPG9), transmembrane 9 superfamily member (A0A8W8MZX0), dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2 (K1QHM2), transglutaminase-like domain-containing protein (A0A8W8JUK3), counting factor associated protein D (A0A8W8NWQ5), proteasome subunit alpha type (A0A8W8J5Q6), proteasome subunit alpha type (A0A8W8JYP9), proteasome subunit beta (K1QBT2), laminin G domain-containing protein (A0A8W8LGH4), ubiquitin carboxyl-terminal hydrolase (K1PX47), heat shock 70 kDa protein 12B (A0A8W8L019), large ribosomal subunit protein uL1 (A0A8W8MU73), argininosuccinate lyase (A0A8W8JQ38), and 60S ribosomal protein L21 (A0A8W8L8N9)). Clusters identified in molecular functions were related to RNA binding, structural molecular activity, extracellular matrix structural constituent, and structural constituent of ribosome, sharing most of the associated proteins (60S ribosomal protein L13 (A0A8W8JYD4), KOW domain-containing protein (A0A8W8IPA8), collagen alpha-2(I) chain (A0A8W8NCS6), fibrillar collagen NC1 domain-containing protein (A0A8W8KPZ6), large ribosomal subunit protein uL1 (A0A8W8MU73), methenyltetrahydrofolate synthetase domain-containing protein (K1QRL6) and 60S ribosomal protein L21 (A0A8W8L8N9)). The overall response of the mussel plantigrades to nocuolin A was characterized by a general stress response, followed by muscle dysregulation, impairment of energy production, and endoplasmic reticulum stress.

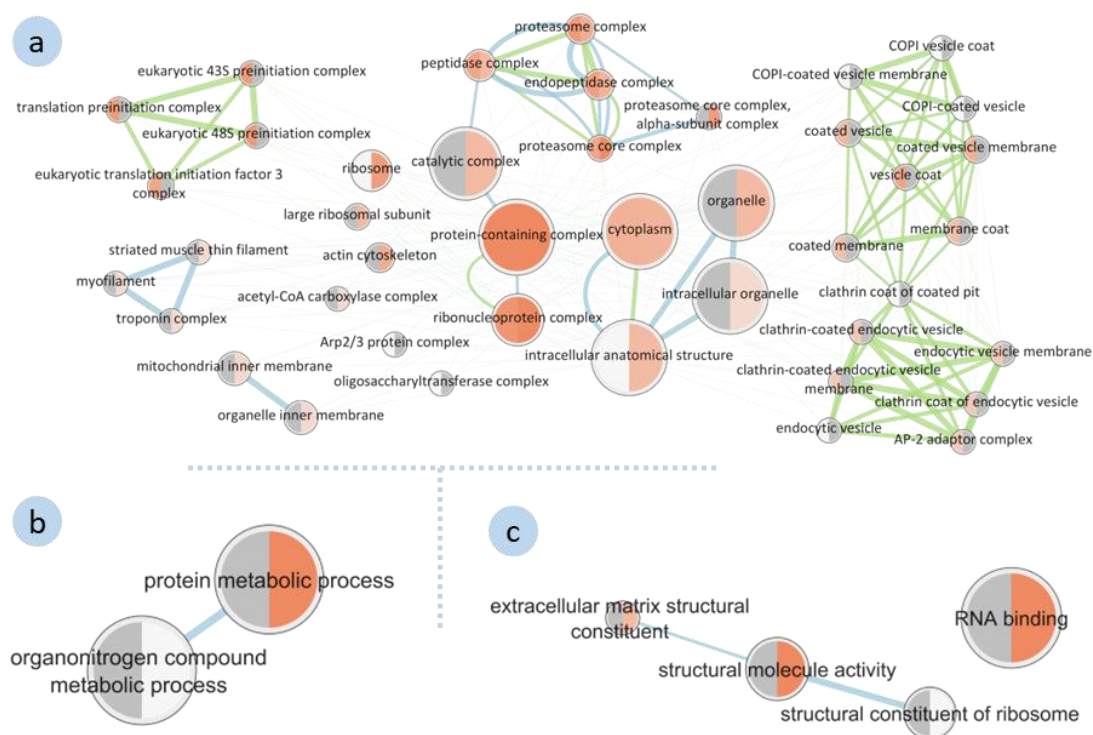


Figure 31. Functional network of differentially expressed proteins in the mussel plantigrades exposed to nocuolin A at 5 and 10 µM. The GO categories represented include (a) Cellular Components, (b) Biological Processes, and (c) Molecular Functions. Node sizes increase with the number of proteins associated with that GO term and colour intensifies with significance (Node cut-off = 0.05; grey means the absence of proteins for the GO term). Nodes are divided, representing the different tested concentrations of nocuolin A, at 5 (left side) and 10 µM (right side). Green edges link functions identified in samples exposed to 5 µM while blue edges connect functions from samples exposed to 10 µM.

5.3.4. Ecotoxicity assessment

5.3.4.1. *In vivo* effects of nocuolin A

In parallel with the AF activity assessment, the toxicity of nocuolin A on target biofouling organisms was also evaluated (Table 14). No lethal effects were observed on the mussel larvae exposed to the compound at any of the tested concentrations. The non-toxic impact on the mussel plantigrades was based on the $LC_{50} > 100 \mu M$ ($> 29.843 \mu g mL^{-1}$) and a therapeutic ratio higher than 15 ($LC_{50}/EC_{50} = 25.61$). Toxicity was also assessed on the macrofouling species *A. amphitrite*, being the nauplii larval stage used for the toxicity tests (Piazza *et al.*, 2012; Rittschof *et al.*, 1992). The compound exhibited a significantly lower LC_{50} ($0.016 \mu M$; $0.00483 \mu g mL^{-1}$) for barnacle larvae when compared to the mussel larvae, indicating higher toxicity to the early stages of barnacle development. The NOEC and LOEC values for barnacle larvae were $0.001 \mu g mL^{-1}$ and $0.239 \mu g mL^{-1}$.

For marine biofilm-forming bacterial strains, no toxic effects were observed as there was no growth inhibition after exposure to nocuolin A (Figure 28.c). However, high mortality was noted in the exposed diatom cells for the screened concentrations (12.5 and 25 μM , 3.730 and 7.461 $\mu\text{g mL}^{-1}$, respectively) with an $\text{LC}_{50} > 3.1 \mu\text{M}$ ($> 0.925 \mu\text{g mL}^{-1}$). The toxicity of nocuolin A towards *Navicula* sp. was further confirmed by the lower therapeutic ratio ($\text{LC}_{50}/\text{EC}_{50} = 1.986$).

Table 14. Antifouling effectiveness *versus* toxicity of nocuolin A towards biofouling organisms. The half maximum effective concentration (EC_{50}), the median lethal concentration (LC_{50}), no observed effect concentration (NOEC), lowest observed effect concentration (LOEC), and the therapeutic ratio ($\text{LC}_{50}/\text{EC}_{50}$) are listed. The EC_{50} , LC_{50} , NOEC, and LOEC are provided in both μM and $\mu\text{g mL}^{-1}$ for comparative purposes. Confidence intervals for EC_{50} values are presented in μM (in parentheses). n.a. – not assessed.

Target species	EC_{50} (μM ; $\mu\text{g mL}^{-1}$)	LC_{50} (μM ; $\mu\text{g mL}^{-1}$)	NOEC (μM ; $\mu\text{g mL}^{-1}$)	LOEC (μM ; $\mu\text{g mL}^{-1}$)	$\text{LC}_{50}/\text{EC}_{50}$
<i>M. galloprovincialis</i>	3.905 (95 % CI: 2.866 – 4.860); 1.165	> 100 ; > 29.843	n.a.	n.a.	25.61
<i>A. amphitrite</i>	n.a.	0.0162 (95 % CI: 0.0138 - 0.0187); 0.00483	0.00335; 0.001	0.00838; 0.0025	n.a.
<i>Navicula</i> sp.	1.561 (95 % CI: 1.460 – 1.671); 0.466	> 3.1 ; > 0.925	n.a.	n.a.	1.986

Beyond considering the target organisms, two more species (*A. salina* and *D. rerio*) were selected to evaluate the environmental toxicity of nocuolin A. All bioassays met the requirements for control survival and normal development ($\geq 90\%$). No significant differences were observed between the control and solvent control groups; thus, treatments were compared to the respective DMSO control (0.1%). Nominal concentrations were used throughout this study. LC_{50} values are summarized in Table 15.

Table 15. Toxicity of nocuolin A towards marine and freshwater organisms. The median lethal concentration (LC_{50}), no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) are presented in both μM and $\mu\text{g mL}^{-1}$ for comparative purposes. Confidence intervals are displayed in μM , in parenthesis, for the LC_{50} values. n.a. – not assessed.

Species	LC_{50} (μM ; $\mu\text{g mL}^{-1}$)	NOEC (μM ; $\mu\text{g mL}^{-1}$)	LOEC (μM ; $\mu\text{g mL}^{-1}$)
<i>Artemia salina</i>	2.480 (95% CI: 2.321-2.648); 0.740	0.8; 0.239	1.5; 0.448
<i>Danio rerio</i>	0.0584 (95% CI: 0.0462-0.0768); 0.0174	0.0503; 0.015	n.a.

The crustacean *A. salina* nauplii, a model species widely used in ecotoxicology, exhibited an LC₅₀ value of 2.480 µM (0.740 µg mL⁻¹), with NOEC and LOEC values of 0.0025 µg mL⁻¹ and 0.448 µg mL⁻¹, respectively. In comparison to the LC₅₀ values of *A. salina* and *A. amphitrite* nauplii, the barnacle nauplii displayed higher sensitivity to the compound, with LC₅₀ values lower by two orders of magnitude). The zebrafish assay revealed a 96h-LC₅₀ of 0.0584 µM; 0.0174 µg mL⁻¹, with almost no sublethal effects detected.

5.3.4.2. *In silico* predictions of nocuolin A

The predicted data obtained from the EPI Suite™ for nocuolin A is shown in Table 16. In ecotoxicology, the octanol/water partition coefficient (also known as log K_{ow} or Log P) indicates the bioconcentration potential of a substance. It has been demonstrated that a log K_{ow} > 4.5 may pose a risk for marine organisms since suggests a high affinity for lipids in an organism (Gimeno *et al.*, 2024). Nocuolin A's log K_{ow} is precisely at this threshold. Nevertheless, the BCF value obtained for nocuolin A is much lower than 2000 L Kg⁻¹, which is the threshold used to classify a bioaccumulative substance. The BCF value is the result of a balance between the rate of chemical uptake from water and its elimination from the organism, indicating a reduced bioconcentration potential in marine organisms. BCF should not be used alone to model bioaccumulation of contaminants; however, it is very useful, especially for initial screening (Wang, 2016).

Table 16. Predicted data for nocuolin A from EPI Suite™ regarding biodegradability, sediment-water partition (Log K_{oc}), octanol-water partition (Log K_{ow}), and bioconcentration factor (BCF) of nocuolin A. ¹DNBF: Does Not Biodegrade Fast.

BIOWIN™			KOCWIN™	KOWWIN™	BCF™
Ultimate biodegradation	Aerobic conditions	Anaerobic conditions	Log K _{oc}	Log K _{ow}	BCF (LKg/wet-wt)
Days-Weeks (3.2963)	Days (4.0848)	DNBF ¹ (0.3536)	3.0155	4.53	19.1 (1.28)

In addition, we used ECOSAR, which is a computerized predictive system that estimates aquatic toxicity, to obtain LC₅₀ calculated using a QSAR (quantitative structure-activity relationship) model for baseline toxicity, for several model organisms. By comparing the estimated LC₅₀ with the experimental value, for the same species, one can obtain a Toxic Ratio (TR). This ratio can be used to distinguish “specifically toxic chemicals” from “baseline toxicants” considering a threshold of TR = 10 (Maeder *et al.*, 2004). In this study, we used zebrafish for these calculations and obtained a TR of 20.4 or 79.5 depending on if nocuolin A is considered more similar to a hydrazine or an amide,

respectively. The threshold of 10 was surpassed, although not by much, meaning that this compound may reveal a specific mode of toxic action.

To perform a quantitative risk characterization, we employed an established approach by calculating the ratio between the predicted environmental concentration (PEC; calculated with MAMPEC) and the predicted no-effect concentrations (PNEC; derived from toxicity data). If this ratio is > 1 , then the compound has the potential to threaten wildlife and ecosystems (European Commission, 2003). Assuming that nocuolin A would be used as an AF biocide in marine coatings, the marine antifoulant model MAMPEC was used to predict the maximum and minimum theoretical environmental concentrations (PEC_{MAX} and PEC_{MIN}). Values varied between a PEC_{MAX} of $8.56 \times 10^{-5} \mu\text{g mL}^{-1}$ and a PEC_{MIN} of $5.55 \times 10^{-6} \mu\text{g mL}^{-1}$ (Table 17). For PNEC calculations, all available toxicity data can be used. Regardless, the use of safety factors is strongly recommended to ensure that the potential for adverse effects on the environment is identified (European Commission, 2003). It should be noted that the safety factors to be applied are strongly associated with the type of toxicity data available. Guidance on this issue is provided in the Technical Guidance Document on Risk Assessment, in support of several directives relating to the risk assessment of new and/or existing substances and relating to the placing of biocidal products on the market (European Commission, 2003). According to this document, the PNEC was thus derived from the lowest LC_{50} or EC_{50} of the most sensitive species, on which a safety factor of 1000 times was applied. The guidelines suggest higher safety values whenever there is scarce information on toxicity (short-term LC_{50} of freshwater or saltwater representatives of three taxonomic groups). However, we consider that if this information is based on highly sensitive tests using early life stages of organisms including 3 trophic levels (algae, crustacean, and fish) plus toxicity to larvae of other marine species, the choice of a safety factor of 1000 seems more appropriate.

Table 17. Putative Risk Assessment of nocuolin A based on the maximum and minimum MAMPEC-predicted environmental concentrations (PEC_{MAX} and PEC_{MIN} , (in $\mu\text{g mL}^{-1}$) in a marine commercial harbour, divided by the PNEC to which an assessment factor of 1000 was applied.

Compound	Endpoint used for PNEC estimation ($\mu\text{g mL}^{-1}$)	PEC Commercial harbour ($\mu\text{g mL}^{-1}$)		Risk Assessment	
		MAX	MIN	$PEC_{MAX}/PNEC$	$PEC_{MIN}/PNEC$
Nocuolin A	0.00483	8.56×10^{-5}	5.55×10^{-6}	17.72	1.15

5.4. Discussion

This is the first report considering nocuolin A as a potential AF agent. In this work, nocuolin A's AF activity was assessed against a panel of microfouling species (five

strains of marine biofilm-forming bacteria and a strain of a marine biofilm-forming diatom) as well as a widely occurring macrofouling species. Despite the inherent challenge of developing a leading AF agent effective across diverse biological targets acting against different fouling species, it is widely assumed that broad-spectrum and non-toxic AF agents are difficult to achieve. To ensure efficacy, mixtures of narrow-spectrum bioactive components might be applied in new AF technologies (Cahill *et al.*, 2024). The prevention of marine bacteria and diatoms growth and, therefore, the formation of biofilms can interfere with further colonization of submerged surfaces by marine invertebrate larvae and macroalgal spores, since natural marine biofilms can modulate the colonization cascade of biofouling communities (Qian *et al.*, 2022; Vinagre *et al.*, 2020). Nocuolin A exhibited different effects on the growth of marine biofilm-forming bacteria and diatoms. No significant growth inhibition was found for any of the five tested marine bacterial strains. However, the compound proved to be active against the diatom *Navicula* sp. Xu and colleagues (Xu *et al.*, 2019) also found active natural compounds against diatoms, namely cochliomysin G, a resorcylic acid lactone isolated from the culture broth of the marine-derived fungus *Cochliobolus lunatusthe* ($EC_{50} = 0.61 \mu\text{g mL}^{-1}$ for *Navicula exigua*), and zeaenol, its analogue (EC_{50} values of 3.20 and $8.71 \mu\text{g mL}^{-1}$, for *Navicula levissima* and *Navicula exigua*, respectively). After comparing the EC_{50} values of these compounds with the one obtained for nocuolin A ($EC_{50} = 0.466 \mu\text{g mL}^{-1}$), our compound exerted a more potent activity towards the diatom. Approved AF compounds that are reported to exert antialgal toxicity (Irgarol 1051, Dichlofluanid, Tolyfluanid, Chlorothalonil, Sea-Nine 211) are described as acting as photosynthesis and carbon incorporation inhibitors and/or cell membrane disruptors through apoptosis (L. Chen & Qian, 2017). Regarding antibacterial activity, the most reported specific targets are related with quorum sensing inhibition (N-acyl homoserine lactone autoinducers) (L. Chen & Qian, 2017). A specific mode of action associated with microalgae (e.g. photosynthesis-related) could explain the differential response observed between bacteria and microalgae in this study. In addition, from a chemical ecology perspective, the demonstrated AF activity profile of nocuolin A against microalgae may offer insights into its natural ecological function in cyanobacteria, likely conferring allelopathic properties to the host strains against competing eukaryotic microbial communities (Leão *et al.*, 2009; Scholz & Liebezeit, 2012).

Alongside the microfouling screening, nocuolin A's activity was also assessed against plantigrades of the macrofouling species *M. galloprovincialis*. In addition to being among the most prominent macrofouling organisms worldwide, it is considered a model macrofouling species for settlement inhibition bioassays (Almeida *et al.*, 2015, 2017,

2018; Antunes *et al.*, 2019; Mabrouk *et al.*, 2020; Vilas-Boas *et al.*, 2020, 2021). Regarding the anti-settlement activity, nocuolin A exhibited an EC_{50} of $1.165 \mu\text{g mL}^{-1}$ which is considerably lower than the reference threshold ($EC_{50} < 25 \mu\text{g mL}^{-1}$) defined by the U.S. Navy program. When comparing the activity against macrofouling organisms (*Amphibalanus amphitrite* cyprids) of natural compounds isolated from cyanobacteria, nocuolin A could be ranked among the most potent cyanobacterial compounds, such as lyngbyabellins O and P ($EC_{50} = 0.24\text{-}0.62 \mu\text{g mL}^{-1}$, respectively) isolated from *Okeania* sp. (Petitbois *et al.*, 2017) and majusculamide A and dolastatin 16 ($EC_{50} = 0.54\text{-}0.003 \mu\text{g mL}^{-1}$, respectively) isolated from *Lyngbya majuscula* (L. T. Tan *et al.*, 2010). Compared with phidianidine A, a natural compound exhibiting a 1,2,4-oxadiazole ring linked to the brominated indole system that prevented the settlement of *Amphibalanus improvisus* cyprids at $4.0 \mu\text{g mL}^{-1}$ (Labriere *et al.*, 2020), nocuolin A was also found to be a more potent AF compound. It is important to note that the model macrofouling species used in those bioassays was *Amphibalanus amphitrite* cyprids, rather than the *M. galloprovincialis* plantigrades used in this study, which implies different sensitivities, as mussel plantigrades usually more robust. When directly compared with a compound derived from cyanobacteria tested on the same target organism, such as portoamides ($EC_{50} = 4.86 \mu\text{g mL}^{-1}$), cyclic peptides isolated from *Phormidium* sp. LEGE 05292 in our group (Antunes *et al.*, 2019), nocuolin A demonstrated significantly stronger activity. Nocuolin A's activity against *M. galloprovincialis* plantigrades is also comparable in potency to other natural compounds isolated from marine actinomycetes *Streptomyces aculeolatus*, namely napyradiomycins ($EC_{50} = 0.1\text{-}6.34 \mu\text{g mL}^{-1}$), madeirone and neomadeirone ($EC_{50} = 1.76$ and $0.12 \mu\text{g mL}^{-1}$, respectively) (F. Pereira *et al.*, 2020; Wissner *et al.*, 2024). A similar conclusion can be drawn by comparing the activity of the commercial AF agent ECONEA® ($EC_{50} = 1.40 \mu\text{g mL}^{-1}$) on mussel plantigrades (Almeida *et al.*, 2017).

As previously discussed, the distinctive effect of nocuolin A on the tested bacteria (non-active) compared to diatom species and mussel larvae (active) suggests that the underlying mechanistic action and biological targets of nocuolin A may involve different eukaryotic pathways. A similar multi-action behavior has been reported for alkyl triphenylphosphonium salts, which act as non-toxic quorum sensing disruptors in marine bacteria and tyrosinase inhibitors for byssal production in the mussel *M. galloprovincialis* (Martín-Rodríguez *et al.*, 2015), as well as for the AF compounds 7-Hydroxy-4-methylcoumarin (M. Pérez *et al.*, 2016), the bromotyrosine derivative ianthelline (Hanssen *et al.*, 2014), 3-Alkylpyridinium oligomers and polymers (3-APS) (Grandič *et al.*, 2012), among others. Given this complexity, elucidating the mechanisms of action

for AF compounds has become essential for the development of new AF agents (L. Chen & Qian, 2017), though a single molecular target or pathway may not always be identifiable due to the intricate biological processes involved in biofouling colonization (Cahill *et al.*, 2024). In this study, we focused on investigating the AF protein targets of nocuolin A in mussel plantigrades, based on its observed ability to inhibit settlement. A well-known pathway in the production of mussel biological adhesives (byssal threads) involves the 3,4-dihydroxyphenyl-L-alanine (L-DOPA) metabolism, which is crucial for the production of DOPA-containing byssal plaques. This process is catalysed by the enzyme tyrosinase, which converts DOPA precursors into DOPA residues (Lianguo Chen & Qian, 2017; Martín-Rodríguez *et al.*, 2015). Additionally, certain molecules, such as the enzyme acetylcholinesterase (AChE), are affected by AF compound exposure in fouling organisms, as AChE is involved in cholinergic neural signalling during settlement (L. Chen & Qian, 2017). Two natural compounds isolated from marine organisms, territrem A and pulmonarin, were found to have AF activity associated with AChE inhibition (Nong *et al.*, 2014; Tadesse *et al.*, 2014). The commercial booster biocide Sea-Nine 211 also functions through this mechanism (Lianguo Chen *et al.*, 2014, 2017). Regarding compounds that act on the tyrosinase pathway, the cyclo-L-Trp-Ala, isolated from the sponge-associated fungus *Eurotium chevalieri* MUT 2316, was found to exert its AF activity via tyrosinase inhibition (Bovio *et al.*, 2019). However, our results suggested that nocuolin A AF activity may not involve these metabolic pathways, as no modulation was found for either enzyme *in vitro*. Characterizing the molecular targets of AF compounds through untargeted shotgun proteomics is a complex process particularly when performed in non-model organisms such as the case of the mussel *M. galloprovincialis*. The absence of annotated genomes in the available databases used by gene set enrichment analysis online programs implies using homologous sequences from other represented organisms, namely the oyster *Crassostrea gigas*. Despite the similarities, differences in adhesion mechanisms among species may result in an imperfect representation of the mussel adhesive proteins in gene set enrichment. Nonetheless, untargeted shotgun proteomics was performed on mussel plantigrades exposed to nocuolin A. Of the 1718 proteins, only precollagen NG and precollagen D were found to be related to mussel adhesive proteins. These prepolymerized collagens (preCOLs), secreted by the collagen gland located on the mussel foot, are core components of the byssal threads (H. Waite *et al.*, 1998). Depending on their distribution along the thread, the preCOLs are classified as distal (preCOL-D), connected to the byssal plaque, non-gradient (preCOL-NG), located at the centre of the thread, and proximal (preCOL-P) found in the region closer to the mussel. The different preCOLs

also confer different properties to the byssal thread. For example, preCOL-NG which grants resistance to pepsin digestion and mediates the interaction between the other two preCOLs, while preCOL-D, with its silk-fibroin domains, allows extensibility to the thread (Silverman & Roberto, 2007; H. Waite *et al.*, 1998). Thus, alterations in these precollagens would greatly impair the efficiency of the byssal threads and, therefore, the adhesion of the mussel larvae to substrates. Nonetheless, despite being identified in the mussel larvae, none of these precollagens were considered differentially expressed in the samples exposed to nocuolin A compared to the control group, after applying the $\log_2$ fold change of 1.5 threshold. Of the proteins that were considered differently expressed when compared to the control ($\log_2$ fold change of 1.5), some were common to both concentrations of nocuolin A, while the majority were significantly altered by each concentration.

Proteins associated with basic biological processes such as vesicle transportation (coatamer subunit delta, and AP-2 complex subunit alpha) and eukaryotic translation complex (translation initiation factor 3 subunit A, eukaryotic translation initiation factor 3 subunit B) seemed to be upregulated by nocuolin A, at the concentration of 5 μ M. The coatamer subunit delta is found in the Golgi apparatus, as part of the coatamer protein complex I (COPI), which is responsible for forming COPI-coated vesicles and the retrograde transport from the Golgi apparatus to the Endoplasmic Reticulum (ER), as well as transport within Golgi compartments (Beck *et al.*, 2009). The AP-2 complex subunit alpha integrates the adaptor protein 2 (AP-2) complex and is involved in the formation of clathrin-coated vesicles, facilitating clathrin-mediated endocytosis (Traub, 2009). Vesicular carriers are fundamental for maintaining cellular homeostasis, and interferences in proteins associated with this process could lead to ER stress and disruption of cellular homeostasis. Eukaryotic translation initiation factors are essential for translation start site specificity, facilitating the assembly of the ribosome on the mRNA, and/or being involved in the hydrolysis of GTP for energy production during ribosomal assembly (Sonnenberg & Dever, 2003). Alterations in these translation factors could reflect cellular stress, impairing protein synthesis and, therefore, overall cellular function. At the higher concentration, nocuolin A seemed to alter proteins associated with muscle contraction (troponin T, myosin regulatory light chain, and myosin-XV) and mitochondrial function (cytochrome c oxidase subunit 5A, calcium-binding mitochondrial carrier protein SCA2 and succinate dehydrogenase). Troponin T is one of the three subunits that comprise of the troponin complex, along with troponin I and troponin C, which exhibit similar functions to their vertebrate homologous proteins, albeit with structural differences (Hooper *et al.*, 2008; A. Inoue *et al.*, 1996; Lehman, 1981). The

troponin complex, found in the striated muscle of the adductor muscle on mussels, is a key regulatory protein on muscle contraction that initiates with the binding of Ca^{2+} (Funabara *et al.*, 2013). The myosin subunit, myosin regulatory light chain, is found on smooth muscle fibres of the muscle foot, where it regulates myosin contractile activity (Kato *et al.*, 2002). Downregulation of muscle regulation proteins could lead to an overall dysfunctional muscle activity. If the mussels' foot muscle function were compromised, the adhesion process could be undermined, as mussel adhesion proteins are synthesized, secreted, and modulated by the mussel foot (DeMartini *et al.*, 2017). Both succinate dehydrogenase and cytochrome c oxidase are involved in the electron transport chain on the mitochondrial inner membrane (as complex II and complex IV respectively), which is essential for ATP synthesis (Osellame *et al.*, 2012). The calcium-binding mitochondrial carrier protein SCA2 is a mitochondrial transporter of ATP-Mg/Pi responsible for ADP/ATP transport, calcium binding, and regulation of mitochondrial bioenergetics regulation (del Arco *et al.*, 2016; Satrustegui *et al.*, 2007). Overexpression of these proteins could impair ATP production and promote oxidative stress and metabolic dysregulation, consistent with a general cytotoxicity/ROS mode of action. Previous findings describe nocuolin A's activity in cancer cell lines as a promoter of ER stress, as shown by increased expression of the p-EIF2 α protein, and as a disruptor of bioenergetic balance by reducing cellular ATP levels (M. L. Sousa *et al.*, 2019), which aligns with our findings in mussel larvae. Moreover, other AF compounds have been shown to modulate ATPase activity, although via different pathways, suggesting that bioenergetic metabolism modulation may be a common mechanism of action among AF agents (Antunes *et al.*, 2019; Dash *et al.*, 2012; Han *et al.*, 2013; Qian, Wong, *et al.*, 2010; Y. F. Zhang *et al.*, 2012). Overall, the results of this study suggest that nocuolin A's AF activity in mussel plantigrades likely results from interference with vesicle transportation, mitochondrial ATP production, and muscle regulation. These general targets appear to reflect a global stress response in the mussel to the stress induced by nocuolin A, categorizing its mode of action as a protein expression regulator, as observed with other natural compounds (Antunes *et al.*, 2019; Dash *et al.*, 2012; Han *et al.*, 2013; Oliveira, Groh, *et al.*, 2016).

New AF compounds should also have a minimal impact on the environment, in addition to preventing the attachment and/or growth of fouling organisms. In this work, the toxicity of nocuolin A was studied in both target and non-target organisms. For target organisms, the therapeutic ratio ($\text{LC}_{50}/\text{EC}_{50}$) higher than 15 was used to assess the effectiveness of the compound relative to its toxicity, recognizing that these are working values and not absolutes (Dan Rittschof *et al.*, 2003). No mortality was observed in the five biofilm-

forming marine bacteria or the mussel plantigrades when exposed to the compound at tested concentrations. Furthermore, in the mussel larvae, nocuolin A displayed an $LC_{50} > 29.843 \mu\text{g mL}^{-1}$ and a therapeutic value higher than 15 (LC_{50}/EC_{50} value of 25.61). However, toxicity was observed in other target species, including *Navicula* sp. ($LC_{50} > 0.925 \mu\text{g mL}^{-1}$ and $LC_{50}/EC_{50} = 1.986$) and *A. amphitrite* nauplii ($LC_{50} = 0.00483 \mu\text{g mL}^{-1}$), as well as non-target marine and freshwater organisms represented by *A. salina* nauplii ($LC_{50} = 0.740 \mu\text{g mL}^{-1}$) and *D. rerio* embryos ($LC_{50} = 0.0174 \mu\text{g mL}^{-1}$). A similar profile was observed for the compound portimine, which was isolated from the dinoflagellate *Vulcanodinium rugosum*, towards macrofouling species larvae (*M. galloprovincialis* D-veliger and *A. improvisus* cyprids), where larval development and metamorphosis were inhibited via apoptosis (Brooke *et al.*, 2018). The same mechanism could be responsible for the toxicity observed in *A. amphitrite* nauplii, since apoptosis induction has already been described as nocuolin A's mode of action on colon cancer cells in both monolayer and spheroid cultures (M. L. Sousa *et al.*, 2019). Moreover, since the mussel plantigrades are at a later developmental stage and are more robust than D-veliger and barnacle nauplii, this could also explain the resistance of the plantigrades to the compound. The furanone derivatives omF1 and omF2, isolated from *Streptomyces violaceoruber* SCH-09, were also active but non-toxic towards *Navicula annexa* ($LC_{50}/EC_{50} = 173\text{-}290$, respectively), and *A. salina* ($LC_{50} = 118.9\text{-}81.9 \mu\text{g mL}^{-1}$, respectively) (Hong & Cho, 2013). These values were far higher than those obtained for nocuolin A, confirming the high toxicity of our compound towards these organisms. Another example is the AF compound maculalactone A, isolated from the cyanobacteria *Kyrtuthrix maculans*, which was found to be toxic to the larvae of various barnacles' species ($LC_{50} = 1.1\text{-}5.2 \mu\text{g mL}^{-1}$) and to *A. salina* nauplii ($LC_{50} = 3.7 \mu\text{g mL}^{-1}$) (Brown *et al.*, 2004). Nonetheless, our LC_{50} values for the same organisms were even lower. In fact, the toxicity exerted by nocuolin A was most comparable to that of already-known biocides, such as Irgarol 1051 ($LC_{50}/EC_{50} = 6$ for *Navicula annexa* and $LC_{50} = 0.51 \mu\text{g mL}^{-1}$ for *A. salina*) (Hong & Cho, 2013) and tralopyril ($LC_{50} = 0.00502 \mu\text{g mL}^{-1}$ for *D. rerio* embryos) (Oliveira *et al.*, 2017). These results highlighted the toxicity of our compound towards several organisms, including the early development stages of a target macrofouling species, which delays its potential as an environmentally compatible AF agent. An example of a compound with low risk is Capsaicin, the natural chilli pepper extract that has been commonly used as an animal repellent (EPA, 1992). Studies suggest that the risk of capsaicin as an AF biocide is relatively low for the marine environment (Oliveira *et al.*, 2014, 2017; Wang *et al.*, 2014) and based on degradation, bioaccumulation, toxicity and accumulation in sediments, additional studies may not be

necessary (Wang *et al.*, 2014). Since then, other compounds inspired by capsaicin's structure have emerged (An *et al.*, 2022; Xuan Wang *et al.*, 2020, 2022). Nocuolin A, on the other hand, raises some concerns regarding some of the parameters analysed, including the risk characterization that indicates a potential threat to the environment. However, essential information, such as the compound's half-life and persistence, is still missing. Conducting such studies early may help avoid future problems, as the history of AF usage is full of examples of environmentally friendly compounds later found to be harmful. Thus, a potential path could involve structural optimization of nocuolin A into less toxic analogues, especially for non-target organisms, while maintaining its bioactivity against fouling organisms (L. L. Liu *et al.*, 2020). Following this lead, and after chemical optimization, future work should also include a broader screening of AF activity against other important macrofouling species (Vinagre *et al.*, 2020), to provide a more comprehensive AF profile of the compounds.

5.5. Conclusions

This study explored the biotechnological potential of nocuolin A as an AF agent by investigating its activity against organisms from different levels of biological organization, identifying molecular targets in *M. galloprovincialis* plantigrades, and assessing its overall environmental toxicity and risk. Nocuolin A effectively inhibited the settlement of plantigrade mussel post-larvae without demonstrating toxicity towards this species. The molecular targets of this AF activity appear to involve alterations in mitochondrial energetic metabolism (cytochrome c oxidase, calcium-binding mitochondrial carrier protein SCaMC-2, and succinate dehydrogenase) and muscle regulation (troponin T), through the upregulation of these proteins. Additionally, nocuolin A successfully inhibited the growth of *Navicula* sp. However, despite its broad-spectrum AF activity against both micro and macrofouling organisms, nocuolin A exhibited high toxicity toward the marine biofilm-forming diatoms, barnacle larvae, and non-target organisms, such as *A. salina* and *D. rerio*. The simulations of environmental nocuolin A concentrations, combined with ecotoxicity results, suggest a significant ecological risk. Given that biocompatibility is a key requirement for new compounds to be integrated into AF technologies, nocuolin A's toxicity limits its immediate potential. Further research, including structure optimization and structure-activity relationship (SAR) studies, may help to identify less toxic analogues that retain AF activity while minimizing environmental risks.

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CRediT authorship contribution statement

Sandra Pereira: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. Isabel B. Oliveira: Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. Maria Lúcia Sousa: Writing – review & editing, Validation, Investigation. Catarina Gonçalves: Writing – review & editing, Investigation, Formal analysis. Marco Preto: Writing – review & editing, Validation. Maria V. Turkina: Writing – review & editing, Validation, Methodology, Investigation. Vitor Vasconcelos: Writing – review & editing, Validation, Supervision, Resources, Investigation. Alexandre Campos: Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition. Joana R. Almeida: Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest.

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Data availability

Data will be made available on request.

6. Engineered coatings containing cyclic peptides from cyanobacteria delay the development of a stable macrofouling community

The content of this chapter corresponds to a published original research article:

Gonçalves, C., Pereira, S., Oliveira, I. B., Preto, M., Morais, J., Gomes, L., Romeu, M. J., Semedo, M., Mergulhão, F., Silva, E. R., Vasconcelos, V., Almeida, J. R. 2025. Engineered coatings containing cyclic peptides from cyanobacteria delay the development of a stable macrofouling community. *Trends in Biotechnology*, 44 (5), 1330-1353. DOI: 10.1016/j.tibtech.2025.11.018

Sandra Pereira performed cyanobacterial culture, up-scaling and isolation of portoamides; lab-scale anti-settlement bioassays (*Mytilus galloprovincialis*) at CIIMAR.

Isolation and leaching assays of portoamides were conducted by Marco Preto and Tiago Ribeiro at CIIMAR.

The zebrafish embryo acute toxicity test (ZFET), as well as *in silico* predictions were performed by Isabel Oliveira at CIIMAR.

Portoamides incorporation in coating formulations was performed by Elisabete R. Silva at BIOISI-FCUL/CERENA-IST-UL.

Laboratory-scale anti-biofilm performance and wettability assays of the coatings were conducted by Maria João Romeu, Luciana Gomes e Filipe Mergulhão at LEPABE/ALiCE-FEUP.

Antifouling performance bioassays *in situ* and biological colonization assessment were conducted by Joana R. Almeida and Catarina Gonçalves at CIIMAR.

The amplicon sequencing approach was conducted by Miguel Semedo and Joana R. Almeida at CIIMAR.

Engineered coatings containing cyclic peptides from cyanobacteria delay the development of a stable macrofouling community

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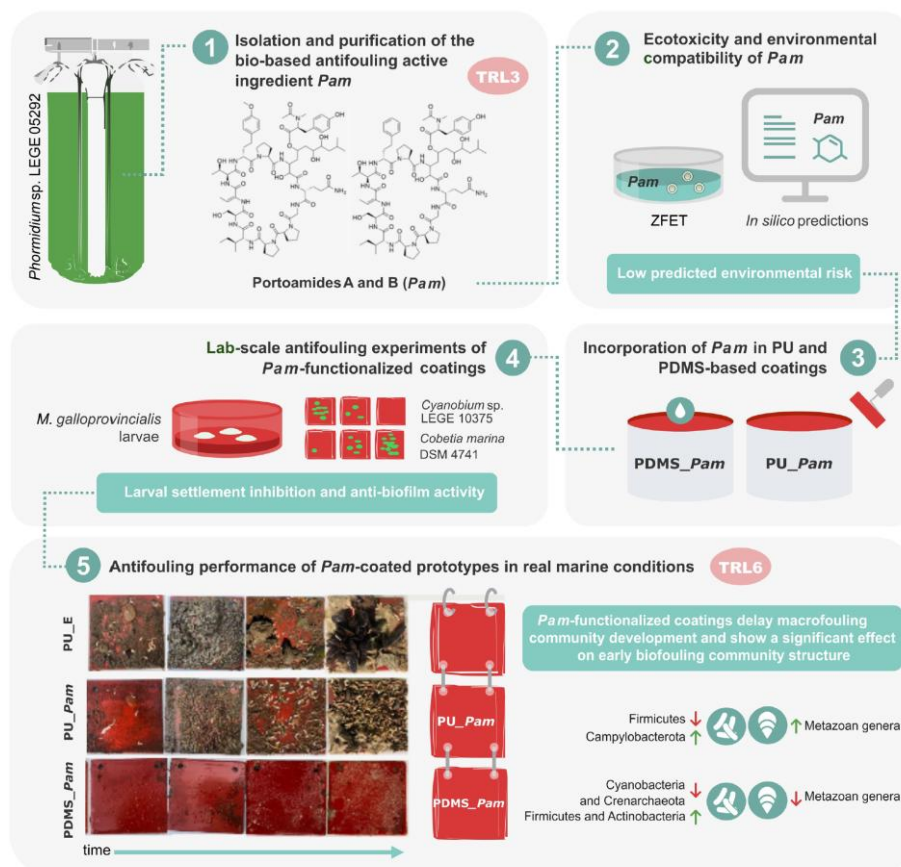
‡These authors contributed equally.

Abstract

Biofouling – the adhesion of organisms and their by-products to submerged surfaces – poses economic and environmental challenges, highlighting the need for sustainable antifouling solutions. This study reports a proof-of-concept investigation into the environmental compatibility and field validation of natural cyclic peptides portoamides A and B (*Pam*), as bio-based antifouling alternative. *Pam* have demonstrated antifouling activity by inhibiting mussel larval settlement and disrupting biofilm formation. Herein, the antifouling performance of *Pam*-engineered coatings was evaluated through anti-settlement, anti-biofilm, as well as marine field tests. Lab-scale tests revealed that *Pam*-based coatings (0.7 wt.%) effectively reduced biofilm thickness, surface coverage and mussel larval settlement. Field trials showed that *Pam*-functionalized coating prototypes outperformed a commercial biocide in use (Econea®), delaying macrofouling community establishment and contributing to enhanced antifouling effectiveness. Overall, this

work supports further development of antifouling engineered systems using *Pam*, representing a significant technological advance (from TRL 3 to 6) toward sustainable marine coating systems.

Graphical Abstract



Keywords

Biofouling Communities; Bio-Based Antifouling Technologies; Natural Products; Coatings; Marine Environmental Health.

6.1. Introduction

Marine biofouling is a natural dynamic process that refers to the reversible or irreversible colonization and adhesion of a diversity of marine organisms – such as bacteria, algae and invertebrates – and their by-products on virtually all submerged structures (Amara *et al.*, 2018). The formation of biofilms and **early-stage biofouling communities** (see [Glossary](#)) relies on the surrounding marine community structure, but is shaped by the nature of the colonization substrates as their intrinsic properties may inhibit certain types of bioadhesion. Still, the first colonizers that successfully settle on a given substrate play a crucial role in driving subsequent colonization. **Microbial cues**, including secondary

metabolites and macromolecular structures, mediate interactions between microorganisms, other benthic organisms, and their environment, ultimately defining the composition and dynamics of the biofouling community (Qian *et al.*, 2022).

Established biofouling communities represent an economic burden to ocean-related industries, with aquaculture and shipping being particularly affected (Demirel *et al.*, 2017; Schultz *et al.*, 2011). Preventive measures used to date, namely the continuous use and release of aggressive AF agents to the marine environment, constitute an environmental problem contributing to the loss of marine biodiversity and damage to ecosystems (Galil *et al.*, 2019). Traditional **AF coatings** have raised significant environmental concerns due to the toxicity of their biocidal components (Lejars *et al.*, 2012). Some biocides were banned (e.g. Tributyltin- TBTs) and the Biocidal Products Regulation (BPR) EU 528/2012 was issued, forcing EU members to invest in the development of environmentally compatible AF strategies (Almeida & Vasconcelos, 2015; Kiosidou *et al.*, 2017; E. R. Silva *et al.*, 2019). Current AF biocide-based coating technologies (95% of total market size) still rely on the controlled release of biocides (primarily copper or metal-based) from the coating matrix. These remain effective from 6 months to 5 years (Amara *et al.*, 2018; Faÿ *et al.*, 2023) and are harmful to marine ecosystems. Fouling release systems (5% market share) are coatings made of crosslinked polydimethylsiloxane (PDMS) matrix and/or doped with 6-10% of PEG (Poly(ethylene glycol)-PDMS copolymer (Holberg *et al.*, 2020), that don't rely on biocidal action. Such systems are not specifically regulated, despite the toxicity of their leachates (microparticles), which seems to be comparable to conventional biocide-release AF paints (Donnelly *et al.*, 2022). The growing recognition of these detrimental effects has encouraged research and development of environmentally-compatible alternatives (Carrier *et al.*, 2023; Paz-Villarraga *et al.*, 2022). Still, fully eco-friendly solutions are not yet available, and the market continues to rely on copper and booster biocides-based polluting coatings (Amara *et al.*, 2018).

Several submerged biological surfaces provide effective protection against **epibiosis** (Liren Chen *et al.*, 2021). Such natural strategies in the marine environment include chemical defence through the production of bioactive metabolites, structural and textural arrangement of outer surfaces, microbial symbiosis, among other (Harder *et al.*, 2004; Z. Li *et al.*, 2023; Qian *et al.*, 2022). Such bio-based strategies hold significant promise as inspiration for the next generation of AF technologies, offering both effectiveness and environmental compatibility (Almeida & Vasconcelos, 2015; Liren Chen *et al.*, 2021). Marine-derived natural products have been demonstrating targeted AF efficacy (Almeida & Vasconcelos, 2015; Cahill *et al.*, 2024; M. Liu *et al.*, 2021; Neves *et al.*, 2024).

Examples include zosteric acid and 6-bromindole-3-carbaldehyde, identified in the eelgrass (*Zostera marina*) and coral-associated bacterium *Acinetobacter* sp., respectively (Magin *et al.*, 2010; Satheesh *et al.*, 2016), synoxazolidinones A and C and pulmonarins A and B, from the sub-arctic ascidian *Synoicum pulmonaria* (Trepas *et al.*, 2014), barettin from the sponge *Geodia barretti* and phidianidine A and B from the nudibranch gastropod *Phidiana militaris* (Labriere *et al.*, 2020; Sjögren *et al.*, 2004). However, a non-biocidal broad-spectrum solution for commercial use is yet to be reached. Recent perspectives point out that engineering surface features to interfere with organisms sensing, thereby modulating the adherence of specific bacterial biofilms and potentially preventing exponential community development, should be a research priority (X. Liu *et al.*, 2024). High-throughput sequencing studies have shown that commercial AF coatings are associated with a lower diversity of early colonizing communities (Briand *et al.*, 2017; Leary *et al.*, 2014), but the potential of bio-based active principles to create such privileged stable AF communities is still unexplored.

Our group has recently assessed the potential of the cyanobacterial cyclic dodecapeptides, portoamides A and B (*Pam*, Figure 32), as a bio-based active ingredient in AF compositions (Antunes *et al.*, 2019). This natural ingredient (*Pam*), isolated from the cyanobacterium *Phormidium* sp. LEGE 05292, showed high effectiveness in the prevention of mussel larvae settlement and was also active towards marine biofouling bacterial strains, while not showing toxicity towards both target and non-target species (Antunes *et al.*, 2019).

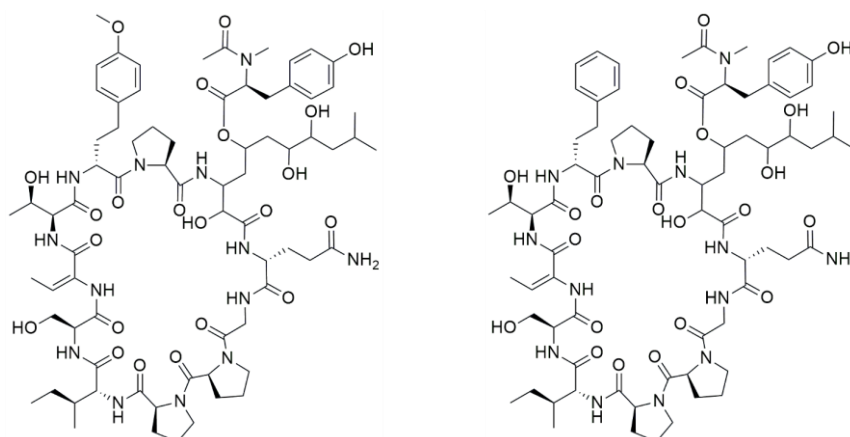


Figure 32. Portoamides A (left) and B (right).

Here, we further explored the potential of *Pam* as a valuable AF ingredient, to advance from early conceptual experimental level (TRL 3) to near-application in relevant environment readiness (TRL 6), establishing an effective and environmentally

responsible alternative to conventional coatings. We hypothesized that *Pam*-coated surfaces could foster the colonization of a specific biofouling community capable of maintaining a stable, low-diversity, and **AF-effective biome** in real marine conditions, while posing minimal environmental risk to marine ecosystems. To reach this, pilot-scale production and incorporation of *Pam* in commercial polyurethane and silicone (PDMS)-based marine coatings were carried out. Produced coatings were evaluated through laboratory-scale experiments and proof-of-concept trials conducted under real sea conditions at Leixões Port (Portugal). *In situ* tests investigated the composition and diversity of microbial and early macrobiota communities through *in vivo* macroscopic inspections and high-throughput sequencing analyses. Environmental risk of *Pam* was further explored including predictive risk parameters and potential leaching from the prepared coating formulations. This integrated approach illuminated our understanding of the biological diversity within early-stage biofouling communities associated with bio-based AF surfaces, contributing to the development of more sustainable AF technologies.

6.2. Method Details

6.2.1. Isolation and Purification of portoamides (*Pam*)

The cyanobacterial strain *Phormidium* sp. LEGE 05292 (LEGE-CC) was cultured in Z8 medium and upscaled to 100 L bioreactors (sleeve bag), where it was grown for 6 months under 14 h light :10 h dark photoperiod ($10\text{--}30\ \mu\text{mol photons s}^{-1}\text{m}^{-2}$), 25 °C and air bubbling. After cell harvesting through centrifugation (4700 rpm), and lyophilization, the resulting biomass (99.5 g d.w.) was extracted with 100% methanol under constant stirring. The resulting crude extract (23.3 g d.w.) was fractionated by normal-phase, Vacuum Liquid Chromatography, and two reversed-phase chromatography steps to afford a 3:1 mixture of portoamides A and B (*Pam*, Figure 32 and Supplementary Figure S2). Silica Gel 60, 0.015-0.040 mm, approximately 300 g), using the following solvent gradient: Fraction A: 500 mL of 10% hexane / 90% ethyl acetate; Fraction B: 500 mL 100% ethyl acetate; Fraction C: 500 mL 100% ethyl acetate; Fraction D: 500 mL 100% ethyl acetate; Fraction E: 500 mL 90% ethyl acetate/10% methanol; Fraction F: 500 mL 90% ethyl acetate/10% methanol; Fraction G: 500 mL 70% ethyl acetate/30% methanol; Fraction H: 500 mL 70% ethyl acetate/30% methanol; Fraction I: 500 mL 50% ethyl acetate/50% methanol; Fraction J: 500 mL 50% ethyl acetate/50% methanol; Fraction K: 1000 mL 100% methanol; Fraction L: 500 mL 100% methanol.

Fractions from J to L were confirmed by LC-MS/MS analysis to contain *Pam* and were thus further fractionated using a prepacked SPE C18 (50 g) cartridge as the stationary phase. The material adsorbed to the cartridge was eluted according to the following

sequence: 150 mL 25% water/75% methanol; 150 mL 10% water/90% methanol; 250 mL 100% methanol; 200 mL 50% ethyl acetate/50% methanol; 100 mL 100% ethyl acetate; 50 mL dichloromethane; 100 mL 100% methanol; 50 mL ethyl acetate; 150 mL 100% methanol; 100mL isopropanol. The eluate was collected in 10 mL glass tubes, the contents of which were pooled according to their TLC profiles, resulting in 17 fractions (fraction 1 to 17). Fractions 1 until 11 were confirmed to contain *Pam* by LC-MS/MS analysis (all eluted with methanol). These were subsequently merged (resulting in a mass of 4.7 g) and fractionated on Buchi Pure C-850 FlashPrep (Buchi Labortechnik AG, Flawil, Switzerland). Briefly, the sample was loaded onto a C18 column (220 g) with a flow of 50 mL min⁻¹, for 65 minutes, using a gradient program, from a mixture of 50% methanol/50% water, to 100% methanol and then to a mixture of 50% methanol/ 50% dichloromethane. The eluate was collected in 20 mL test tubes whose contents were pooled according to their UV (absorbance detector at 254, 265, 276 and 650 nm) and ELS profiles, resulting in 11 fractions (A to K). LC-MS/MS analysis confirmed that fraction D (1.1 g) contained a mixture of portoamides A and B (*Pam*) in a proportion of 3:1 (Supplementary Figure S2). This defined mixture of naturally produced portoamides (*Pam*) was maintained and used in all the following experiments.

6.2.2. Ecotoxicity and Environmental Compatibility of *Pam*

6.2.2.1. Zebrafish Embryo Acute Toxicity Test (ZFET)

The OECD guideline 236 also known as Fish Embryo Acute Toxicity test (FET; OECD, 2013) (OECD, 2013), using zebrafish (*Danio rerio*) embryos, with modifications, was used to assess the lethal and sub-lethal effects of *Pam* (S. Pereira *et al.*, 2024). The certified Bioterium of Aquatic Organisms (BOGA, CIIMAR, Porto) performed fish maintenance, breeding and embryo collection. All experiments were conducted in accordance with ethical guidelines of the European Union Council (Directive 2010/63/EU) and the Portuguese Ministry of Agriculture, Sea, Environment, and Spatial Planning (Decreto-Lei no. 113/2013, 7 August) for the protection of animals used for experimental and other scientific purposes. Fish embryo exposures were conducted in E3 medium (pH 8.6), at 28 °C, and adapted to 96-well plates. Briefly, ten newly fertilized eggs up to 1-hour post fertilization (hpf) were exposed, per condition, to a range of concentrations (4.0 to 7.5 mg L⁻¹) of 1 until 96 hpf. Regular inspections of the embryos were performed under the microscope every 24 hours, with solution renewal after each inspection. Indicators of lethality included coagulation of fertilized eggs, absence of heartbeat or somite formation, and failure of tail detachment from the yolk sac. Recorded sub-lethal endpoints included hatching time, tail deformities, presence of heart or yolk sac oedema, and locomotion difficulties. Controls and solvent controls (DMSO 0.1%)

were included in the assays. Zebrafish experiments met the requirements for control survival and normal development of $\geq 90\%$. The assay was repeated three times. Zebrafish median lethal concentration (LC_{50}) estimates were obtained by nonlinear fitting to a four-parameter logistic equation through the least squares statistical method using Prism V. 8.2.1 (Graph Pad, CA, USA).

6.2.2.2. *In silico* predictions

The Toxicity Ratio, estimated LC_{50} vs the experimental LC_{50} value for a given species (Maeder *et al.*, 2004), was calculated to distinguish *Pam* between a baseline toxicant of a specifically toxic substance, as PBT (persistence, bioaccumulation, and toxicity) criteria of *Pam* are not available. The generated predictions were based on the molecular structure of the most abundant peptide of *Pam* - portoamide A. Input values included compound name, molecular weight, chemical formula (Leão *et al.*, 2010) and SMILES notation. To understand the potential ecological risk of *Pam*, we used the Marine Antifoulant Model to Predict Environmental Concentrations (MAMPEC; Version 3.0; Deltares, Netherlands), by considering three different descriptors - environments, emission scenarios, and physicochemical properties (Oliveira *et al.*, 2014; Oliveira, Groh, *et al.*, 2016; Van Hattum *et al.*, 2006). MAMPEC is able to calculate Predicted Environmental Concentrations (PEC), used for risk characterization (OECD-EU working group for the risk assessment of antifoulants) (OECD, 2004). The worst-case scenario was used for risk characterization. Input values included the molecular formula and mass (Leão *et al.*, 2010; Ribeiro *et al.*, 2017).

6.2.3. Incorporation of *Pam* in polyurethane (PU) and polydimethylsiloxane (PDMS)-based coatings

Commercial polyurethane (PU) and polydimethylsiloxane (PDMS)-based marine polymeric matrices were used to prepare and optimize coating formulations incorporating *Pam*, according to previously developed methodologies (Neves, Gomes, *et al.*, 2022; Elisabete R. Silva *et al.*, 2021). To enhance compatibility and ensure homogeneous formulations, *Pam* was first dissolved in *N*-methyl-2-pyrrolidone (NMP, 99.5%, Acros Organics, Geel, Belgium) at *Pam*/solvent ratios of 0.06 ± 0.01 and 0.04 ± 0.01 for PU- and PDMS-based formulations, respectively. These bioactive solutions were blended into two distinct bi-component coating systems: a PU-based system comprising base resin F0032 and curing agent 95,580 (Hempel, A/S, Copenhagen, Denmark), and a foul-release PDMS-based coating system, HEMPASIL X3+ 87500 paint (Hempel, A/S, Copenhagen, Denmark), which consists of a base resin (reference 87509) and a curing

agent (reference 98950). The bioactive solutions were added in exact amounts to achieve the desired contents in the wet formulation systems (Table 18).

For comparison purposes, coating formulations containing the commercial biocide Ecomea® (E) were prepared. Biocide E was firstly dissolved in methyl ethyl ketone (MEK, > 99.0%, Sigma-Aldrich) to obtain biocidal solutions with a biocide/solvent ratio of 0.45 ± 0.02 . The resulting biocidal solutions were then incorporated into the bi-component PU- and PDMS-based coating systems, following the same procedure described above.

The optimized coating formulations were further applied to coat several acrylic substrates (Acrilcorte, Portugal) for lab-scale assessment of anti-settlement (mussel larvae) and anti-biofilm activities, as well as AF performance under real marine conditions, following the procedure recommended by the paint supplier Hempel A/S.

For the larval settlement assessment, 24 well-acrylic plates (4 wells/formulation) were coated using conventional brush painting (Vilas-Boas *et al.*, 2021). Coated acrylic slides (1 cm², 100 slides/formulation) and larger prototypes (10 cm²) for lab-scale anti-biofilm testing and real marine AF performance assessments, respectively, were prepared using a 4-sided film applicator (300 µm) with a reservoir (TQC Sheen). Formulations selected for each of the AF assessment experiments are indicated in Table 18.

Specifically, for the PDMS-based formulations, substrates were pre-coated with a universal tie-coat (base resin XA17-17310 and curing agent XA18-RD003), followed by an 8-hour curing step at room temperature (approximately 23 °C). After this step, a second coating with the HEMPASIL X3+ system was applied, with an additional curing step of about 1 week to ensure the complete curing. For PU-based formulations, substrate coating involved a single coating step and curing at room temperature (approximately 23 °C) for about 1 week.

6.2.4. Lab-scale AF experiments of *Pam*-functionalized coatings

6.2.4.1. Anti-settlement activity of *Pam* in polyurethane (PU) and polydimethylsiloxane (PDMS)-based coating

For a preliminary efficacy assessment of the coating formulations, *M. galloprovincialis* plantigrade larvae were used to evaluate their ability to settle in the presence of the different coatings under laboratory-controlled conditions. Larvae were collected from Memória beach (N41°13'51.5", W8°43'15.5") at low tide. Competent plantigrades with exploring behaviour (i.e., moving their foot searching for the appropriate substrate to settle) were selected in the laboratory and transferred to the coated wells with different formulations (PU and PDMS). All the coated wells were filled with filtered natural

seawater to reduce any interferents. The coating formulations (PU_*Pam* 0.3 and 0.7 wt.% and PDMS_*Pam* 0.7 wt.%) were tested in four replicates (wells) with five plantigrades per well with a total of 20 larvae per treatment. A negative control (base coating) was included. After 15 and 40 h, the percentage of larval settlement as well as thread production was determined by the presence/absence of efficiently attached byssal threads (on the surface of the coating or within larvae) produced by each individual in each formulation condition. Larvae that did not settle (i.e., did not produce byssal threads) remained alive during the assays, but unattached to the substrate throughout the assay period. The best-performing polymeric matrix was selected for further lab-scale analysis on anti-biofilm properties.

6.2.4.2. Leaching assessment of *Pam*-functionalized coating formulations

After 40 h of exposure, seawater from the coated wells containing *Pam*-functionalized formulations (PU_*Pam* 0.35% and 0.7 wt.% and PDMS_*Pam* 0.7 wt.%) and controls (PU_C and PDMS_C) were analysed by LC-MS. Negative (blanks) and a positive control containing *Pam* were included. Samples were injected in a Liquid Chromatograph Thermo Finnigan Surveyor HPLC System (Thermo Scientific, MA, USA), coupled to a Mass Spectrometry LCQ Fleet™ Ion Trap Mass Spectrometer (Thermo Scientific, MA, USA). Data were acquired and processed in Xcalibur™ version 2. Mass Spectrometer Tune Method parameters optimization was performed through direct injection of purified portoamides A, B, C and D (~5 µg mL⁻¹) dissolved in Methanol (0.1% formic acid). The Mass Spectrometer operated in electrospray positive polarity mode using *Single Ion Monitoring* (SIM). The spray voltage was maintained at 3.50 kV; Capillary temperature at 275 °C; Capillary voltage and tube lens maintained at 300 and 120 kV, respectively. Nitrogen was used as sheath and auxiliary gas and helium as a buffer. Separation was achieved on an ACE Excel C18 (50 × 2.1 mm I.D., 1.7 µm, Batch: V17-1253, Avantor® ACE®) kept at 35 °C, with a flow rate of 0.25 mL/min. and injected volume was 10 µL in a no waste mode. The eluents used were methanol (A) and water (B) both acidified with formic acid at 0.1% (v/v). The gradient program started at 45% A, increasing to 90% A in 8 min, returning to initial conditions in 5 min, and equilibrating for 8 min with 45% A.

6.2.4.3. Wettability measurements of *Pam*-functionalized coating formulations

Wettability is a key surface property supporting the AF behaviour of different polymeric matrices and was evaluated to understand the influence of *Pam* incorporation on the original wettability properties of coatings. The influence of *Pam* on the surface wettability of PDMS and PU coatings was evaluated through wettability tests performed on coated acrylic substrates (10 × 10 mm), by monitoring the hydrophobicity of the developed

coating formulations (static water contact angle over time). Static contact angle measurements were recorded over time using the sessile drop method, following the ASTM standard D5725-99 (2003). The sessile drop method was applied at 25 °C, and images were recorded and analysed from 0 to 600 seconds using a contact angle meter (Dataphysics OCA 25, Filderstadt, Germany) and dpiMAX software. Water contact angle values represent the average of at least 10 independent measurements per formulation

6.2.4.4. Anti-biofilm properties of *Pam* polydimethylsiloxane (PDMS)-based coating

Cyanobium sp. LEGE 10375 (Synechococcales order) was used as a model marine bacterium to study the AF performance of the functionalized coatings at laboratory scale. This picocyanobacterium has been used in marine biofouling studies due its strong biofilm-forming capacity and ecological relevance (Faria *et al.*, 2020; Faria, Gomes, *et al.*, 2021; Faria, Teixeira-Santos, *et al.*, 2021). Cyanobacteria are dominant constituents of marine phototrophic biofilms, known for initiating biofilm formation and secreting large amounts of EPS, which contribute significantly to biofilm structure and resilience (Rossi & De Philippis, 2015). Additionally, members of the Synechococcales order, including *Cyanobium* sp., are widely distributed across diverse geographic regions (Africa, America, Antarctica, and Europe) and thrive in marine, brackish, and freshwater environments (Callieri, 2008). Together with *Synechococcus*, *Cyanobium* represents one of the dominant picocyanobacterial genera in these ecosystems (Callieri, 2008; Faria, Gomes, *et al.*, 2021; Ramos *et al.*, 2018; Rossi & De Philippis, 2015). The *Cyanobium* sp. LEGE 10375 strain was obtained from the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) (Ramos *et al.*, 2018). Bacterial cells were grown in 750 mL of Z8 medium supplemented with 25 g L⁻¹ of synthetic sea salts (Tropic Marin) and vitamin B₁₂ (Sigma Aldrich, Merck, Saint Louis, MO, USA) (Kotai, 1972). Cultures were incubated under a cycle of 14 h of light (10–30 µmol photons/m²/s), followed by 10 h of darkness at 25 °C (Romeu *et al.*, 2019).

Cyanobacterial biofilm development was performed using 12-well microtiter plates (VWR International) in a long-term assay under controlled hydrodynamic conditions to mimic marine environments (Romeu *et al.*, 2019). Borosilicate glass slides (1 cm²) were used as pristine control surfaces. The PDMS_C coated slides (negative control), *Pam*-functionalized coatings (PDMS_ *Pam* (0.3 wt.%), PDMS_ *Pam* (0.5 wt.%), PDMS_ *Pam* (0.7 wt.%)), and glass slides were sterilized by ultraviolet radiation for 30 min before use. After fixing the surfaces with double-sided adhesive tape to 12-well microtiter plates, 3 mL of the adjusted cyanobacterial suspension at a final concentration of 1 × 10⁸ cells

mL⁻¹ was added into each well, and the microtiter plates were incubated at 25 °C and 185 rpm in an orbital shaker with a 25 mm diameter (Agitorb 200ICP, Norconcessus). In this platform, an average shear rate of 40 s⁻¹ is achieved at the beginning of biofilm formation, which is similar to the values of shear rate that a ship in a harbour is subjected to (50 s⁻¹) (Alexandrou, 2001; Bakker *et al.*, 2003). During biofilm development, the shear rates at the biofilm surface were shown to depend on the biofilm architecture (Romeu *et al.*, 2024). Biofilm development was followed for 49 days (seven weeks) since it is accepted that a two-month interval for maintenance is the minimum period for economically viable underwater monitoring systems (Blain *et al.*, 2004; Romeu *et al.*, 2019). During this time, the medium was replaced twice a week, and to mimic real light exposure periods, a photoperiod of 14 h light (8–10 µmol photons m⁻² s⁻¹)/10 h dark cycles was applied.

After 49 days of biofilm development, cyanobacterial biofilms formed on the control surfaces (Glass and PDMS_C), and the *Pam*-functionalized coatings (PDMS_*Pam*) were analysed by CLSM. Coupons were removed from the microplates, washed with saline solution, and the biofilms were stained for 10 min with 6 µM cell-permeant nucleic acid marker SYTO®9 (Thermo Fisher Scientific, Waltham, MA, USA). Each stained biofilm was mounted on a microscopic slide and image acquisition was performed using a Leica TCS SP5 II confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with a 40× water objective lens (LEICA HCX PL APO CS 40.0x/1.10WATER UV) (Faria *et al.*, 2020; Faria, Gomes, *et al.*, 2021; Romeu *et al.*, 2023). Three-dimensional (3D) projections of the biofilms were constructed from the CLSM acquisitions using the “Easy 3D” function of the IMARIS 9.1 software (Bitplane, Zurich, Switzerland). Additionally, biofilm structural parameters such as biovolume (µm³/µm²), biofilm thickness (µm), and surface coverage (%) were determined using COMSTAT image analysis software (National Institutes of Health, Bethesda, MD, USA) (Heydorn *et al.*, 2000). A minimum of five stacks of horizontal-plane images (512 × 512 pixels, corresponding to 387.5 µm × 387.5 µm) with a z-step of 1 µm were acquired per sample, and a total of three samples coming from two biological assays were analysed (n = 6).

To expand the microbial scope of the anti-biofilm assays, the marine heterotrophic bacteria *Cobetia marina* DSM 4741 was tested as an additional microfouler organism. Colonies of *C. marina*, previously grown on the salt-rich Våatanen Nine Salt Solution (VNSS) marine medium supplemented with 15 g/L agar (VWR International, Leuven, Belgium), were inoculated into 100 mL of liquid VNSS medium. After overnight incubation at 25 °C and 120 rpm (Agitorb 200ICP, Norconcessus, Ermesinde, Portugal), the

bacterial suspension was centrifuged for 10 min at 3100× *g* (Eppendorf Centrifuge 5810R, Eppendorf, Hamburg, Germany). The resulting pellet was resuspended in fresh VNSS medium and adjusted to a final concentration of 1×10^8 cells/mL prior to biofilm formation assays.

Biofilm development was assessed on PDMS_C-coated slides (control) and on the most promising *Pam*-functionalized coating (PDMS_*Pam* (0.7 wt.%)) under the same conditions. Biofilms were allowed to develop over 14 days, with the culture medium replaced twice weekly. After 14 days, biofilms were characterized by using Optical Coherence Tomography (OCT) to quantify biofilm thickness and total volume, while the number of biofilm cells was determined by colony-forming unit (CFU) enumeration. The culture medium was removed, and the microplate wells were gently rinsed with sterile saline solution (8.5 g L⁻¹ NaCl) to eliminate loosely attached cells. Fresh sterile saline was then added, and 3D OCT acquisitions were performed using a Thorlabs Ganymede Spectral Domain OCT system with a central wavelength of 930 nm (Thorlabs GmbH, Dachau, Germany) (Romeu *et al.*, 2019). Image analysis and quantification of biofilm parameters were performed (Romeu *et al.*, 2022). Following imaging, biofilms were detached from the surfaces by immersing the coupons in 2 mL of sterile saline and vortexing for 3 min. The resulting bacterial suspensions were serially diluted and plated on VNSS agar, followed by overnight incubation at 25 °C for CFU enumeration.

6.2.5. Antifouling performance assessment of coated prototypes in real marine conditions

For the analyses of biological colonization in real sea conditions, prototypes (10 × 10 cm) coated with PU and PDMS-based marine coating formulations containing *Pam*, the commercial AF agent Econe[®] and correspondent controls were subjected to immersion tests at a pier in the cruise ship terminal of the Leixões Port, Matosinhos, Portugal (41° 11' N 8° 42' W), in accordance with ASTM D6990 and D3623-78a standards. The *in situ* test was performed from September 2021 to January 2023. Nine timepoints of inspection were applied including weekly inspections for the first month, and then after 2, 3, 5, 7, 9 and 16 months of submersion. During inspections, photographic records of all the prototypes and environmental parameters data were recorded. Biological samples of the biofouling community (biofilm and surface first colonizers) were collected from each prototype according to the level of colonization for each formulation over time. For the PU-coating prototypes, biological samples (4 replicates) were collected by gentle scratching from the surface of the prototypes in the timepoint 8 (after 9 months of submersion). For the PDMS-coating prototypes (3 replicates), the same procedure was

only possible after 16 months of exposure due to the intrinsic AF properties of the PDMS matrix and was conducted only for the PDMS_E and PDMS_Pam formulations. All prototypes were permanently immersed throughout the duration of the test (16 months). At the sampling site and for all time points, seawater physical-chemical parameters such as temperature (°C), salinity, dissolved oxygen concentration (mg L⁻¹), oxygen saturation (%), and pH were measured, with calibrated multiparametric probes (HI98194 Hanna Instruments). Field tests were carried out under an average pH of 7.78 and a salinity ranging from 24 to 38‰, for a water depth of 1.5 to 3 m, with temperatures ranging from 13 to 21 °C, and for a test period of 16 months to include all-season conditions (Table S1).

Photographic records of the prototypes from inspections at different time points were analysed to determine biomass coverage percentages using Fiji/ImageJ software v.1.54. In situ registrations and prototype images were also used for a qualitative assessment of predominant fouling taxa throughout the study. Taxa were classified according to the World Register of Marine Species (WoRMS) nomenclatural system (WoRMS Editorial Board, 2021).

The biological samples of the early biofouling community were used to investigate the microbial and early macrobiota community composition and diversity by HTS of the 16S and 18S rRNA genes, which are universal markers for prokaryotic and eukaryotic taxonomic classification, respectively. Total DNA was extracted using commercial extraction kits (QIAamp DNA Microbiome Kit, Qiagen). Extracted DNA was prepared for Illumina MiSeq PE250 sequencing by PCR amplification of hypervariable regions of 16S (V4-V5) and 18S (V4) rRNA genes, using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') / 907R (5'-CCGTCAATTCCTTTGAGTTT-3') and 528F (5'-GCGGTAATTCCAGCTCCAA-3') / 706R (5'-AATCCRAGAATTTACCTCT-3'), respectively (Supplementary Table S15).

The raw FASTQ files obtained with Illumina MiSeq were trimmed for primer and barcode removal and imported into R, using the dada2 package (Callahan *et al.*, 2016). Sample filtering, dereplication, ASV inference, merging, and chimera removal were performed with default settings for both 16S and 18S reads, similarly to previously described (Semedo & Song, 2020). These pre-processing steps resulted in a mean number of 22,305 16S reads and 44,627 18S reads per sample (Table S3), corresponding to a total of 12,112 and 2,402 ASVs, respectively. Prokaryotic taxonomy was assigned on the 16S final reads using the dada2 naive Bayesian classifier with the Silva v138 reference database (Quast *et al.*, 2012). Taxonomy filtering was applied to the 16S reads by removing eukaryotic, mitochondrial, and chloroplast sequences (201 ASVs were

removed). Eukaryotic taxonomy was assigned on the 18S final reads using the same dada2 classifier with the Silva v132_18S reference database (Quast *et al.*, 2012). Additionally, for disambiguation of some taxa classification, eukaryotic taxonomy was further classified with the PR2 v4.14.0 database (Guillou *et al.*, 2012). and through an NCBI blast on the Core Nucleotide Database (core_nt). Relative abundances of each ASV per sample were calculated in the filtered 16S and 18S datasets by dividing the absolute abundance (counts) of each ASV by the sum of counts of all ASVs, using the phyloseq package (McMurdie & Holmes, 2013). The number of observed ASVs per sample and the Shannon index were used to estimate species richness and α -diversity, respectively. Differences in species richness and α -diversity between the different treatments were tested with a two-way analysis of variance (ANOVA, coating matrix and formulation as categorical factors) and a Tukey's HSD test to perform multiple comparisons. β -diversity among samples was estimated with the Bray-Curtis dissimilarity calculator and a principal coordinate analysis (PCoA). Significant effects of coating matrix and formulation on community dissimilarity were tested by a non-parametric permutational ANOVA (PERMANOVA) using the adonis function of the vegan package in R (Oksanen *et al.*, 2017). Multivariate homogeneity of group dispersion was evaluated using the betadisper function of the same R package. To normalize α - and β - diversity estimates, samples were randomly subsampled to the lowest number of sequences per sample in each dataset.

6.2.6. Quantification and statistical analysis

Biological and environmental data were presented as means with standard deviations or as individual observations. Normality and variance equality were assessed using Kolmogorov-Smirnov and Levene's tests, with appropriate data transformations applied when assumptions were not met. Independent unpaired t-tests ($\alpha = 0.05$, $p < 0.05$) were used to compare lab-scale anti-settlement test results and qualitative biofilm parameters. Statistical analyses were conducted using PASW Statistics 21.0.

LC₅₀ values for zebrafish were estimated through nonlinear regression using a four-parameter logistic model with least-squares fitting in Prism v8.2.1 (GraphPad, CA, USA).

Analysis and downstream processing of DNA sequencing data were conducted within the R statistical computing environment.

6.3. Results and Discussion

6.3.1. Environmental compatibility of the naturally produced active ingredient *Pam*

The cyanobacterial AF bioactive ingredient *Pam* was produced under optimized culture conditions, yielding 1.1 g of purified material over 6 months (Supplemental Information, Figure S1). Its environmental compatibility and AF behaviour in coatings were evaluated. Given the previous indications on the suitable ecotoxicological profile of *Pam* (Antunes *et al.*, 2019; Leão *et al.*, 2010), the Fish Embryo Acute Toxicity test (OECD guideline 236) was performed to assess toxicity at higher biological levels and estimate risk parameters in ecologically relevant scenarios. Treatments were compared to 0.1% DMSO control, a safe concentration in ZFET (Hoyberghs *et al.*, 2021). The LC₅₀ for zebrafish embryos was 6.25 (6.071 – 6.43, 95 CI) mg L⁻¹ (Supplementary Figure S3). This was lower than reported values for the microalgae *Chlorella vulgaris* (IC₅₀= 12.8 mg L⁻¹), *Ankistrodesmus falcatus* (IC₅₀ = 24.7 mg L⁻¹) and *Chlamydomonas reinhardtii* (IC₅₀=12.6 mg L⁻¹) (Leão *et al.*, 2010), or on *Artemia salina*, which showed no mortality up to 24.4 mg L⁻¹ (Antunes *et al.*, 2019).

The experimental LC₅₀ (6.254 mg L⁻¹) was compared to ECOSAR's estimated LC₅₀ for *D. rerio* (1.4x10⁵ mg L⁻¹) to determine the Toxic Ratio (TR). A TR of 1.66 × 10⁷ exceeded the threshold of 10, supporting a specific toxic mode of action (Ribeiro *et al.*, 2017; M. L. Sousa *et al.*, 2020). *Pam* had a low log K_{ow} (-5.488), indicating minimal bioconcentration potential. However, as ECOSAR is unreliable for molecular weights > 1000 g mol⁻¹, which is the case of *Pam* (MW of portoamides A ≈ 1532 g mol⁻¹ and B ≈ 1502 g mol⁻¹), results remain indicative (Ribeiro *et al.*, 2017).

Quantitative risk assessment compared the predicted environmental concentration (PEC, from MAMPEC; Supplementary Table S17) to the predicted no-effect concentrations (PNEC from zebrafish LC₅₀). A PEC/PNEC >1 suggests ecological risk (European Commission, 2003). Simulated environmental concentrations obtained here (Supplementary Table S17) were significantly lower than those of known AF compounds, such as tralopyril (Econea®) and tributyltin (Oliveira *et al.*, 2014). A comparative analysis between *Pam* and a set of reference AF biocides and natural alternatives, regarding persistence, bioaccumulation, and toxicity (PBT criteria), together with regulatory status, key biological effects, and risk characterization data (Supplementary Table S18), shows that traditional AF biocides such as tributyltin and copper exhibit high persistence, strong bioaccumulation potential, and significant toxicity across trophic levels, resulting in historically high environmental risks (Antizar-Ladislao, 2008; A. C. A. Sousa *et al.*, 2014).

Tralopyril, used in this study as a reference compound under its commercial designation Econe[®], as well as DCOIT, although approved under the Biocidal Products Regulation (BPR), exhibit elevated toxicity and a high ecological risk ($PEC/PNEC > 1$) (B. G. de Campos *et al.*, 2022; Oliveira *et al.*, 2017; Oliveira, Groh, *et al.*, 2016; Santos *et al.*, 2020). In turn, medetomidine seems to fulfil the requirements of a good biocide (low persistence; rapid elimination rate and low ecological risk) (Cai, Apell, *et al.*, 2021; Fox *et al.*, 2014; Hilvarsson *et al.*, 2009). although more studies on the effect of this compound are needed due to their possible effects in some organisms. In contrast, naturally derived compounds with available ecotoxicological data, such as capsaicin, and *Pam*, present a more favourable ecotoxicological profile, characterized by low acute toxicity, low persistence, and limited bioaccumulation potential. Specifically, *Pam* demonstrated no mortality in *Artemia salina*, reversible effects on mussel larvae settlement, and the highest ZFET LC_{50} , indicating an overall low hazard potential for non-target organisms. Recent AF candidates of natural origin – such as barettin (Labriere *et al.*, 2020; Sjögren *et al.*, 2004), phidianidine A/B (Labriere *et al.*, 2020; Q. Xu *et al.*, 2022), adenosine and 2'-deoxyadenosine (S. Pereira *et al.*, 2025), and actinobacterial metabolites including napyradiomycins (F. Pereira *et al.*, 2020), madeirone, and neomarinone (Wissner *et al.*, 2024) – also seem to show low acute toxicity, reinforcing the environmental compatibility trend observed for natural product-based antifoulants. However, data on the PBT criteria are still missing to fully infer about these compounds' toxicity. Among the selected natural compounds, only nocuolin A exhibited higher ecological risk, limiting its immediate potential as an environmentally compatible option (S. Pereira *et al.*, 2024).

Taken together, this comparative framework highlights that *Pam* combines strong biological efficacy with an improved environmental safety margin, supporting its potential as a sustainable AF candidate relative to both currently used and emerging biocides.

6.3.2. Bio-based coating formulations containing *Pam* preserve the inherent anti-biofilm and larval settlement inhibition potential

Commercial polyurethane (PU) and polydimethylsiloxane (PDMS)-based marine polymeric matrices were used to optimize coating formulations incorporating *Pam* (Neves, Gomes, *et al.*, 2022; Elisabete R. Silva *et al.*, 2021). Bioactive solutions were added in defined amounts to reach the target concentrations, and formulations containing the commercial biocide Econe[®] (E) were included for comparison (Table 18).

For lab-scale pre-validation of the *Pam*-functionalized coating formulations regarding AF properties, their ability to inhibit the settlement and/or thread production ability of *M.*

galloprovincialis larvae were evaluated in coated 24-well microplates for PU_*Pam* (0.35 and 0.7 wt.%) and PDMS_*Pam* (0.7 wt.%) formulations (Figure 33.A).

Table 18. Coating formulations.

*Coating	Polymeric matrix	Formulation		Average ratio of Base/Curing Agent (w/w)	Content in wet formulation (wt.%)	
					<i>Pam</i>	Econea®
PU	Polyurethane	PU_C	+ ∴	8.9 ± 0.1	-	-
		PU_ <i>Pam</i> (0.3 wt.%)	+	8.8 ± 0.1	0.34 ± 0.01	-
		PU_ <i>Pam</i> (0.7 wt.%)	+ ∴	8.9 ± 0.1	0.72 ± 0.01	-
		PU_E	∴	8.8 ± 0.1	-	0.56 ± 0.01
PDMS	Polydimethylsiloxane	PDMS_C	+ • ∴	7.6 ± 0.1	-	-
		PDMS_ <i>Pam</i> (0.3 wt.%)	•	7.7 ± 0.1	0.33 ± 0.01	-
		PDMS_ <i>Pam</i> (0.5 wt.%)	•	7.7 ± 0.1	0.51 ± 0.01	-
		PDMS_ <i>Pam</i> (0.7 wt.%)	+ • ∴	7.7 ± 0.1	0.67 ± 0.01	-
		PDMS_E	∴	7.5 ± 0.1	-	0.56 ± 0.01

* PU – polyurethane-based marine paint (F0032, Hempel A/S); Econea® (E)– 4-bromo-2-(4-chlorophenyl)-5-(trifluoromethyl)-1H pyrrole-3-carbonitrile (biocide); PDMS – polydimethylsiloxane (PDMS) based marine paint (Hempasil X3+, Hempel A/S).

Formulations used in each experimental section are marked by the symbols: + anti-settlement assays; • anti-biofilm assays; ∴ in situ prototypes.

PU marine coatings containing *Pam* seem to be effective against the settlement of mussel larvae on coated wells. PU_*Pam* 0.35 wt% and 0.7 wt% formulations significantly inhibited larval settlement after 40 h (10 and 0% of larval settlement, respectively) when compared to the control (PU_C). PU_*Pam* 0.7 wt.% formulation seems particularly effective as results show 0% larval settlement even after 15 h of exposure (Figure 33.A). Regarding PDMS-based coating formulations, 0% of larval settlement was observed in the control (PDMS_C), which did not allow gauging the AF effect of *Pam*. This result is consistent with previous studies using lab-scale anti-settlement bioassays testing this base-coating (Neves, Vilas Boas, *et al.*, 2022; Resende *et al.*, 2021; Vilas-Boas *et al.*, 2021), and emphasizes the fact that the matrix itself acts as a fouling release surface, as further supported by the more dynamic wettability behaviour observed for the PDMS formulations (Supplementary Figure S4).

Regarding the byssal threads production endpoint (Figure 34.A), for both the PU and PDMS coating matrices, test larvae were competent in the ability to produce byssal threads in all tested conditions. This serves as test validation because larvae seem to maintain the purpose of settlement by producing the structures for fixation but are unable to fix on selected coated surfaces, namely in PDMS and PU_*Pam* 0.7 wt.% formulations.

Analysis of the seawater media used in the coated wells for the controls and *Pam*-functionalized formulations, by LC-MS, indicated that the leaching of *Pam* over the 40 h period was negligible for all the formulations tested (Supplementary Figure S2).

The antibiofilm potential of *Pam*-functionalized PDMS coatings (PDMS_*Pam* (0.3 wt.%), PDMS_*Pam* (0.5 wt.%), and PDMS_*Pam* (0.7 wt.%)) was evaluated under controlled hydrodynamic laboratory conditions after 49 days of cyanobacterial *Cyanobium* sp. LEGE 10375 biofilm development, and 14 days of bacterial *Cobetia marina* biofilm development. Only PDMS formulations were used on these bioassays, given the observed superior anti-settlement performance compared to PU formulations. Regarding cyanobacterial biofilms, quantitative and qualitative structural differences between biofilms formed on the different coating formulations and the control slide surfaces (Glass and PDMS_C) were analysed by confocal laser scanning microscopy (CLSM) (Figure 34.B). A reduction in the average biofilm biovolume was observed for all functionalized surfaces compared to glass (although with no statistically significant differences), and a significant effect was found for PDMS_*Pam* (0.7 wt.%) compared to PDMS_C, reaching almost 40% reduction (Figure 33.B).

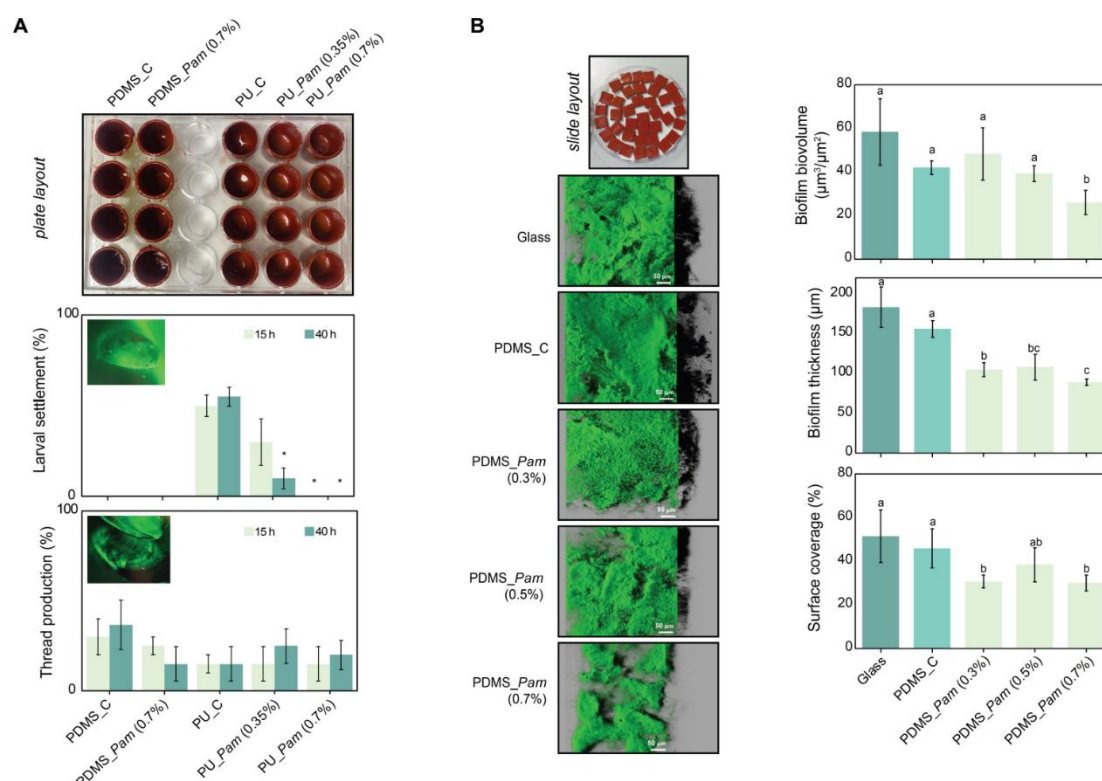


Figure 33. Antifouling performance of *Pam*-functionalized coating formulations in macro (A) and microfouling (B) bioassays. (A) Larval settlement and byssal threads production of *Mytilus galloprovincialis* larvae in the presence of the *Pam*-functionalized coating formulations (PDMS_*Pam* 0.7 wt.%; PU_*Pam* 0.35 wt% and PU_1 0.7 wt%) and control formulations (PDMS_C and PU_C) *indicates significant differences against the respective control formulation ($p < 0.05$, t-test). (B) Representative three-dimensional (3D) cyanobacterial biofilm images and quantitative parameters (biofilm

biovolume, biofilm thickness, and surface coverage) obtained from CLSM analysis of 49-days biofilms formed of *Cyanobium* sp. LEGE 10375 grown on control surfaces (Glass and PDMS_C) and functionalized coatings (PDMS_*Pam* 0.3 wt.%, PDMS_*Pam* 0.5 wt.%, and PDMS_*Pam* 0.7 wt.%). 3D images show the biofilm aerial view, with the virtual dark shadow projection on the right (illustrative of biofilm thickness). White scale bars are 50 μm . Means and standard deviations (SD) are shown (letters show statistical differences at $p < 0.05$, t -test).

Thinner *Cyanobium* sp. LEGE 10375 biofilms were formed on the functionalized coatings (PDMS_*Pam* (0.3 wt.%), PDMS_*Pam* (0.5 wt.%), and PDMS_*Pam* (0.7 wt.%)) compared to those formed on Glass and PDMS_C (thickness reductions ranging from 30 to 43% compared to non-functionalized PDMS. Similar reductions in surface coverage were also observed on the functionalized coatings (Figure 33.B). For all functionalized coatings (PDMS_*Pam* (0.3 wt.%), PDMS_*Pam* (0.5 wt.%), and PDMS_*Pam* (0.7 wt.%)), a significant reduction in the total biofilm amount vs. control surfaces was observed.

Considering bacterial *Cobetia marina* biofilms, a reduction in all parameters (biofilm total volume, biofilm thickness, and biofilm cells) was observed on PDMS_*Pam* (0.7 wt.%) compared to PDMS_C (Supplementary Figure S5). The biofilm biovolume decreased by up to 18% ($p < 0.05$) for PDMS_*Pam* (0.7 wt.%) compared to PDMS_C (Supplementary Figure S5.A). Similar to the observed with *Cyanobium* sp. LEGE 10375, thinner *Cobetia marina* biofilms were developed on PDMS_*Pam* (0.7 wt.%) compared to those formed on PDMS_C ($p < 0.05$), with overall reductions round 21% (Supplementary Figure S5.B). Moreover, a significant decrease in biofilm cell number was observed on PDMS_*Pam* (0.7 wt.%), reaching 82% reduction ($p < 0.05$; Supplementary Figure S5.C), confirming the anti-biofilm potential of PDMS_*Pam* (0.7 wt.%).

The performance of the foul-release silicone-based paint (PDMS_C) was previously evaluated for its potential to inhibit marine biofilm formation by common microfoulers (Faria *et al.*, 2020; Faria, Teixeira-Santos, *et al.*, 2021). Compared with a commercial epoxy resin used for hull coating, PDMS_C surfaces exhibited higher efficacy in decreasing biofilm formation during its maturation stage (from day 35 onwards) (Faria *et al.*, 2020). These preliminary lab-scale anti-biofilm assays revealed that the most promising results against biofilms were obtained with *Pam*-functionalized PDMS at 0.7 wt.%, with significant reductions in biovolume, biofilm thickness, and surface coverage after 49 days of biofilm development, respectively, compared to biofilms formed on PDMS_C.

Overall, the potential of *Pam* after incorporation in coatings was confirmed in those anti-macro- and microfouling preliminary tests, which indicated that PU_*Pam* 0.7 wt.% and

PDMS_ *Pam* 0.7 wt.% formulations were the most promising formulations to be used in the *in situ* prototypes.

6.3.3. *Pam*-functionalized coating prototypes inhibit early biofilm formation, delaying hard biofouling community development under real marine conditions

Biological colonization of the test prototypes became visible after two months, showing distinct trends among formulations (Figure 34.A; Supplementary Table S19). By this time, PU_C and PU_E prototypes started to be colonized by a microbial biofilm, alongside early macrofoulers visible by the presence of calcareous tubes of polychaeta (Annelida) and small colonies of erect hydrozoans (Cnidaria). After three months, biofilm coverage and thickness increased on PU_C and PU_E prototypes. In contrast, the PU *Pam*-functionalized coating prototype, only showed polychaeta tubes, with low abundant biofilm appearing after five months (Figure 34.A).

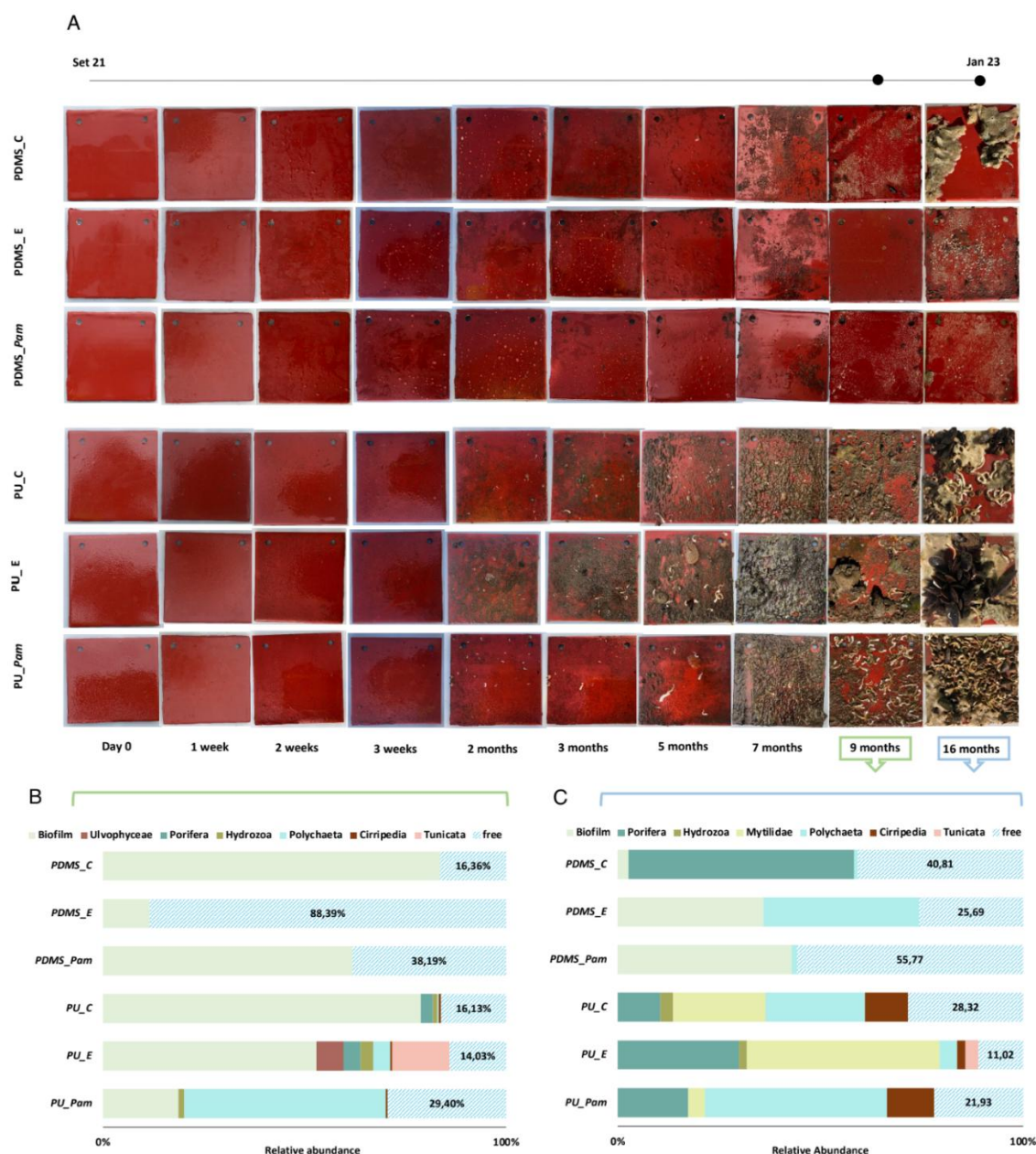


Figure 34. *In situ* biological colonization of the *Pam*-functionalized (PDMS_*Pam* and PU_*Pam*) prototypes, control formulations (PDMS_C and PU_C) and a positive control including the commercial AF agent Ecomea® (PDMS_E and PU_E). Temporal progression of biofouling communities (A); Surface colonization (free area in the prototypes is represented by blue stripes as given by the ImageJ analyses), relative abundance (%) and morpho-taxonomic classification of prototypes communities 9 (B) and 16 (C) months after the first immersion.

After five months, biofilm first appeared on the PU *Pam*-functionalized prototype, while macrofouling increased on the PU_C and PU_E prototypes (Figure 34.A). These findings evidenced that PU *Pam*-functionalization effectively inhibited biofilm development for 5-7 months under real marine conditions, outperforming both the PU control and the commercial Ecomea®-based coatings. By month 7, microbial biofilms had developed across all PU formulations, while a thin biofilm started to form on the PDMS prototypes,

with more noticeable colonization on the PDMS_C and PDMS_E prototypes (Figure 34.A see Supplementary Table S19 for detailed morpho-taxonomical analyses).

At nine months, PU prototypes were colonized by a wide diversity of organisms (Figure 34.B). PU_C exhibited abundant biofilm, low abundance of polychaeta calcareous tubes, a few barnacles (Balanidae, Arthropoda), and erect hydroids. PU_E prototype lacked hydrozoans but featured a colony of encrusting bryozoan *Watersipora subtorquata*. Surface coverage (ImageJ) analysis showed that PU_Pam had the lowest surface colonization (70.6%) with 29% free area, compared to PU_C (16%) and PU_E (18%) (Figure 34.B). Even after 16 months, PDMS prototypes exhibited only a thin biofilm and sparse polychaeta calcareous tubes, and a predominant encrusting sponge (*Halichondria* sp., Demospongiae, Porifera) in the PU_C prototype (Figure 34.A and C; Supplementary Table S19). PDMS coatings showed lower surface coverage than PU, with 62% and 44% for PDMS_Pam at 9 and 16 months, respectively (Figure 35.B and C). At the end of the study (Figure 34.C), PDMS formulations outperformed PU formulations, as expected, while differences in surface coverage among PU formulations (69 to 89%) were not significantly different. The time-dependent wettability measurements (Supplementary Figure S4) further elucidate the distinct surface behaviours of PU- and PDMS-based coatings. PU formulations exhibited relatively stable contact angles over 600 s, indicating minimal dynamic changes in surface properties. In contrast, PDMS coatings showed more pronounced decreases in contact angles over time, consistent with hydrogel formation, surface reorganization, and the influence of incorporated additives such as *Pam*. These findings provide mechanistic insight into the higher time-dependent variability of PDMS surfaces relative to PU, which may contribute to the enhanced and sustained fouling-release performance of PDMS_Pam coatings during prolonged seawater exposure.

In summary, visual inspections and morpho-taxonomic analysis confirmed that *Pam*-functionalized formulations improved the efficacy of both PU and PDMS coatings in reducing biomass and diversity. These effects lasted up to 9 months for PU_Pam and persisted throughout the entire exposure period for PDMS_Pam, outperforming E-conea®-based formulations.

6.3.4. Coating prototypes containing *Pam* show a significant effect on early biofouling community structure

The amplicon sequencing approach targeting prokaryotic and eukaryotic organisms in early biofouling communities, allowed the assessment of 12,112 amplicon sequence variants (ASVs) for the 16S rRNA gene marker and 2,402 ASVs for the 18S rRNA gene

marker in test prototypes (Figure 35 and Figure 36). No significant differences among treatments were observed for the effect of the coating formulations on prokaryotic species richness and α -diversity (Supplementary Figure S6). Within the communities of the PDMS formulations, the species richness of the biofilm associated with the *Pam*-functionalized prototype was lower (1104 ± 32 ASVs) compared to the biofilm grown in the PDMS_E prototype (1224 ± 34 ASVs). Regarding the β -diversity of the same communities (Supplementary Figure S7), significant effects of both the coating matrix and the formulation were detected (PERMANOVA, $p < 0.05$). There was a clear spatial separation between PU and PDMS coatings ($R^2 = 0.37$, $p = 0.001$) while the formulation appeared to have a finer effect on prokaryotic community dissimilarity ($R^2 = 0.13$, $p = 0.026$). The differences detected in prokaryotic β -diversity were visible in the taxonomic composition of these communities, even at the phylum level (Figure 35.A). The major difference was observed for the relative abundance of Firmicutes, which was substantially higher in the biofilms grown on PDMS prototypes when compared to PU prototypes. Particularly in biofilms from the PDMS *Pam*-functionalized prototype (PDMS_*Pam*), an increase of Firmicutes (129 ASVs) and also Actinobacteriota (153 ASVs) relative abundance (12.9 and 19.4, respectively) was observed together with a noticeable decrease in the relative abundance of Crenarchaeota (9 ASVs) and Cyanobacteria (14 ASVs) in this prototype (relative abundance of 1.1 and 0.57, respectively). Conversely, in PU_*Pam* prototype, a decrease in the relative abundance of Firmicutes (1.1; 19 ASVs) was accompanied by an increase in the relative abundance of Campylobacterota (8.7; 28 ASVs).

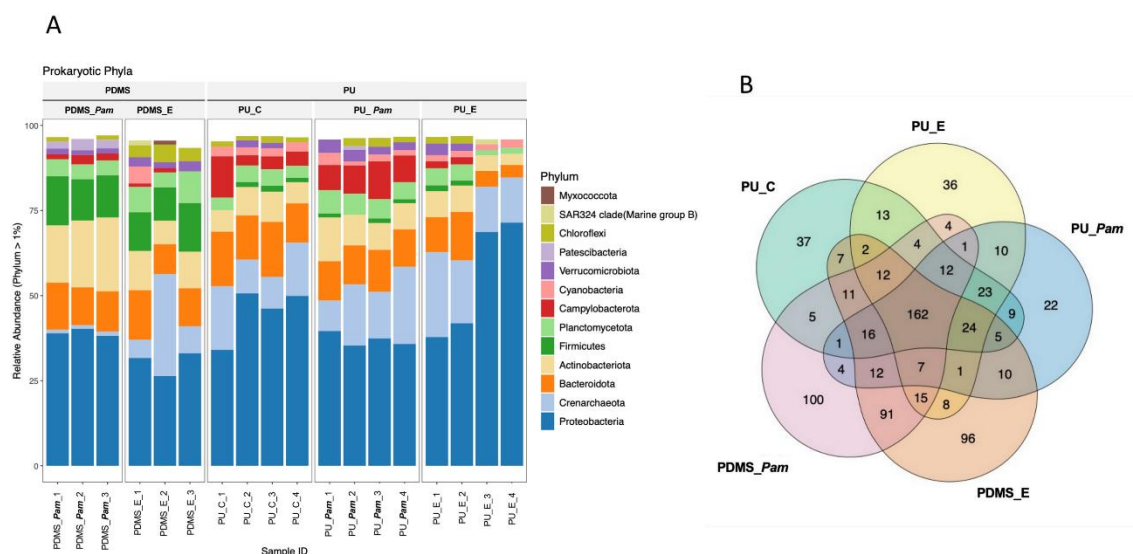


Figure 35. Prokaryotic community composition at the phylum level (A) and Venn diagram showing the number of exclusive and shared genera across the different treatments (B), determined by 16S rRNA gene sequencing. Only phyla with relative abundance higher than 1% are shown in the stacked barplot.

According to the sequence analysis for all the prokaryotic genera present in the different prototypes (Figure 35.B), PDMS_ *Pam* prototype was the most favourable for microbial growth, given the detected number of exclusive genera (100), while PU_ *Pam* showed the lowest (22).

When considering the eukaryotic communities, species richness and α -diversity were not significantly affected by the different coating matrix nor formulation (Supplementary Figure S8). Regarding β -diversity, a significant effect of the coating matrix and the formulation was observed in the eukaryotic communities, similarly to what was observed for the prokaryotic communities (Figure 36, Supplementary Figure S8).

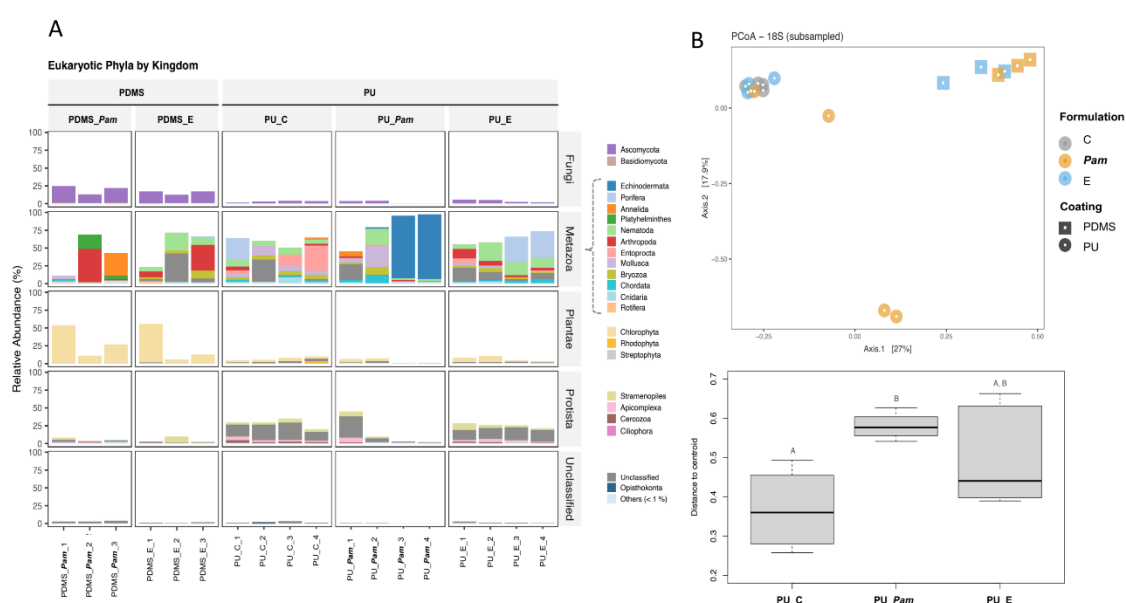


Figure 36. Eukaryotic community composition at the phylum level (A), β -diversity representation by a PCoA plot (B), and boxplot representation of distances to centroid for prototypes treated with PU coating (C). The boxes represent the first and third quartiles, with median value bisecting each box. The whiskers extend to the largest/smallest value, excluding outliers (data beyond 1.5 inter-quartile range). Dissimilar letters in the boxplot of distances denote a significant difference between the formulations (one-way ANOVA, $p < 0.05$). The effect of the coating matrix ($R^2 = 0.25$, $p = 0.001$) was stronger than that of the formulation ($R^2 = 0.16$, $p = 0.006$) in shaping eukaryotic community structure.

It is interesting to note that within the PU prototypes, the PU_ *C* and PU_ *E* formulations clustered very close to each other, while the PU_ *Pam* formulation dispersed away from the PU cluster and had significantly greater beta dispersion than the control samples (Figure 36.B). This observation strongly suggests that PU_ *Pam* formulation has a significant effect on the eukaryotic community structure of the early colonizers.

In agreement with visual inspections, higher relative abundances of Fungi (Ascomycota) and Plantae (Chlorophyta) were detected in PDMS prototypes (Figure 36.A), while higher relative abundances of protists and metazoans were detected in PU prototypes. In

addition, within PU prototypes, PU_ *Pam* was considerably less impacted by the higher abundance of protistan sequences (dominated by dinoflagellates) when compared to PU_C and PU_E formulations. Within the metazoan communities, a high intra-group variability was observed across all treatments, in particular for the PU_ *Pam* prototype, where two of the replicates (PU_ *Pam*_3 and PU_ *Pam*_4) are dominated by Echinodermata. Only two Echinodermata ASVs were responsible for the drastic increase observed in these two samples; both are likely members of the genus *Antedon*, sessile crinoids that were not detected during visual inspections. This highlights- as previously advocated (Azevedo *et al.*, 2020) - that morphological identification of taxa should be complemented metabarcoding analysis as important biofouling species, and vice versa.

Besides these overall and coarse trends, we also explored the treatment effects at the genus level for all eukaryotic kingdoms identified (Supplemental Information, Figure S8). Regarding fungal organisms, a higher number of different genera was detected in PDMS prototypes (15 genera above 0.3%) than in PU (3 genera above 0.3%). A higher number of different metazoan genera in PU (35 genera above 0.3%) than in PDMS (21 genera above 0.3%) prototypes. Particularly, in PDMS_ *Pam* prototype only seven metazoan genera were detected with relative abundances above 0.3%, in contrast to 15 detected in the PDMS_E prototype, and 18 in PU_ *Pam*. Among these seven, only four correspond to genera of known fouling species, namely *Botryllus* and *Styela* (Tunicata), *Musculus* (Mytilidae) and *Lumbrineris* (Polychaeta). Notably, in the PDMS_ *Pam* prototype, only polychaetes were identified in the visual morpho-taxonomical analysis, evidenced by the presence of a few scattered calcareous tubes. In contrast, nine genera associated with fouling communities were detected in PU_ *Pam* (Supplementary Figure S9), including *Mytilus* (Mytilidae), *Caberea*, *Membranipora* and *Scrupocellaria* (Bryozoa), *Botryllus*, *Molgula* and *Styela* (Tunicata) and *Eumida* (Polychaeta), although only *Mytilus* individuals and polychaeta tubes were observed in the visual assessment. Interestingly, no genera belonging to *Balanidae* family (barnacles) were detected by the sequencing analysis in any prototype, despite their visual identification in PU-based formulations.

Regarding Plantae genera (Supplementary Figure S9), *Prasiola*, one of the most abundant plantae genera in this dataset, was substantially more abundant in PDMS than in PU prototypes. No apparent trends regarding formulation effects on Plantae genera were detected.

Overall, our results indicate that both *Pam*-containing formulations enhanced the AF performance of the prototypes, but through distinct and selective ecological mechanisms Figure 37. PU_ *Pam* formulation significantly influenced the early eukaryotic community

structure, privileging a specific macrofouling assemblage (metazoan, protistan and plantae) while supporting a low microbial exclusive diversity (22 ASVs). Visual analyses revealed that PU_*Pam* supports only a small subset of macrofouling species and minimal biofilm growth. However, the high relative abundance of metazoan genera detected through DNA-based high-throughput sequencing (HTS) suggests potential inhibition of larval settlement and/or metamorphosis in response to certain chemical cues present. Campylobacterota-rich biofilms, as those detected in PU_*Pam* prototypes, typically develop in sulphide- and redox-altered microenvironments, and are associated with low oxygen, high sulphide and variable pH, conditions that are generally unfavourable for larval settlement that typically prefer more oxic and chemically stable surfaces (Grosche *et al.*, 2025). This may explain the discrepancy between the reduced fouling diversity observed through morpho-taxonomic analysis and the higher richness revealed by sequencing, likely reflecting early colonizers that failed to successfully settle or metamorphose. In contrast, PDMS_*Pam* formulation favoured the establishment of microbial communities and exclusive genera, characterized by high relative abundance of Firmicutes and Actinobacteria, and a reduced representation of Crenarchaeota and Cyanobacteria, while simultaneously deterring colonization of macrofouling taxa (metazoan). Members of the Firmicutes phylum, such as *Bacillus* and related genera, are known to produce antimicrobial peptides and biofilm-degrading enzymes, whereas Actinobacteria are prolific producers of antibacterial, anti-quorum sensing, and biofilm-inhibitory metabolites (Freckelton *et al.*, 2022; Muras, Larroze, *et al.*, 2021). The ability of *Pam*-functionalized PDMS coatings to inhibit cyanobacterial biofilms was also validated under laboratory conditions in this study (Figure 33.B). A biofilm dominated by Firmicutes and Actinobacteria, and lacking strong inductive settlement cues, such as extracellular polymeric substances or secondary metabolites typically released by cyanobacteria or certain Gram-negative bacteria), may therefore contribute to inhibit selected metazoan larval settlement (Dobretsov & Rittschof, 2020; Jagannathan *et al.*, 2021; Rajitha *et al.*, 2020). Together, these selective microbial and ecological effects underscore the potential of *Pam* to modulate coating performance by limiting harmful macrofouling and shaping early biofilm community assembly.

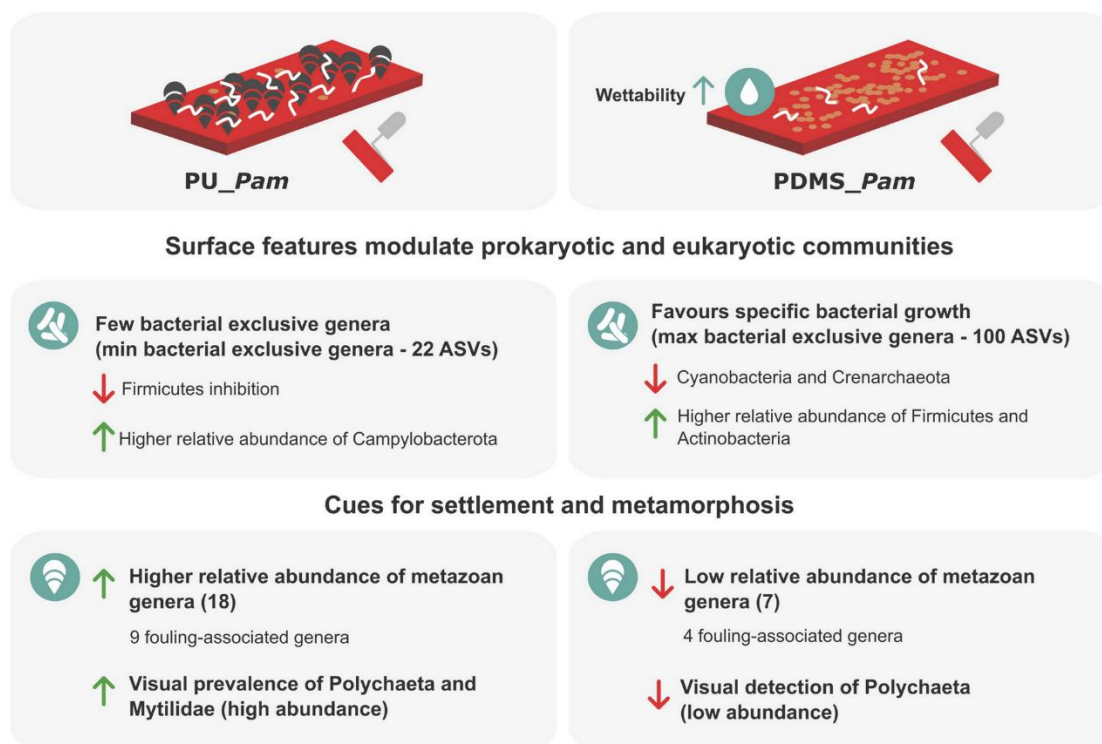


Figure 37. Putative selective ecological mechanisms underlying the differential behaviour of *Pam*-engineered coating prototypes. The superior wettability of PDMS_*Pam* modulates the establishment of early colonizers, selecting specific prokaryotic phyla that favour biofilm growth, while limiting the abundance of macrofouling-associated genera. In contrast, PU_*Pam* supports only a few macrofouling species, as supported by visual analyses, and exhibits low biofilm development. The high relative abundance of metazoan genera detected by DNA-based HTS may suggest settlement and metamorphosis impairment on PU_*Pam* prototypes.

6.4. Concluding remarks

In this study, the natural cyclic peptides *Pam*, derived from cyanobacteria, used as active ingredients in engineered coatings, act as effective antifouling mediators, while posing minimal risk to marine ecosystems. ERA models predicted negligible impact, and lab-scale assays confirmed its ability to reduce cyanobacterial and bacterial biofilms' biovolume, thickness, and surface coverage, as well as to inhibit mussel larval settlement. *In situ* proof-of-concept tests further validated the antifouling effectiveness of *Pam*-functionalized PU and PDMS coatings, which delayed macrofouling colonization, outperforming control and commercial Econe® formulations. Overall, the integration of *Pam* as a bioactive ingredient in new engineered AF systems, represents a technological advance from early proof-of-concept to near-application readiness (TRL 3 to 6), bridging the gap between laboratory innovation and near-application readiness (see **Outstanding Questions**). Unlike conventional antifouling agents, which rely on toxic biocides with high environmental persistence and regulatory costs, this bio-based

alternative combines high AF chemo-ecological efficacy with enhanced environmental safety, offering a sustainable alternative aligned with current regulatory and ecological priorities. Beyond environmental benefits, the use of natural, biodegradable active ingredients such as *Pam* may also reduce long-term operational and compliance costs, supporting the transition toward next-generation, eco-friendly antifouling technologies.

Materials availability

Materials will be made available upon request from the lead contact, following standard Material Transfer Agreement (MTA) procedures.

Data availability

All data are available in the main text or in the supplemental information section. Raw data used to generate Figures 2 and 3 have been deposited in Zenodo (10.5281/zenodo.17648153). The 16S and 18S amplicon sequencing data, used to generate Figures 4 and 5, have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession numbers PRJEB103959 (ERP185275), and PRJEB103962 (ERP185278), respectively.

Author Contributions

Conceptualization: J.R.A.; data curation: C.G., S.P., I.B.O., L.C.G., M.J.R, M.S.; F.J.M., E.R.S., J.R.A.; methodology: C.G., S.P., I.B.O., M.P., J.M., L.C.G., M.J.R, M.S.; F.J.M., V.V., E.R.S., J.R.A.; investigation: C.G., S.P., I.B.O., T.R., F.J.M., E.R.S., J.R.A.; resources: L.C.G., M.J.R, M.S., V.V., E.R.S., J.R.A.; validation: F.J.M., V.V., E.R.S., J.R.A.; supervision: F.J.M., E.R.S., J.R.A; funding acquisition: J.R.A; writing - original draft: J.R.A.; writing - review & editing: all authors.

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Technology Readiness

Pam-functionalized coatings, a bio-based antifouling technology engineered to modulate the early biofouling community colonization and delay the establishment of macrofouling organisms, have reached the Technology Readiness Level (TRL) 6. This technology has been successfully tested under both laboratory and relevant marine conditions, demonstrating high effectiveness in preventing biofilm formation and mussel larval settlement at lab-scale, as well as sustained antifouling performance in marine field trials. To progress toward higher TRLs, key challenges include optimizing cost-effective large-scale production of the cyanobacterial metabolites, demonstrating efficacy across diverse marine environmental settings worldwide, and adapting coating matrices to meet the needs of different end users. Reaching these milestones will support the transition toward next-generation, eco-friendly antifouling technologies.

Glossary

AF-effective biome: surface-associated biotic assemblage shaped by the presence of antifouling cues.

Antifouling coatings: engineered surface treatments applied to structures intended for submersion to prevent or reduce the settlement and growth of biofouling organisms, by providing physical and chemical surface properties that deter adhesion and biofilm formation, thereby enhancing material durability, operational efficiency, and environmental performance.

Early-stage biofouling communities: initial multispecies assemblages dominated by microorganisms—such as bacteria, microalgae, and fungi— along with larval stages of macroorganisms that rapidly colonize submerged surfaces. Their composition, succession, and functional interactions are shaped by environmental factors and substrate characteristics, while their intrinsic biochemical activity conditions the surface and drives subsequent stages of biological colonization.

Epibiosis: colonization of a living host's surface by other organisms, resulting in the formation of surface-associated communities that can influence the host's ecological and functional processes.

Microbial cues: biochemical signals or byproducts produced by microorganisms—such as metabolites, extracellular polymeric substances, or surface-bound molecules—that modulate the behavior, settlement, or developmental processes of other organisms within a biofilm or biofouling community.

Outstanding Questions

- How can large-scale, cost-efficient and sustainable production of cyanobacterial secondary metabolites such as *Pam* be optimized to support commercial antifouling applications?
- How broadly effective are *Pam*-functionalized coatings across different biogeographic regions, seasons, and substrate types commonly found in ocean-

related industries? And which environmental parameters most strongly influence their efficacy?

- Can *Pam*-based formulations be further improved by integrating them with advanced polymer matrices or surface-engineering strategies to enhance durability, controlled *Pam* release, and compatibility with diverse end-user applications?
- Which regulatory, standardization, and validation frameworks are needed to accelerate the transition from TRL 6 to market-ready, eco-safe marine antifouling technologies based on natural products?
- Which molecular and chemo-ecological interactions enable *Pam*-based coatings to steer early biofouling assemblages towards an AF-effective biome? And can these mechanisms be generalized to engineer AF-effective biomes across diverse and dynamic marine environments?

7. General Discussion

The preventive coatings currently used to control marine biofouling still rely heavily on copper and booster biocides, despite their well-documented detrimental impacts on marine ecosystems (Amara *et al.*, 2018). In response, the European Union has been fostering the transition towards more environmentally sustainable AF solutions (European Commission: Deltares *et al.*, 2021). The Biocidal Products Regulation (BPR, EU 528/2012) requires that newly developed AF agents demonstrate target-specific efficacy, a clearly defined mode of action, minimal toxicity to non-target species, and low potential for bioaccumulation and environmental persistence (European Commission, 2024). In parallel, the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) framework establishes key endpoints for assessing long-term environmental effects— including physicochemical properties, environmental fate, toxicology, and ecotoxicology— to assess whether substances meet the criteria of being persistent, bioaccumulative, and toxic (PBT) (ECHA, 2017).

Within this regulatory context, the present thesis investigated cyanobacteria as a source of natural products with potential AF applications, in line with the guidelines set by BPR and REACH. An initial screening of fifty-nine cyanobacterial strains (Chapter 2) identified more than twelve with settlement-inhibiting activity in at least three fractions. This work ultimately led to: (a) the bio-guided isolation of the nucleosides adenosine and 2'-deoxyadenosine from *Leptothoe* sp. LEGE 181152; (b) the identification of hydroxypheophytins from the unidentified filamentous Synechococcales LEGE 06144 as potential AF leads; and (c) the recognition of Synechococcales strain LEGE 15546 as a promising candidate for AF applications.

The AF potential of selected cyanobacterial metabolites was then investigated in greater detail. These included: (a) the nucleosides adenosine and 2'-deoxyadenosine, isolated from *Leptothoe* sp. LEGE 181152, and disclosed in the context of the present thesis; (b) the oxadiazine nocuolin A, previously isolated from *Nodularia* sp. LEGE 06071 and highlighted as a potential biotechnological resource (M. L. Sousa *et al.*, 2019); and (c) the cyclic peptides portoamides, previously isolated from *Phormidium* sp. LEGE 05292 (Leão *et al.*, 2010) and investigated for their AF potential (Antunes *et al.*, 2019). In addition, two nucleoside analogues – hypoxanthine arabinoside and 2'-deoxyinosine – were evaluated to establish structure–activity relationships (Figure 38).

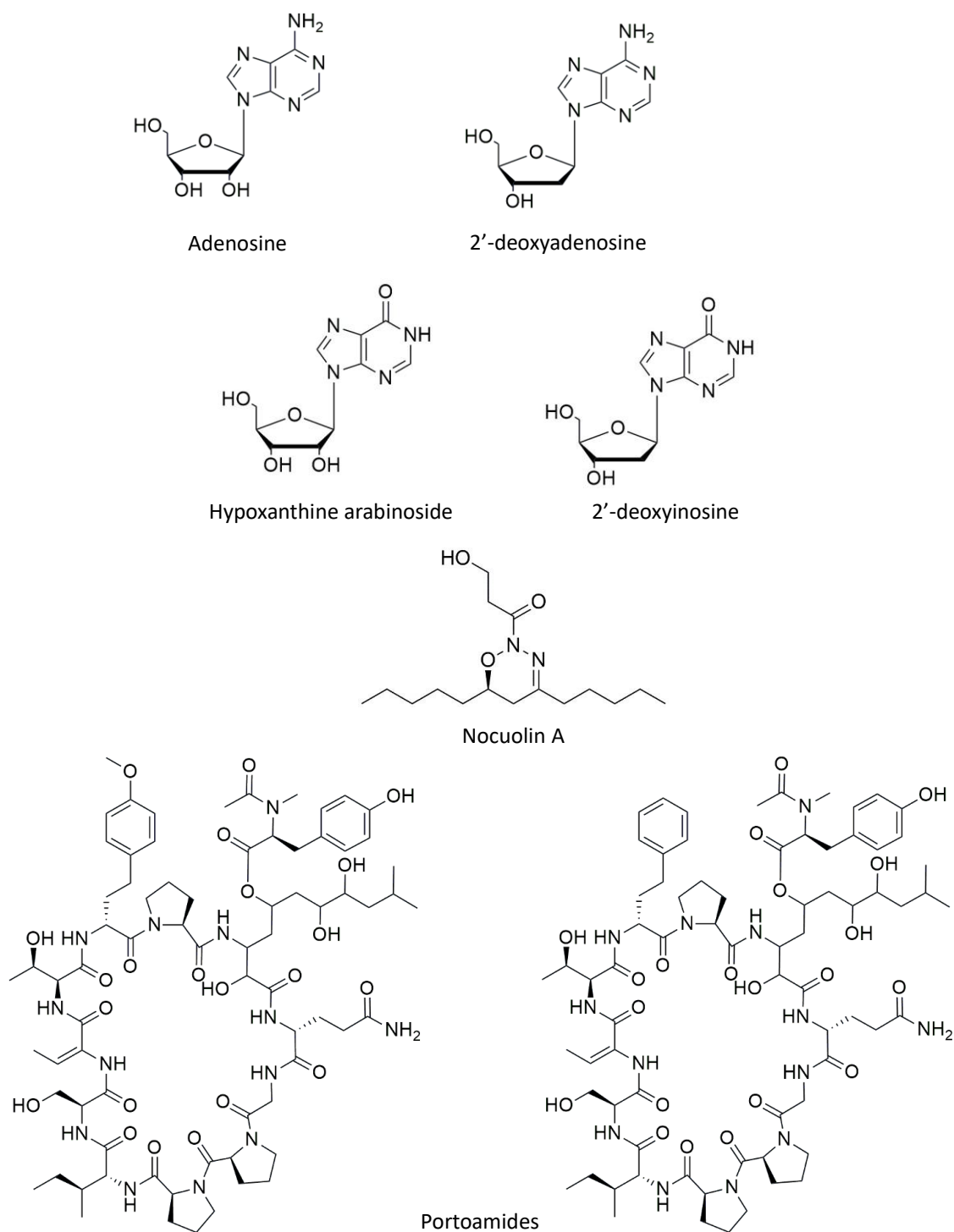


Figure 38. Chemical structure of all six compounds studied for AF purposes.

The AF discovery pipeline implemented in this work comprised several sequential stages: (i) evaluation of AF efficacy against representative micro- and macrofouling species; (ii) identification of putative molecular targets through *in vivo* and *in vitro* assays;

(iii) assessment of ecotoxicity towards both target and non-target organisms; (iv) environmental fate predictions, including affinity for soils, sediments, and lipids; potential for bioconcentration in aquatic organisms; biodegradation under aerobic and anaerobic conditions; and predicted environmental concentrations; and (v) incorporation into marine coatings, evaluated through both laboratory-scale formulations and *in situ* field tests.

All six compounds advanced through multiple stages of this discovery pipeline/technology readiness; however, only one of the most promising candidates, portoamides, successfully progressed to *in situ* field evaluation. A consolidated overview of the main findings is presented in Table 19 and will be further elaborated in the following sub-chapters (7.1–7.4).

Table 19. Overview of the main results obtained for the six compounds studied for AF purposes. (1) Previously determined by (Antunes *et al.*, 2019).

Compounds	AF activity	Putative molecular targets on <i>M. galloprovincialis</i>	General Ecotoxicity	Marine coatings incorporation
Adenosine	<i>M. galloprovincialis</i> EC ₅₀ = 6.63 µM LC ₅₀ > 200 µM	Increased AChE activity No effect on TYR activity	<i>A. amphitrite</i> LC ₅₀ > 32 µM <i>A. salina</i> LC ₅₀ > 50 µM	-
	<i>Navicula</i> sp. ≈ 40% inhibition LC ₅₀ > 200 µM	No DEPs for 12.5 µM	Log K _{oc} = 0.19 Log K _{ow} = -1.38 BCF = 3.16 L Kg ⁻¹	
	Marine biofilm-forming bacteria No effect	-	Biodegrades fast	
2'-deoxyadenosine	<i>M. galloprovincialis</i> EC ₅₀ = 8.74 µM LC ₅₀ > 200 µM	Increased AChE activity No effect on TYR activity	<i>A. amphitrite</i> LC ₅₀ > 32 µM <i>A. salina</i> LC ₅₀ > 50 µM	-
	<i>Navicula</i> sp. ≈ 60% inhibition LC ₅₀ > 200 µM	vWFA EGF-like proteins	Log K _{oc} = 0.50 Log K _{ow} = -0.71 BCF = 3.16 L Kg ⁻¹	
	Marine biofilm-forming bacteria No effect	ATP metabolism Ciliary movement Oxidative stress	Does not biodegrade fast	
Hypoxanthine arabinoside	<i>M. galloprovincialis</i> EC ₅₀ = 5.50 µM LC ₅₀ > 200 µM	Increased AChE activity Increased TYR activity	<i>A. salina</i> LC ₅₀ > 50 µM	-
	<i>Navicula</i> sp. ≈ 60% inhibition LC ₅₀ > 200 µM	vWFA / vWFD EGF-like proteins	Log K _{oc} = -1.00 Log K _{ow} = -1.84 BCF = 3.16 L Kg ⁻¹	
	Marine biofilm-forming bacteria No effect	ATP metabolism Ciliary movement Oxidative stress	Biodegrades fast	
2'-deoxyinosine	<i>M. galloprovincialis</i> EC ₅₀ = 8.54 µM LC ₅₀ > 200 µM	Increased AChE activity Increased TYR activity	<i>A. salina</i> LC ₅₀ > 50 µM	-
	<i>Navicula</i> sp. ≈ 60% inhibition LC ₅₀ > 200 µM	vWFA	Log K _{oc} = -0.79 Log K _{ow} = -1.18 BCF = 3.16 L Kg ⁻¹	
	Marine biofilm-forming bacteria No effect	ATP metabolism Oxidative stress	Does not biodegrade fast	
Nocuolin A	<i>M. galloprovincialis</i> EC ₅₀ = 3.90 µM LC ₅₀ > 100 µM	No effect on AChE activity No effect on TYR activity	<i>A. amphitrite</i> LC ₅₀ = 0.016 µM <i>A. salina</i> LC ₅₀ = 2.48 µM <i>D. rerio</i> LC ₅₀ = 0.058 µM	-
	<i>Navicula</i> sp. EC ₅₀ = 1.56 µM LC ₅₀ > 3.1 µM	troponin T myosin-XV SCaMC-2	Log K _{oc} = 3.01 Log K _{ow} = 4.53 BCF = 19.1 L Kg ⁻¹	
	Marine biofilm-forming bacteria No effect	Endoplasmic reticulum stress Muscle regulation ATP metabolism Oxidative Stress	Does not biodegrade fast	
Portoamides	<i>M. galloprovincialis</i> (1) EC ₅₀ = 3.16 µM LC ₅₀ > 32 µM	Pedal retractor muscle myosin heavy chain (1) Proton-transporting ATPase (1)	<i>D. rerio</i> LC ₅₀ = 4.10 µM	PU 0.7 wt% PDMS 0.7 wt% Inhibited biofilm formation and larval settlement at lab scale and microbiota settlement <i>in situ</i>
	Marine biofilm-forming diatoms (1) No effect			
	<i>C. marina</i> , <i>H. aquamarina</i> , <i>P. atlantica</i> (1) EC ₃₀ = 13.59-14.83 µM	ATP metabolism (1) Ciliary movement (1) Muscle regulation (1)	Log K _{ow} = -5.49	

7.1. Antifouling efficiency

Marine biofouling is a complex process involving multiple levels of biological organization within the fouling community. To address this complexity, the AF efficacy of adenosine, 2'-deoxyadenosine, hypoxanthine arabinoside, 2'-deoxyinosine, and nocuolin A was evaluated against five strains of marine biofilm-forming bacteria, the biofilm-forming diatom *Navicula* sp., and *M. galloprovincialis* mussel plantigrades.

The four nucleosides exhibited comparable activity against mussel plantigrades (EC_{50} = 5.50–8.74 μ M). Against *Navicula* sp., however, their effects diverged: adenosine produced $\approx$ 40% inhibition, while 2'-deoxyadenosine reached $\approx$ 60%. This stronger activity may stem from its increased lipophilicity due to the absence of the 2'-hydroxyl group in the ribose (Novotny *et al.*, 2000). Substitution of the purine amino group ($-NH_2$) with a carbonyl ($C=O$) at the 6-position, as in hypoxanthine arabinoside and 2'-deoxyinosine, appeared to reduce this difference in effect, with inhibition remaining at $\approx$ 60%. Notably, no clear dose-response relationships were established, indicating that nucleoside inhibition may follow a threshold-like pattern. Further mechanistic studies are needed to clarify this non-linear activity.

Nocuolin A displayed stronger AF activity, with EC_{50} values of 3.90 μ M in mussel plantigrades and 1.56 μ M in *Navicula* sp.

Importantly, none of the tested compounds inhibited the growth of marine bacteria (5 strains tested), suggesting selectivity towards eukaryotic targets. This selective activity profile contrasts with that of broad-spectrum synthetic biocides, which often exhibit high marine toxicity, and is generally regarded as a desirable feature of safer and more environmentally compatible AF agents (Qiu *et al.*, 2024).

7.2. Putative molecular targets for AF activity

According to the environmental regulation BPR (EU 528/2012), newly developed AF agents must undergo precise characterization of their AF mechanistic processes. To this end, the molecular targets of adenosine, 2'-deoxyadenosine, hypoxanthine arabinoside, 2'-deoxyinosine, and nocuolin A were investigated using complementary approaches, including *in vitro* enzyme assays (AChE and TYR activity), *in silico* docking studies (AChE binding) and *in vivo* analyses (whole-organism proteome and functional analysis in mussel plantigrades) Table 19.

At their effective concentrations, all four nucleosides increased AChE activity, an enzyme implicated in cholinergic signalling during larval settlement (L. Chen & Qian, 2017). Docking studies supported this affinity, revealing multiple stabilizing contacts and

interactions within the AChE binding site that varied according to nucleoside structural differences. However, the ecological implications of AChE activation in mussels remain unclear, as most studies to date focused on AChE inhibition rather than activation (D. Pereira, Palmeira, *et al.*, 2024; S. Pereira *et al.*, 2024).

In addition, hypoxanthine arabinoside and 2'-deoxyinosine enhanced TYR activity. TYR is essential for catalysing reactions in byssal plaques and threads that enable firm attachment (Silverman & Roberto, 2007). A similar effect has been reported for a xanthone derivative, proposed to reflect either immune system activation or increased demand for cytoskeleton- and adhesion-related proteins (Almeida *et al.*, 2020). Consistent with this, the proteomic analysis conducted here revealed altered expression of cytoskeleton-associated and TYR-related proteins, particularly in response to hypoxanthine arabinoside. Notably, adenosine and 2'-deoxyadenosine did not affect TYR activity, suggesting that structural substitution of the amino group (-NH₂) by carbonyl group (C=O) in these nucleosides may underlie this mechanistic divergence.

Proteome analysis also identified shared differentially expressed proteins (DEPs) among the nucleosides, including vWFA domain-containing proteins and EGF-like proteins. These are structural components of mussel adhesion proteins – proximal thread matrix protein-1 (PTMP1) (Sun *et al.*, 2002) and mussel foot protein-2 (Mfp-2) (Hwang *et al.*, 2010), respectively. These findings point to vWFA and EGF-like proteins as putative effectors of nucleoside activity, with altered expression potentially disrupting the adhesion process. Functional enrichment further highlighted effects on ciliary movement, ATP metabolism, and oxidative stress.

In contrast, nocuolin A did not affect AChE or TYR activity. Instead, its AF activity in mussel plantigrades appeared to be associated with muscle regulation (e.g., troponin T, myosin-XV), ATP metabolism (e.g., SCaMC-2), and endoplasmic reticulum stress (e.g., coatomer subunit delta). These results parallel observations in cancer cell lines, where nocuolin A has been reported to disrupt ATP metabolism and induce endoplasmic reticulum stress (M. L. Sousa *et al.*, 2019), supporting a conserved mode of action across biological systems.

The effect of portoamides on the proteome of mussel plantigrades was previously investigated by Antunes *et al.*, (2019). The study associated portoamides activity with disruptions in ATP metabolism, particularly through the impairment of proton-transporting ATPase activity. Additionally, alterations in gill structure, including changes in tubulin isoforms, were observed, and was suggested that portoamides may also influence protein and gene regulatory mechanisms.

Together, these results suggest distinct mechanistic pathways for the studied compounds: nucleosides primarily interfere with adhesion-related proteins and cholinergic signalling, whereas nocuolin A disrupts muscle functions, cellular energy metabolism and protein homeostasis. The identification of putative targets such as AChE, TYR, vWFA, and EGF-like proteins provides valuable insights into the molecular basis of AF activity. Importantly, these findings illustrate how structurally diverse compounds can exert AF effects through multiple molecular routes, underlining the need for integrative mechanistic studies in early-stage AF development.

7.3. Ecotoxicological assessment of bioactive compounds

One of the objectives of this work was to assess the environmental compatibility of the bioactive compounds in both target and non-target organisms, a key requirement for the development of new AF agents. *In vivo* assays on target species (*M. galloprovincialis* plantigrades and *Navicula* sp.) and non-target organisms (*A. salina* nauplii, *A. amphitrite* nauplii, and *D. rerio* embryos) were complemented by *in silico* predictions of environmental concentrations in harbours, as well as assessments of persistence, bioaccumulation, and toxicity (PBT).

Despite structural differences, the four nucleosides exhibited a consistently low ecotoxicological profile. No lethality was observed at tested concentrations for *A. salina* nauplii and *A. amphitrite* nauplii ($LC_{50} > 50 \mu M$ and $LC_{50} > 32 \mu M$, respectively), or for the target species ($LC_{50} > 200 \mu M$ for *M. galloprovincialis* and *Navicula* sp.) (Table 19). The therapeutic ratio in mussel plantigrades was also higher than 15, further supporting low toxicity. *In silico* analyses also indicated a low potential for bioaccumulation (low BCF, log K_{ow} values) and minimal environmental persistence in soils and sediments (low log K_{oc} values), reinforcing their environmentally compatible profile.

In contrast, nocuolin A presented a significant ecological risk. It was highly toxic to both target and non-target species, causing lethal effects in *Navicula* sp. ($LC_{50} > 3.1 \mu M$), *A. amphitrite* nauplii ($LC_{50} = 0.016 \mu M$), *A. salina* nauplii ($LC_{50} = 2.48 \mu M$), and *D. rerio* embryos ($LC_{50} = 0.058 \mu M$). A log Kow value greater than 4.5 indicated strong lipophilic bioaccumulation potential, while a PEC/PNEC ratio exceeding 1 confirmed a high ecological risk profile.

Portoamides, on the other hand, exhibited low toxicity, consistent with previous reports (Antunes *et al.*, 2019). This was supported by the LC_{50} value obtained for *D. rerio* embryos ($LC_{50} = 4.10 \mu M$), higher than the effective concentration against mussel plantigrades ($EC_{50} = 3.16 \mu M$). A negative log Kow (-5.49) indicated negligible

bioaccumulation potential, while a low PEC/PNEC ratio further confirmed their environmental compatibility.

In summary, the ecotoxicological assessment demonstrated that nucleosides and portoamides exhibit low toxicity, minimal bioaccumulation potential, and favourable PEC/PNEC ratios, supporting their environmental compatibility as AF candidates. In contrast, nocuolin A poses significant ecological risks due to its high toxicity and persistence. These contrasting profiles emphasize the importance of early stage ecotoxicological evaluation in the AF discovery pipeline, ensuring that promising compounds combine biological efficacy with environmental safety, in line with current regulatory frameworks. Furthermore, the exploration of synthetic analogues of toxic AF compounds offers a promising strategy to mitigate toxicity while preserving AF potency through targeted chemical modifications.

7.4. Incorporation and functionalization in marine coatings

The incorporation of new AF compounds into coating matrices is a critical step in assessing their real-world commercial applicability. Accordingly, the cyclic dodecapeptides portoamides A and B were further evaluated as potential active ingredients in marine coating formulations, first at laboratory scale and then *in situ* in a real marine scenario. This compound had previously demonstrated a well-characterized AF profile (Antunes *et al.*, 2019), exhibiting activity against both micro- and macrofouling species, inducing specific proteomic alterations in mussel plantigrades and showing non-toxic effects on target and non-target organisms. Its AF performance was assessed in PU and PDMS coatings.

At lab scale, after 40 h of exposure, PU (0.35 and 0.7 wt%) and PDMS (0.7 wt%) formulations significantly inhibited settlement of *M. galloprovincialis* plantigrades. Leaching of portoamides was negligible across all formulations. Functionalized PDMS coatings (0.3, 0.5, and 0.7 wt%) were also tested for anti-biofilm activity under controlled hydrodynamic conditions during 49 days of cyanobacterial biofilm development. The 0.7 wt% PDMS formulation showed the strongest effects, reducing biovolume, thickness, and surface coverage by 38%, 43%, and 35%, respectively, compared with controls (glass and non-functionalized PDMS).

In situ marine proof-of-concept assays were conducted at Leixões cruise ship terminal pier over 16 months. Portoamides enhanced the performance of both PU and PDMS coatings, reducing biofilm formation, surface colonization, biomass, and macrofouler biodiversity compared with controls. Effects persisted up to 9 months for PU coatings

and throughout 16-month period for PDMS. Analyses of early biofouling community structure of prokaryotic and eukaryotic assemblages further revealed a deterrent effect on macrofouling diversity and abundance (metazoan, protistan, and plantae) in the coatings functionalized with portoamides compared to controls and the commercial biocide ECONEA®.

Overall, portoamides maintained their AF properties after incorporation into coatings, highlighting their potential as active ingredients in bio-based, more environmentally friendly AF formulations.

8. Final Remarks

This thesis advances the understanding of cyanobacteria as a promising source of natural products for AF applications. Through a large-scale screening of fifty-nine cyanobacterial strains, Synechococcales and Oscillatoriales emerged as particularly promising orders, yielding several bioactive hits against *M. galloprovincialis*. Among these, three strains were particularly relevant: *Leptothoe* sp. LEGE 181152, from which nucleosides were isolated through a bio-guided approach; unidentified filamentous Synechococcales strain LEGE 06144, where hydroxypheophytins were identified as potential AF leads; and Synechococcales LEGE 15546, whose AF potential was recognized but requires further investigation.

The discovery of adenosine and 2'-deoxyadenosine metabolites represent a key contribution of this work, establishing nucleosides for the first time as effective AF agents. Furthermore, the activity of analogues such as hypoxanthine arabinoside and 2'-deoxyinosine demonstrated how structural differences can modulate AF efficacy, underscoring the importance of structure–activity relationship studies in the rational design of safer AF candidates.

This study also emphasizes the necessity of early-stage ecotoxicological assessment, ensuring that promising AF compounds combine biological effectiveness with environmental safety, in accordance with European regulatory requirements for sustainable AF solutions. Importantly, the successful incorporation of selected metabolites into experimental coating formulations demonstrated that natural products of cyanobacterial origin can be functionally translated into applied AF strategies.

Altogether, the findings presented in this thesis underscore the biotechnological potential of cyanobacteria as reservoirs of AF metabolites. By integrating compound isolation, mechanistic studies, ecotoxicological profiling, and proof-of-concept application in coatings, this work contributes to the development of environmentally responsible alternatives to copper- and booster-based biocides in use. These results lay the groundwork for advancing cyanobacterial compounds from promising laboratory discoveries toward practical, field-ready solutions for marine biofouling management.

8.1. Future Perspectives

This thesis provided valuable hints into the AF potential of cyanobacterial strains and their metabolites, and opened several avenues and opportunities for further exploration:

Antifouling potential of bioactive metabolites from the cyanobacterium *Synechococcales* LEGE 15546

The *Synechococcales* cyanobacterium LEGE 15546 emerged as one of the most promising strains in the initial screening. Future studies should therefore prioritize this strain, applying a combined bioassay- and MS-guided approach to isolate the active metabolites detected in settlement inhibition assays, followed by spectroscopic analysis for structural elucidation. Their AF efficacy should be systematically assessed against marine biofilm-forming bacteria, diatoms, and mussel plantigrades, complemented with mechanistic studies. Such profiling is critical to determine their suitability as natural alternatives to currently used biocides in preventive coatings.

Hydroxypheophytins from the filamentous cyanobacterium *Synechococcales* LEGE 06144

Chlorophyll derivatives such as hydroxypheophytins represent a largely unexplored class of compounds in the AF context. Since 13²-R-hydroxypheophytin A and its 13²-S isomer, were identified as major constituents in the settlement-active fractions of the unidentified filamentous *Synechococcales* strain LEGE 06144, future studies should aim to comprehensively characterize their AF potential, including efficacy assays, identification of molecular targets, and ecotoxicological evaluation.

Cyanobacterial nucleosides as promising AF agents: mechanistic and *in situ* validation

The novel AF activity reported here for adenosine, 2'-deoxyadenosine, hypoxanthine arabinoside, and 2'-deoxyinosine warrants deeper mechanistic validation. Future studies should include *in vivo* analyses of AChE and TYR activity in mussel plantigrades and transcriptomic approaches (e.g., RNA sequencing, qPCR) to confirm the involvement of EGF-like and vWF domain proteins. Additionally, incorporation of these nucleosides into polymeric coatings followed by laboratory and *in situ* functionalization tests represents the next step in translating these findings into practical applications.

Synthesis and structure-activity relationship of nocuolin A analogues for AF purposes

Nocuolin A showed a harmful ecotoxicological profile, precluding its use as an AF agent. Nonetheless, structure–activity relationship studies with its biosynthetic precursors and tailored synthetic analogues could provide valuable less toxic alternatives with AF potential.

Development of biogenic matrices for novel environmentally sustainable antifouling coatings

Optimization of coating formulations and extended *in situ* assays will be critical to assess the long-term performance and stability of cyanobacteria-derived AF agents under realistic marine conditions. A crucial step towards environmentally compatible alternatives to AF coating technologies is the development of matrices of biogenic origin (natural polymers or synthetic derivatives) to replace conventional plasticized matrices for the incorporation of eco-friendly active principles. Biogenic systems provide renewable and biodegradable platforms that can enhance the stability and controlled release of natural AF agents, while reducing leaching and minimizing non-target toxicity. Moreover, their adjustable physicochemical properties – such as porosity, hydrophobicity, and mechanical strength – can be tailored to optimize coating performance under dynamic marine conditions. The replacement of plasticized matrices and other polluting systems with bio-based alternatives therefore represents a promising strategy to align AF innovation with environmental sustainability and regulatory goals.

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Attachments

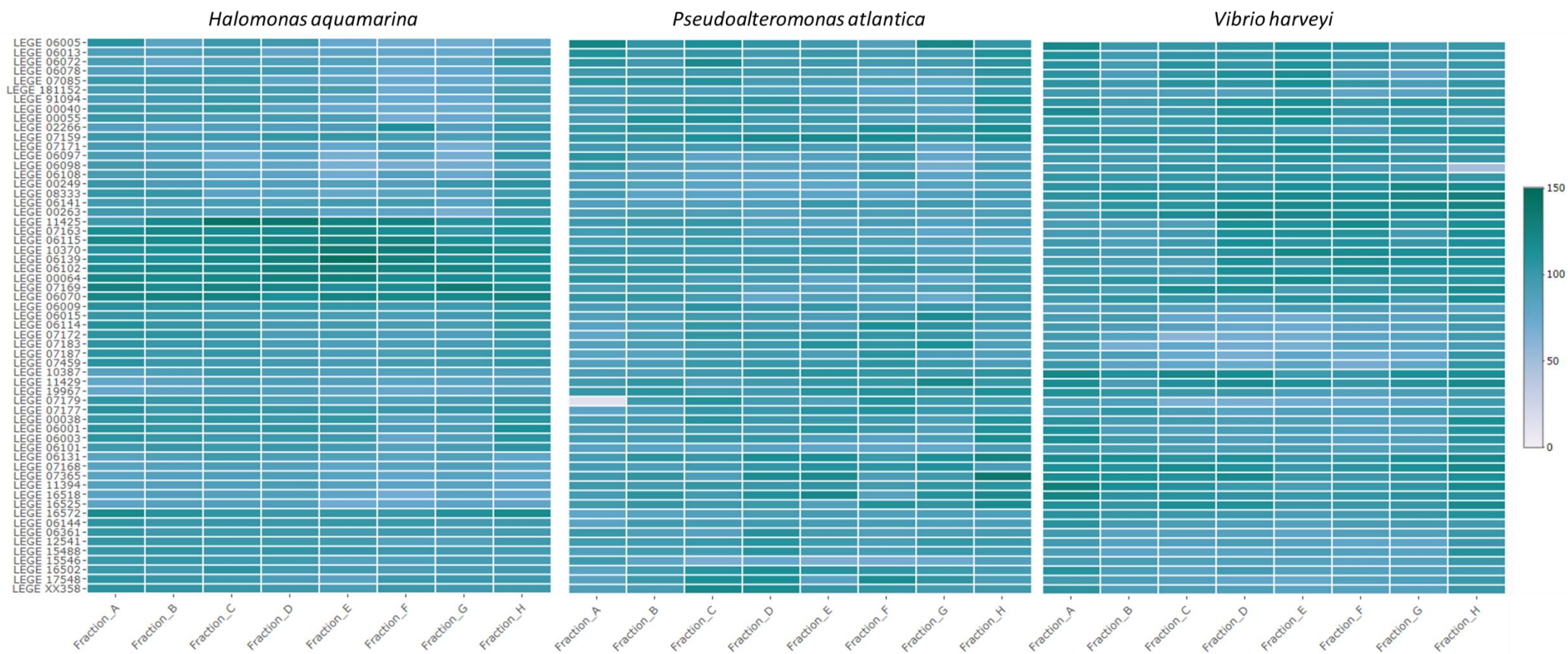
Supplementary Material of Chapter 2

Supplementary Table S1. List of the 59 LEGE-CC cyanobacterial strains used in this work. Cyanobacterial strains grown during this study are marked with an (*).

Strain	Species	Order	Accession Number	Environment	Growth media	Habitat	Location	Country
LEGE 00038	<i>Synechocystis salina</i>	Chroococcales	KU951816	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, <i>Sabellaria alveolata</i> reef	Praia do Mindelo, Vila do Conde	Portugal
LEGE 00040	<i>Synechocystis salina</i>	Chroococcales	KU951817	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	Intertidal zone, on a <i>Patella</i> sp. shell	Praia de Vila Praia de Âncora, Caminha Portugal	Portugal
LEGE 00055	<i>Phormidium cf. irriguum</i>	Oscillatoriales	KU951790	Freshwater	Z8	Water sample	Imfout reservoir, Oum Rabiaa basin	Morocco
LEGE 00064	<i>Phormidium sp.</i>	Oscillatoriales	n/a	Freshwater	Z8	unknown	Lake Takerkoust	Morocco
LEGE 00249	<i>Sphaerospermopsis sp.</i>	Nostocales	KC989701	Freshwater	Z8	water sample	Maranhão dam reservoir, campsite at Montargil	Portugal
LEGE 00263	<i>Dolichospermum sp.</i>	Nostocales	KU951721	Freshwater	Z8	water sample	Maranhão dam reservoir, Benavila	Portugal
LEGE 02266	<i>Sphaerospermopsis sp.</i>	Nostocales	KU951801	Freshwater	Z8	water sample	Maranhão dam reservoir, Benavila, Avis	Portugal
LEGE 06001	<i>Nodosilinea sp.</i>	Synechococcales	KU951758	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	sea water sample, coastal, surf zone	Praia de Buarcos	Portugal
LEGE 06003	<i>cf. Romeria sp.</i>	Synechococcales	KU951865	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone	Praia de Buarcos	Portugal
LEGE 06005	<i>Synechocystis sp.</i>	Chroococcales	KU951820	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	sea water sample, coastal, surf zone	São Pedro de Moel	Portugal
LEGE 06009	<i>Nodosilinea sp.</i>	Synechococcales	JF708121	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, epilithic	Praia da Foz do Arelho, Caldas da Rainha	Portugal
LEGE 06013	<i>cf. Romeria sp.</i>	Synechococcales	KU951673	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, wave-exposed rock	Praia da Foz do Arelho, Caldas da Rainha	Portugal
LEGE 06015	<i>Cyanobium sp.</i>	Synechococcales	KU951688	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, on a <i>Patella</i> sp. Shell	Baleal Norte, Ferrel	Portugal
LEGE 06070	unidentified filamentous <i>Synechococcales</i>	Synechococcales	HM217074	Brackish	Z8	mesotidal zone, benthic	Douro estuary, Porto	Portugal
LEGE 06072	<i>cf. Oxytoma acuminatum</i>	Oscillatoriales	HM217077	Brackish	Z8	mesotidal zone, benthic	Vouga estuary, Ria de Aveiro, São Jacinto	Portugal
LEGE 06078	<i>cf. Oxytoma acuminatum</i>	Oscillatoriales	HM217075	Brackish	Z8	mesotidal zone, benthic	Douro estuary, Vila Nova de Gaia	Portugal
LEGE 06097	<i>Cyanobium sp.</i>	Synechococcales	HQ832928	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, on a green macroalga	Praia do Martinhal, Vila do Bispo	Portugal

LEGE 06098	<i>Cyanobium sp.</i>	Synechococcales	KC469572	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, on a green macroalga	Praia do Martinhal, Vila do Bispo	Portugal
LEGE 06101	<i>Nodosilinea nodulosa</i>	Synechococcales	KU951756	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, on a green macroalga	Praia da Luz, Lagos	Portugal
LEGE 06102	<i>Nodosilinea nodulosa</i>	Synechococcales	HQ832906	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	tide pool, on a submerged stone	Praia de São Bartolomeu do Mar, Esposende	Portugal
LEGE 06108	<i>Toxifilum mysidocida</i>	Synechococcales	HQ832942	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	tide puddle, rock surface scraping	Praia da Luz, Lagos	Portugal
LEGE 06114	<i>Plectonema cf. Radiosum</i>	Oscillatoriales	n/a	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, on a green macroalga	Praia da Luz, Lagos	Portugal
LEGE 06115	<i>Nodosilinea sp.</i>	Synechococcales	KU951765	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, on a green macroalga	Praia da Luz, Lagos	Portugal
LEGE 06131*	<i>Leptolyngbya saxicola</i>	Oscillatoriales	n/a	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, wave-exposed rock, surface scraping	Praia de Burgau, Budens	Portugal
LEGE 06139*	<i>Cyanobium sp.</i>	Synechococcales	KC469574	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, on a <i>Mytilus</i> sp. Shell	Praia da Aguda, Arcozelo	Portugal
LEGE 06141*	<i>cf. Oculatella sp.</i>	Synechococcales	KU951789	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, on green macroalga	Praia da Luz, Lagos	Portugal
LEGE 06144	Unidentified filamentous Synechococcales	Synechococcales	HQ832937	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, wave-sheltered zone, sand	Praia de Burgau, Budens	Portugal
LEGE 06361	<i>Leptolyngbya sp.</i>	Synechococcales	n/a	Freshwater	Z8	biofilm, from a secondary decanter tank bank	wastewater treatment plant at Febros river, Vila Nova de Gaia	Portugal
LEGE 07085	<i>Nodosilinea sp.</i>	Synechococcales	HM217079	Brackish	Z8	mesotidal zone, benthic	Douro estuary, Porto	Portugal
LEGE 07159	<i>Nunduva sp.</i>	Nostocales	HQ832938	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	tide pool, rock surface scraping	Praia de Burgau, Budens	Portugal
LEGE 07163*	unidentified filamentous Synechococcales	Synechococcales	HQ832900	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, on a <i>Mytilus</i> sp. Shell	Praia de Moledo, Caminha	Portugal
LEGE 07168*	<i>Chroococcopsis sp.</i>	Pleurocapsales	n/a	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, on a brown macroalga	Praia de Lavadores, Canidelo	Portugal
LEGE 07169	<i>Pseudanabaena cf. Curta</i>	Synechococcales	HQ832923	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, wave-exposed rock	Praia da Aguda, Arcozelo	Portugal
LEGE 07171	<i>Synechococcus nidulans</i>	Synechococcales	KU951805	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	tide puddle, air-exposed rock surface scraping	Praia de Burgau, Budens	Portugal
LEGE 07172*	<i>Synechococcus sp.</i>	Synechococcales	HQ832950	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	tide puddle, on a submerged stone	Praia de Olhos d'Água, Albufeira	Portugal
LEGE 07177	<i>Rivularia sp.</i>	Nostocales	KU951799	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, on green macroalga	Praia do Martinhal, Vila do Bispo	Portugal
LEGE 07179	<i>Hyella patelloides</i>	Pleurocapsales	HQ832901	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, on a <i>Patella</i> sp. shell, euendolithic	Praia de Moledo, Caminha	Portugal
LEGE 07183*	<i>Cyanobium sp.</i>	Synechococcales	KU951702	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	tide puddle, rock surface scraping	Praia de Olhos d'Água, Albufeira	Portugal
LEGE 07187*	<i>Chroococcopsis sp.</i>	Pleurocapsales	HQ832904	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, on a <i>Gibbula</i> sp. Shell	Praia de Moledo, Caminha	Portugal
LEGE 07365*	<i>Nostoc sp.</i>	Nostocales	KT951672	Brackish	Z8	n/a	n/a	Portugal

LEGE 07459*	<i>Geminobacterium atlanticum</i>	Chroococcales	KU951882	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, on a <i>Patella</i> sp. Shell	Praia do Martinhal, Vila do Bispo	Portugal
LEGE 08333	unidentified unicellular Synechococcales	Synechococcales	KU951884	Freshwater	Z8	water sample	Lake Zumpango	Mexico
LEGE 10370*	<i>cf. Phormidesmis</i> sp.	Synechococcales	JQ927344	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, on a marine sponge	Praia da Memória	Portugal
LEGE 10387*	unidentified unicellular Synechococcales	Synechococcales	KU951886	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, from a dry dark mat, on a rock	Vila Nova de Mil Fontes	Portugal
LEGE 11394*	<i>Synechococcus</i> sp.	Synechococcales	KU951806	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	hypersaline upper layer of a microbial mat	Pitanguinha lagoon (Araruama system), in the state of Rio de Janeiro	Brazil
LEGE 11425*	<i>Leptolyngbya ectocarpi</i>	Synechococcales	KT951671	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	subtidal sample, epilithic (10-13m depth), less than 1 km off the shore	Pêlo Negro', diving spot near Leixões Harbour	Portugal
LEGE 11429*	<i>Nodosilinea</i> sp.	Synechococcales	KU951778	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	subtidal sample, epilithic (13m depth), about 200 m off the shore	A Pedra', diving spot in front of the fort 'Castelo do Queijo'	Portugal
LEGE 12541	<i>Oscillatoriales cyanobacterium</i>	Oscillatoriales	n/a	Freshwater	Z8	water tank, effluent	Oliveira do Hospital	Portugal
LEGE 15488	<i>Phormidium</i> sp.	Oscillatoriales	MF629805	Freshwater	Z8	water sample, euphotic zone	Macapá, Amazon River	Brazil
LEGE 15546	Synechococcales cyanobacterium	Synechococcales	MW790916	Freshwater	Z8	water tank, effluent	Oliveira do Hospital	Portugal
LEGE 16502	<i>Brasilonema</i> sp.	Nostocales	MW790911	Freshwater	Z8	floodgate wall, scraping	Rio Douro, Porto	Portugal
LEGE 16518*	<i>Tildenella</i> sp.	Synechococcales	MW790918	Freshwater	Z8	ditch	Exploratório - Centro Ciência Viva de Coimbra	Portugal
LEGE 16525*	<i>Microcoleus</i> sp.	Oscillatoriales	MW790913	Freshwater	Z8	floodgate wall, scraping	Rio Douro	Portugal
LEGE 16572	<i>Gloeotheca</i> sp.	Chroococcales	MW790910	Freshwater	Z8	fountain	Malhada Quente, Serra de Monchique	Portugal
LEGE 17548	Nostocales cyanobacterium	Nostocales	MW790912	Freshwater	Z8	Lagoon	Lagoa de Mira, Mira	Portugal
LEGE 181152	<i>Leptothoe</i> sp.	Synechococcales	JASATU00 0000000	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	Shallow tide pools	Baía das Gatas	Cape Verde
LEGE 19967*	Oscillatoriales cyanobacterium	Oscillatoriales	n/a	Marine	Z8 + 25‰ Salt TM+ 1‰ B ₁₂	intertidal zone, scraped from an oyster	Cais de Olhão	Portugal
LEGE 91094	<i>Microcystis aeruginosa</i>	Chroococcales	KU950712	Freshwater	Z8	Pond, water sample	Lagoa de Mira	Portugal
LEGE 18358	<i>Limnorphis robusta</i>	Oscillatoriales	KU951735	Freshwater	Z8	unknown	unknown	unknown



Supplementary Figure S1. Screening results against *H. aquamarina*, *P. atlantica* and *V. harveyi*. A total of 472 fractions (A-H) from 59 cyanobacterial strains were screened at 10 $\mu\text{g mL}^{-1}$. Heatmaps demonstrates the activity of the fractions through a colour gradient. Scale bar represents the percentage of bacterial growth. Rows represent the cyanobacterial strains. Columns represent the fractions.

Supplementary Material of Chapter 3

Supplementary Text S1. General Experimental Procedures

Chromatographic separations were performed either using column chromatography with silica gel, or the Pure C-850 FlashPrep chromatography system (Büchi, Switzerland) with prepacked columns, and ACS grade solvents. HPLC was performed on a Thermo Scientific UltiMate 3000 HPLC instrument, with UV detection (254 nm), using an ACE Excel 3 C18-AR (3 μm $\times$ 7.5 mm $\times$ 4.6 mm) column and HPLC gradient grade solvents.

LC-ESI-MS in SIM mode analysis of compounds **1-2** were performed on a Thermo Finnigan Surveyor HPLC System (Thermo Scientific, MA, USA) coupled to an LCQ Fleet™ Ion Trap Mass Spectrometer (Thermo Scientific, MA, USA). Data acquisition and processing were performed using Xcalibur™ software (version 2). The mass spectrometer operated in electrospray ionization (ESI) positive mode, employing Full Scan and Collision-Induced Dissociation (CID) for data acquisition (Table 1). Key parameters included a spray voltage of 3.50 kV, a capillary temperature of 275 °C, and capillary and tube lens voltages of 35 kV and 120 kV, respectively. Nitrogen served as the sheath and auxiliary gas, while helium was used as the damping gas. Chromatographic separation was achieved on an ACE Excel C18 column (50 $\times$ 2.1 mm I.D., 1.7 μm particle size; Batch: V17-1253, Avantor® ACE®, VWR, PT) maintained at room temperature. The flow rate was set at 0.30 mL/min, with a 10 μL injection volume in no-waste mode. An isocratic mode was employed using a mobile phase consisting of 10% methanol acidified with 0.1% (v/v) formic acid.

The optical rotations were obtained using a JASCO P-2000 polarimeter with SpectraManager 2.14.02 software. The infrared spectra were collected on a Nicolet iS5 FTIR spectrometer (ThermoScientific) with OMNIC 9.8.372 software. The UV-Vis spectrum was acquired on a 1600PC spectrophotometer (VWR) with a 1 cm path length quartz cuvette.

NMR spectra were acquired as a service of the Materials Centre of the University of Porto (CEMUP): 1D and 2D NMR data of compounds **1-2** were recorded on a Bruker Avance III HD 400MHz (400.14MHz ^1H : 100.63MHz ^{13}C) controlled by TopSpin 3.6.3. ^1H and ^{13}C chemical shifts were expressed in δ (ppm), referenced to the CD_3OD solvent used, and the proton coupling constants J in hertz (Hz). Spectra were assigned using the appropriate COSY, HSQC-DEPT, HMBC pulse sequences.

Supplementary Table S2. List of differentially expressed proteins (DEPs) in *M. galloprovincialis* plantigrades exposed to 2'-deoxyadenosine (**2**). DEPs were obtained from pairwise comparisons between the control group (0.1% DMSO) and exposed group at 50 μ M, using a threshold of $\log_2(\text{FC}) = 1.5$. Represented are the relative expression of DEPs on the control group (Ctr-) and the exposed group (50 μ M), and the obtained fold change of each DEP.

Reference Sequence	Protein name	Relative expression		Fold Change
		Ctr-	50 μ M	
A0A649Z313	Actin Fragment	-1.59	1.59	3.18
A0A6J8C173	Acyl coenzyme A oxidase	-1.15	1.15	2.29
A0A8B6DSL8	Adenosylhomocysteinase	-0.38	0.38	0.75
A0A8B6GV47	ADP ATP translocase	-1.36	1.36	2.72
A0A8B6D2T4	ADP ribosylation factor	0.52	-0.52	-1.03
A0A8B6GKE0	Aldehyde dehydrogenase NAD	-1.43	1.43	2.87
A0A8S3T4E0	ALDH	-1.12	1.12	2.23
A0A8B6D196	Aminopeptidase	-0.65	0.65	1.29
A0A8B6DAC4	Annexin	-0.41	0.41	0.83
A0A8B6D0T5	Apple domain containing protein	-0.81	0.81	1.61
A0A8B6G933	Aspartate aminotransferase	-1.21	1.21	2.41
A0A6J8EBJ6	Band 7 domain containing protein	-0.48	0.48	0.97
A0A8B6GW86	C1q domain containing protein	2.14	-2.14	-4.28
A0A8B6FBT9	Calmodulin	-0.99	0.99	1.99
A0A8S3R7D4	Calreticulin	-1.42	1.42	2.84
A0A8B6D440	Cathepsin D	-0.73	0.73	1.46
A0A8S3S230	CDC42	-1.01	1.01	2.03
A0A8B6FIN1	Chitin binding type 2 domain containing protein	-1.61	1.61	3.23
A0A8B6CJE0	Chromo domain containing protein	-1.93	1.93	3.86
A0A8B6C153	Citrate synthase	-0.96	0.96	1.92
A0A8B6GX94	Clathrin heavy chain	-0.79	0.79	1.58
A0A8B6HJJ9	Cluster of 14 3 3 protein beta theta zeta	-0.37	0.37	0.73
A0A6J8EHM6	Cluster of 40S ribosomal protein S2	-1.29	1.29	2.58
A0A8S3Q5J1	Cluster of ACTN1_4	0.57	-0.57	-1.14
A0A8B6CRC1	Cluster of ATP synthase subunit alpha	-0.92	0.92	1.85
A0A8B6FHM0	Cluster of Collagen type XIII alpha	0.33	-0.33	-0.65
A0A8B6FGA8	Cluster of Dynein heavy chain axonemal	0.73	-0.73	-1.45
A0A8S3R8F7	Cluster of E2 7 3 3	0.33	-0.33	-0.65
A0A8B6CPN1	Cluster of EGF like domain containing protein	-0.96	0.96	1.92

A0A8B6FTA2	Cluster of Far upstream element binding protein	-1.18	1.18	2.36
A0A8B6BVI8	Cluster of Fructose bisphosphate aldolase Fragment	-0.74	0.74	1.48
A0A8B6E885	Cluster of Gelsolin like domain containing protein	-1.04	1.04	2.08
A0A8S3QB23	Cluster of H3	1.47	-1.47	-2.93
A0A8B6DYX8	Cluster of Heat shock 70kDa protein 5	-0.99	0.99	1.99
C0Z203	Cluster of Heat shock protein 90	-0.93	0.93	1.85
A0A8B6BR27	Cluster of Heat shock protein 90kDa beta	-0.80	0.80	1.60
A0A8B6GPC6	Cluster of Laminin gamma 1	-1.60	1.60	3.20
A0A8S3QM37	Cluster of MYH9s	-0.48	0.48	0.97
A0A8B6H7X6	Cluster of Myosin heavy chain 6 7	0.46	-0.46	-0.92
A0A8B6FPF3	Cluster of Myosin heavy chain 6 7 1	-0.45	0.45	0.90
A0A8B6EJ02	Cluster of Protein disulfide isomerase	-0.68	0.68	1.36
A0A8B6C4T1	Cluster of RNA helicase	-1.54	1.54	3.09
A0A8S3Q9N6	Cluster of SHSP domain containing protein	-0.65	0.65	1.30
A0A8B6EGQ2	Cluster of Small subunit ribosomal protein S14e	-0.82	0.82	1.63
A0A8S3QQF6	Cluster of Sodium potassium transporting ATPase subunit alpha	-0.47	0.47	0.94
A0A8B6D810	Cluster of Spectrin alpha	-0.72	0.72	1.45
Q966V3	Cluster of Transgelin	-0.66	0.66	1.32
A0A076FIR9	Cluster of Tubulin beta chain Fragment	0.70	-0.70	-1.39
A0A8B6ECV1	Cluster of Twitchin	0.57	-0.57	-1.14
A0A8B6C479	Cluster of Uncharacterized protein 12	-0.44	0.44	0.88
A0A8B6FG82	Cluster of Uncharacterized protein 2	0.72	-0.72	-1.43
A0A8B6DC97	Cluster of Uncharacterized protein 5	-0.82	0.82	1.63
A0A8B6GIV6	Cluster of Uncharacterized protein 6	-2.01	2.01	4.01
A0A8B6BSR2	Coatomer subunit gamma	-0.85	0.85	1.69
A0A8B6CP95	Collagen type IV alpha	-0.59	0.59	1.18
A0A8B6EZ83	Collagen type IV alpha 1	0.49	-0.49	-0.99
A0A8B6CQJ1	Copper acquisition factor BIM1 like domain containing protein	-0.31	0.31	0.61
A0A8S3QML7	CTSL 1	-1.37	1.37	2.73
A0A8B6EWU5	Cytosolic fatty acid binding proteins domain containing protein	-2.05	2.05	4.10
A0A8B6D685	Dihydropolyllysine residue succinyltransferase component of 2 oxoglutarate dehydrogenase complex mitochondrial	0.33	-0.33	-0.66
A0A409V740	Dna binding a protein Fragment	-1.41	1.41	2.83
A0A8B6DX16	Dolichyl diphosphooligosaccharide protein glycosyltransferase subunit 2	-1.08	1.08	2.17
A0A8B6BL12	Dolichyl diphosphooligosaccharide protein glycosyltransferase subunit DAD1	-0.79	0.79	1.57
A0A8B6GJJ6	Dynein intermediate chain 1 axonemal	-0.60	0.60	1.19
A0A8B6CHC6	EF hand domain containing protein	-2.41	2.41	4.81
A0A8S3V871	EML1_2	-0.73	0.73	1.47

A0A8B6F1A7	Endoplasmic reticulum transmembrane protein	-1.64	1.64	3.28
A0A8S3VLQ5	F actin capping protein subunit alpha	-1.23	1.23	2.47
A0A8B6DQX4	FABP domain containing protein	-2.60	2.60	5.21
A0A8B6DPU0	FABP domain containing protein 1	-2.35	2.35	4.70
A0A3L5TQZ0	Fc e cd23 receptor Fragment	1.05	-1.05	-2.10
A0A6J8CQ76	FLOT	-1.84	1.84	3.68
A0A0C5Q4G0	Galectin	-1.25	1.25	2.51
A0A8B6DQP9	glutamate dehydrogenase NAD P	0.35	-0.35	-0.71
A0A8B6BF20	Glutathione S transferase	-1.4	1.4	2.81
A0A6J8ENV8	GNB1	-1.07	1.07	2.14
A0A8B6H497	Granulins domain containing protein	-0.82	0.82	1.64
A0A6J8A5H3	H1_5	0.86	-0.86	-1.71
A0A411G5T5	Hemicentin like protein Fragment	0.49	-0.49	-0.98
A0A0B7AUT8	Histone H2A	1.83	-1.83	-3.67
A0A0L8GYE0	Histone H4 Fragment	1	-1	-2.00
A0A8S3RBA2	HNRNPA1_3	-0.70	0.70	1.40
A0A6J8ESL8	HNRNPABD	-0.65	0.65	1.30
A0A8B6GQQ8	Ig like domain containing protein	-0.39	0.39	0.78
A0A8B6DFJ0	Insulin like growth factor 2 mRNA binding protein 1	-1.21	1.21	2.41
A0A8B6GGC2	Large subunit ribosomal protein L5e	-1.05	1.05	2.10
A0A8B6BTE6	Large subunit ribosomal protein LP1	0.39	-0.39	-0.78
A0A8B6GWR5	LIM and SH3 domain protein	-0.99	0.99	1.98
A0A8B6GV25	LIM zinc binding domain containing protein	-0.35	0.35	0.71
A0A8B6GHC2	Lipocalin cytosolic fatty acid binding domain containing protein	-2.36	2.36	4.72
A0A8S3Q6R0	LMNB	-0.97	0.97	1.93
A0A8B6F3V9	Low density lipoprotein receptor related protein 4	-0.41	0.41	0.81
A0A8B6C2Y2	Malate dehydrogenase 1	-0.72	0.72	1.45
A0A8B6FW38	Molecular chaperone DnaK	-0.42	0.42	0.85
A0A0B7B8Z7	Myosin motor domain containing protein	-0.47	0.47	0.95
A0A8B6EU84	Na H exchange regulatory cofactor NHE RF1	-0.84	0.84	1.69
A0A8S3U4W4	NADP dependent oxidoreductase domain containing protein	-1.01	1.01	2.01
A0A3G1CJL2	Nascent polypeptide associated complex subunit alpha like protein Fragment	-2.03	2.03	4.05
A0A8B6DGA0	Outer dense fiber protein 3	-1.48	1.48	2.97
A0A8B6D9K2	P type domain containing protein	1.3	-1.3	-2.60
A0A8B6BIH5	Peptidyl prolyl cis trans isomerase	1.72	-1.72	-3.44
A0A6J8DU89	Peptidyl prolyl cis trans isomerase 2	-1.71	1.71	3.41
A0A6J8EPJ2	Phospholipid scramblase	-1.57	1.57	3.14

A0A8B6C763	Plastin 3	-0.68	0.68	1.37
A0A8B6G5S6	Prohibitin	-1.84	1.84	3.69
A0A8B6DIX4	Prostaglandin H2 D isomerase glutathione transferase	-2.21	2.21	4.43
A0A8B6FXU3	protein disulfide isomerase	-0.78	0.78	1.57
A0A8B6EIQ6	Protein sleepless	-1.6	1.6	3.20
A0A8S3UA94	PSAP	-0.45	0.45	0.90
A0A8S3T3V1	PSAP 1	-0.69	0.69	1.37
A0A8B6BG90	Rab GDP dissociation inhibitor	-1.53	1.53	3.05
A0A6J8CI59	RAB1A	-2.17	2.17	4.34
A0A8B6CVW7	Ricin B type lectin domain containing protein	-1.18	1.18	2.35
A0A8B6CDT6	Rieske domain containing protein	-0.66	0.66	1.33
A0A6J8F0B5	RNA helicase	-1.01	1.01	2.02
A0A3L5TU64	RNA helicase Fragment	-0.80	0.80	1.59
A0A8S3TL45	RSPH1	0.76	-0.76	-1.51
A0A6J8C2N8	RSPH9	-1.74	1.74	3.48
A0A6J8BLD6	S adenosylmethionine synthase	-0.47	0.47	0.94
A0A8S3TQI9	Sarcoplasmic calcium binding protein	-0.30	0.30	0.61
A0A8B6GPC3	SHSP domain containing protein	-1.85	1.85	3.70
A0A8B6F648	Small nuclear ribonucleoprotein Sm D2	-2.72	2.72	5.44
A0A8B6F519	Small subunit ribosomal protein S10e	0.98	-0.98	-1.96
A0A8B6HL62	Small subunit ribosomal protein S19e	-2.1	2.1	4.21
A0A433TP13	Sperm associated antigen 6	-0.71	0.71	1.42
A0A8B6DIG2	T complex protein 1 subunit alpha	-0.62	0.62	1.24
A0A8B6E6D8	T complex protein 1 subunit gamma	-1.62	1.62	3.25
A0A8B6GHP0	Tektin	-1.44	1.44	2.89
A0A8B6GPN2	Tektin 1	-1	1	2.00
A0A8S3SXV7	Tetraspanin	-1.07	1.07	2.14
A0A8S3QFG9	TGFBI	-1.29	1.29	2.59
A0A8B6FQW6	Transaldolase	-0.55	0.55	1.10
A0A8B6H439	Transglutaminase like domain containing protein	-0.69	0.69	1.38
A0A0K2D7M8	TSP_1 domain containing protein 1	0.77	-0.77	-1.55
A0A8S3QSQ2	Ubiquitin 60S ribosomal protein L40	-1.01	1.01	2.02
A0A8B6G8E3	Uncharacterized protein	0.80	-0.80	-1.60
A0A8S3U726	Uncharacterized protein 1	-2.12	2.12	4.23
A0A6J8CCF5	Uncharacterized protein 10	-1.79	1.79	3.58
A0A8B6E104	Uncharacterized protein 11	-1.25	1.25	2.50
A0A8B6GUE8	Uncharacterized protein 12	3.44	-3.44	-6.88

A0A8B6CB58	Uncharacterized protein 13	1.92	-1.92	-3.84
A0A8S3S991	Uncharacterized protein 15	-1.57	1.57	3.14
A0A8B6BNI7	Uncharacterized protein 17	1.79	-1.79	-3.58
A0A8B6DPQ0	Uncharacterized protein 2	1.05	-1.05	-2.11
A0A8S3TWM9	Uncharacterized protein 26	-1.79	1.79	3.59
A0A8B6CU91	Uncharacterized protein 7	-0.78	0.78	1.57
A0A8B6CTG8	Uncharacterized protein 9	-2.68	2.68	5.36
A0A8B6G9T0	UspA domain containing protein 1	-2.19	2.19	4.39
A0A6J8DNW3	VDAC2	-0.794	0.794	1.59
A0A8B6H3D0	Vesicle associated membrane protein associated protein A	-1.06	1.06	2.13
A0A8B6BI55	Vinculin	-0.81	0.81	1.62
A0A8B6GXU1	Vitelline membrane outer layer 1 like protein	1.67	-1.67	-3.33
A0A8B6C9L4	VWFA domain containing protein	1.19	-1.19	-2.38
A0A8B6F1U6	WD_REPEATS_REGION domain containing protein	-0.76	0.76	1.53
A0A8B6HDB4	WD_REPEATS_REGION domain containing protein 1	-1.06	1.06	2.13
A0A8B6CY00	X2 oxoglutarate dehydrogenase E1 component	-0.60	0.60	1.20
A0A6J8EWE8	X40S ribosomal protein S12	-1.11	1.11	2.22
A0A0L8FIY8	X40S ribosomal protein S28	-1.14	1.14	2.28
A0A2L1TGZ7	X40S ribosomal protein S3 Fragment	-1.26	1.26	2.52
A0A8S3QVR3	X40S ribosomal protein SA	-1.26	1.26	2.52
A0A6J8B1B1	X6 phosphofructokinase	0.82	-0.82	-1.65
A0A8S3RZZ6	X60S acidic ribosomal protein P0	-1.06	1.06	2.12
A0A8S3V934	X60S ribosomal protein L18a	1.08	-1.08	-2.15
A0A077GYU2	X60S ribosomal protein L23 Fragment	-1.43	1.43	2.86
A0A077GZK7	X60S ribosomal protein L28 Fragment	-1.5	1.5	2.99
A0A077H0N7	X60S ribosomal protein L7a Fragment	-0.84	0.84	1.68
A0A8B6D5A0	X60S ribosomal protein L9	-2.75	2.75	5.50

Supplementary Table S3. Results of BLASTp analysis for DEPs identified in response to 2'-deoxyadenosine (2). The table lists homologous proteins from *Magallana gigas*, along with their corresponding accession numbers retrieved from the UniProt and Ensembl Metazoa databases.

Reference Sequence	Uniprot			Ensembl	
	Accession number	Annotation	e-Value	Accession number	e-Value
A0A649Z313	A0A8W8LZ95	Actin-2	2.56E-110	G3054.116	2.00E-110
A0A6J8C173	A0A8W8KG37	Acyl-coenzyme A oxidase	9.28E-75	G23748.2	7.25E-75
A0A8B6DSL8	K1RW85	Adenosylhomocysteinase	0	G22300.4	0
A0A8B6GV47	K1R0Y9	ADP/ATP translocase	0	G730.11	0
A0A8B6D2T4	A0A8W8MRV1	ADP-ribosylation factor	5.31E-124	G34817.29	4.15E-124
A0A8B6GKE0	K1QNT7	Aldedh domain-containing protein	0	G12763.28	0
A0A8S3T4E0	K1QNT7	Aldedh domain-containing protein	0	G12763.28	0
A0A8B6D196	A0A8W8IJS1	Aminopeptidase	0	G14427.1	0
A0A8B6DAC4	A0A8W8MW14	Annexin	9.01E-117	G417.11	7.04E-117
A0A8B6D0T5	A0A8W8N549	Apple domain-containing protein	0	G432.1	0
A0A8B6G933	K1R2Q9	Aspartate aminotransferase	0	G20764.3	0
A0A6J8EBJ6	A0A8W8L8X3	Flotillin-1	0	G26928.23	0
A0A8B6GW86	A0A8W8J8Q2	C1q domain-containing protein	1.57E-20	G17851.1	1.23E-20
A0A8B6FBT9	K1Q3D9	Calmodulin	1.89E-23	G32596.6	3.67E-23
A0A8S3R7D4	A0A8W8KPL5	Calreticulin	0	G2451.27	0
A0A8B6D440	A0A8W8NL52	Peptidase A1 domain-containing protein	0	G5738.16	0
A0A8S3S230	K1PJ53	Cell division control protein 42 homolog	9.09E-134	G22524.23	7.10E-134
A0A8B6FIN1	K1QY92	Chitin-binding type-2 domain-containing protein	5.43E-49	G22619.1	4.24E-49
A0A8B6CJE0	A0A8W8N0M4	Chromo domain-containing protein	6.56E-20	G35434.7	5.13E-20
A0A8B6C153	A0A8W8MWR9	Citrate synthase	0	G35409.40	0
A0A8B6GX94	A0A8W8KCV2	Clathrin heavy chain	0	G23360.1	0
A0A8B6HJJ9	K1P9N7	14-3-3 protein zeta	1.23E-114	G23866.50	6.66E-102
A0A6J8EHM6	A0A8W8HYQ8	Small ribosomal subunit protein uS5	1.77E-160	G1172.7	1.39E-160
A0A8S3Q5J1	K1RH58	Alpha-actinin, sarcomeric	0	G13016.1	2.24E-112

A0A8B6CRC1	K1R6Z7	ATP synthase subunit alpha	0	G11926.23	0
A0A8B6FHM0	K1RH30	Collagen alpha-1(IV) chain	1.65E-39	G9790.1	1.61E-37
A0A8B6FGA8	K1PAG1	Dynein beta chain, ciliary	0	G761.1	0
A0A8S3R8F7	A0A8W8JPQ6	Arginine kinase	1.14E-178	G19705.1	8.89E-179
A0A8B6CPN1	K1R0G7	Fibropellin-1 (Fragment)	7.73E-83	G5363.1	4.06E-80
A0A8B6FTA2	K1RKC1	Far upstream element-binding protein 3	2.43E-169	G12312.1	2.51E-12
A0A8B6BVI8	A0A8W8MQD3	Fructose-bisphosphate aldolase	0	G34649.12	0
A0A8B6E885	A0A8W8LLG1	Gelsolin-like domain-containing protein	2.41E-160	G28864.12	1.88E-160
A0A8S3QB23	K1R2L4	Histone H2B	2.28E-66	G22555.2	1.03E-62
A0A8B6DYG8	K1QIR8	78 kDa glucose-regulated protein	0	G29360.3	0
C0Z203	A0A8W8HTN3	Histidine kinase/HSP90-like ATPase domain-containing protein	0	G10994.4	0
A0A8B6BR27	A5LGG7	Glucose-regulated protein 94	0	G30100.1	0
A0A8B6GPC6	K1QIU9	Laminin subunit gamma-1	0	G26184.1	0
A0A8S3QM37	K1QXX7	Myosin heavy chain, non-muscle (Fragment)	0	G24734.1	0
A0A8B6H7X6	A0A8W8KP76	Myosin heavy chain, striated muscle	0	G24734.1	0
A0A8B6FPF3	K1QTC1	Paramyosin	0	G24734.1	1.00E-127
A0A8B6EJ02	K1Q6X5	Protein disulfide-isomerase	0	G1339.29	0
A0A8B6C4T1	A0A8W8MU34	RNA helicase	0	G35350.2	0
A0A8S3Q9N6	K1QZZ5	Stress-induced protein 1	1.00E-33	G17984.2	1.30E-33
A0A8B6EGQ2	A0A8W8JKH8	40S ribosomal protein S14	3.59E-98	G1900.4	2.81E-98
A0A8S3QQF6	K1R0L4	Sodium/potassium-transporting ATPase subunit alpha	0	G25027.37	0
A0A8B6D810	K1R401	Spectrin alpha chain	0	G25515.3	0
Q966V3	A0A8W8LCL2	Transgelin	0	G27652.16	0
A0A076FIR9	K1R7V7	Tubulin beta chain	0	G15286.68	0
A0A8B6ECV1	A0A8W8N7M5	Twitchin	0	G4997.11	0
A0A8B6C479	A0A8W8LS81	Ependymin-related protein 1	5.49E-24	G294.3	4.29E-24
A0A8B6FG82	K1R1A5	Collagen alpha-1(IV) chain	4.72E-38	G9790.1	8.19E-36
A0A8B6DC97	A0A8W8NN09	Metalloendopeptidase	6.29E-128	G6474.29	4.91E-128
A0A8B6GIV6	K1QJ28	Mammalian ependymin-related protein 1	1.09E-89	G12752.5	7.87E-63
A0A8B6BSR2	A0A8W8M032	Coatomer subunit gamma	0	G30403.12	0
A0A8B6CP95	K1R1A5	Collagen alpha-1(IV) chain	0	G15188.2	9.46E-120
A0A8B6EZ83	K1RSS9	Collagen alpha-5(VI) chain	7.16E-50	G3584.8	5.59E-50

A0A8B6CQJ1	K1QYS9	Reelin domain-containing protein	1.23E-43	G21989.2	9.60E-44
A0A8S3QML7	A0A8W8P484	Cathepsin L	1.20E-140	G9704.7	9.39E-141
A0A8B6EWU5	K1PSR5	Fatty acid-binding protein, heart	2.37E-60	G21524.4	1.85E-60
A0A8B6D685	A0A8W8I7C9	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	1.36E-159	G12941.5	1.06E-159
A0A409V740	-	-	-	-	-
A0A8B6DX16	A0A8W8N7H8	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	0	G4985.2	0
A0A8B6BL12	A0A8W8K2R5	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1	3.21E-63	G22173.1	2.50E-63
A0A8B6GJJ6	A0A8W8IB39	Dynein intermediate chain 2, ciliary	0	G13194.4	0
A0A8B6CHC6	A0A8W8MAQ3	EF-hand domain-containing protein	4.15E-22	G32594.10	3.24E-22
A0A8S3V871	K1PWP8	Echinoderm microtubule-associated protein-like 1	0	G22103.1	4.60E-60
A0A8B6F1A7	K1Q5J2	Endoplasmic reticulum transmembrane protein	1.34E-86	G29058.1	1.93E-85
A0A8S3VLQ5	K1PLV6	F-actin-capping protein subunit alpha	1.48E-172	G29303.22	1.16E-172
A0A8B6DQX4	K1QM68	Cytochrome P450 3A29	4.19E-09	G35510.1	3.28E-09
A0A8B6DPU0	A0A8W8N171	FABP domain-containing protein	1.58E-20	G35511.6	1.24E-20
A0A3L5TQZ0	A0A8W8HZA6	C-type lectin domain-containing protein	3.96E-44	G11780.1	3.09E-44
A0A6J8CQ76	A0A8W8KED7	Band 7 domain-containing protein	0	G23529.2	0
A0A0C5Q4G0	A0A8W8I5I4	Galectin	1.06E-130	G12471.17	8.26E-131
A0A8B6DQP9	K1QRQ2	glutamate dehydrogenase	0	G3271.17	0
A0A8B6BF20	A0A8W8NBH2	glutathione transferase	4.33E-86	G5106.4	3.38E-86
A0A6J8ENV8	K1PWZ3	Guanine nucleotide-binding protein subunit beta	0	G22114.37	0
A0A8B6H497	A0A8W8MQI2	Granulins domain-containing protein	0	G34862.1	0
A0A6J8A5H3	K1Q264	Putative histone H1.6	1.86E-35	G34589.4	1.34E-34
A0A411G5T5	A0A8W8KZI1	Ig-like domain-containing protein	2.17E-10	G25938.1	1.70E-10
A0A0B7AUT8	A0A8W8NPB5	Histone H2A	1.79E-84	G661.4	1.40E-84
A0A0L8GYE0	A0A8W8K8G0	Histone H4	6.13E-49	G22554.1	4.79E-49
A0A8S3RBA2	A0A8W8NEU6	RRM domain-containing protein	1.01E-71	G5522.28	7.86E-72
A0A6J8ESL8	A0A8W8HSK9	RRM domain-containing protein	4.20E-82	G1087.10	3.28E-82
A0A8B6GQQ8	A0A8W8HSI3	Ig-like domain-containing protein	2.49E-13	G10784.34	1.95E-13
A0A8B6DFJ0	A0A8W8M908	RRM domain-containing protein	0	G31955.2	0
A0A8B6GGC2	A0A8W8KZ78	Large ribosomal subunit protein uL18 C-terminal eukaryotes domain-containing protein	8.25E-166	G25620.1	6.44E-166
A0A8B6BTE6	A0A8W8N7H9	Large ribosomal subunit protein P1	1.62E-32	G5649.7	1.26E-32
A0A8B6GWR5	K1QNP7	LIM and SH3 domain protein Lasp	4.25E-112	G3888.9	5.91E-92

A0A8B6GV25	K1QT91	LIM domain and actin-binding protein 1	0	G26782.9	5.36E-13
A0A8B6GHC2	K1P2H1	Fatty acid-binding protein, adipocyte	8.48E-57	G21693.2	6.63E-57
A0A8S3Q6R0	K1RFA3	Lamin Dm0	0	G11465.2	0
A0A8B6F3V9	A0A8W8LAY2	SRCR domain-containing protein	0	G26744.2	0
A0A8B6C2Y2	A0A8W8K8K1	Malate dehydrogenase, mitochondrial	2.45E-162	G22856.16	1.91E-162
A0A8B6FW38	A0A8W8J1A5	Stress-70 protein, mitochondrial	0	G16610.7	0
A0A0B7B8Z7	A0A8W8NAI3	Myosin-lb	0	G5341.2	0
A0A8B6EU84	A0A8W8MQE5	PDZ domain-containing protein	1.25E-109	G35457.24	9.80E-110
A0A8S3U4W4	A0A8W8MK96	NADP-dependent oxidoreductase domain-containing protein	3.54E-175	G32718.3	2.76E-175
A0A3G1CJL2	A0A8W8NR61	NAC-A/B domain-containing protein	1.34E-118	G6859.4	1.05E-118
A0A8B6DGA0	K1PSE7	Outer dense fiber protein 3	2.64E-159	G18379.13	2.06E-159
A0A8B6D9K2	-	-	-	-	-
A0A8B6BIH5	A0A8W8N8J6	Peptidyl-prolyl cis-trans isomerase	3.77E-90	G5794.10	2.94E-90
A0A6J8DU89	A0A8W8HZN8	Peptidyl-prolyl cis-trans isomerase	2.94E-121	G11826.2	2.30E-121
A0A6J8EPJ2	A0A8W8LD13	Phospholipid scramblase	3.96E-123	G27559.1	3.09E-123
A0A8B6C763	A0A8W8NS22	Plastin-2	0	G6498.41	0
A0A8B6G5S6	A0A8W8L3N4	Prohibitin	5.25E-155	G26216.2	4.10E-155
A0A8B6DIX4	A0A8W8LQN5	Glutathione S-transferase 1	1.40E-55	G29525.1	1.09E-55
A0A8B6FXU3	A0A8W8MXV3	Thioredoxin domain-containing protein	0	G4424.4	0
A0A8B6EIQ6	K1PQY0	Protein sleepless	4.44E-34	G2186.6	3.47E-34
A0A8S3UA94	A0A8W8LP70	Proactivator polypeptide	9.39E-164	G2909.4	7.33E-164
A0A8S3T3V1	A0A8W8KAL0	Saposin B-type domain-containing protein	4.71E-101	G2310.4	3.68E-101
A0A8B6BG90	A0A8W8LWI7	Rab GDP dissociation inhibitor	0	G30133.24	0
A0A6J8CI59	A0A8W8HWK6	Ras-related protein Rab-1A	4.79E-139	G11385.6	3.74E-139
A0A8B6CVW7	A0A8W8L0E1	Profilin	1.75E-54	G26073.12	1.36E-54
A0A8B6CDT6	A0A8W8KNR2	Rieske domain-containing protein	0	G23926.11	0
A0A6J8F0B5	A0A8W8IZS0	RNA helicase	0	G16238.2	0
A0A3L5TU64	K1QT21	RNA helicase	0	G3197.24	0

A0A8S3TL45	A0A8W8L4Z2	Radial spoke head 1-like protein	6.40E-138	G26404.4	5.00E-138
A0A6J8C2N8	A0A8W8ICW5	Radial spoke head protein 9 homolog	9.09E-152	G13591.5	7.10E-152
A0A6J8BLD6	K1PVW0	S-adenosylmethionine synthase	0	G3241.4	0
A0A8S3TQI9	A0A8W8MDY5	EF-hand domain-containing protein	2.18E-82	G32504.8	1.71E-82
A0A8B6GPC3	A0A8W8JB64	SHSP domain-containing protein	2.01E-33	G17984.2	1.57E-33
A0A8B6F648	A0A8W8IE99	Small nuclear ribonucleoprotein Sm D2	7.72E-80	G13575.2	6.03E-80
A0A8B6F519	A0A8W8N6N8	Plectin/eS10 N-terminal domain-containing protein	5.15E-83	G4582.13	4.02E-83
A0A8B6HL62	K1QC22	40S ribosomal protein S19	7.29E-81	G26391.10	5.70E-81
A0A433TP13	K1PQU8	Sperm-associated antigen 6	0	G32192.7	0
A0A8B6DIG2	A0A8W8LAX1	T-complex protein 1 subunit alpha	0	G27437.1	0
A0A8B6E6D8	A0A8W8I1T4	T-complex protein 1 subunit gamma	0	G12090.2	0
A0A8B6GHP0	A0A8W8IGH4	Tektin	0	G1416.3	0
A0A8B6GPN2	A0A8W8JPK9	Tektin	0	G20358.2	0
A0A8S3SXV7	A0A8W8NSM9	Tetraspanin	3.52E-73	G6559.4	2.75E-73
A0A8S3QFG9	K1R2K2	Periostin	3.41E-92	G29366.1	2.66E-92
A0A8B6FQW6	A0A8W8J501	Transaldolase	0	G17273.18	0
A0A8B6H439	A0A8W8ILL9	Transglutaminase-like domain-containing protein	0	G14594.1	0
A0A0K2D7M8	A0A8W8HUR9	Protocadherin Fat 4	2.52E-29	G11053.1	1.97E-29
A0A8S3QSQ2	K1PD36	Ubiquitin-ribosomal protein eL40 fusion protein	2.89E-92	G26976.2	2.26E-92
A0A8B6G8E3	A0A8W8MB95	SCO-spondin	4.99E-125	G32173.6	3.89E-125
A0A8S3U726	K1QJ28	Mammalian ependymin-related protein 1	4.49E-73	G12308.3	1.04E-58
A0A6J8CCF5	A0A8W8KT22	NodB homology domain-containing protein	7.72E-120	G24491.7	6.03E-120
A0A8B6E104	K1PSR5	Fatty acid-binding protein, heart	3.26E-34	G21524.4	2.90E-34
A0A8B6GUE8	-	-	-	-	-
A0A8B6CB58	A0A8W8JRD3	Apextrin C-terminal domain-containing protein	2.36E-08	G20570.1	1.84E-08
A0A8S3S991	A0A8W8LR11	Mammalian ependymin-related protein 1	5.94E-22	G287.2	4.64E-22
A0A8B6BNI7	-	-	-	-	-
A0A8B6DPQ0	K1R459	SCO-spondin	0	G35504.1	1.30E-133
A0A8S3TWM9	-	-	-	-	-
A0A8B6CU91	-	-	-	-	-
A0A8B6CTG8	-	-	-	-	-
A0A8B6G9T0	A0A8W8LQ90	UspA domain-containing protein	1.66E-43	G29172.5	1.29E-43

A0A6J8DNW3	A5LGH1	Voltage-dependent anion channel	1.73E-137	G1931.16	1.13E-48
A0A8B6H3D0	A0A8W8KJT0	MSP domain-containing protein	2.61E-105	G23892.18	2.04E-105
A0A8B6BI55	A0A8W8MX28	Vinculin	0	G35429.17	0
A0A8B6GXU1	A0A8W8JME6	Vitelline membrane outer layer protein 1	2.26E-48	G19499.2	1.76E-48
A0A8B6C9L4	A0A8W8KJ84	VWFA domain-containing protein	8.69E-106	G23385.8	6.79E-106
A0A8B6F1U6	K1RDT2	Cilia- and flagella-associated protein 52	0	G3365.1	0
A0A8B6HDB4	K1Q662	Actin-interacting protein 1	0	G15632.1	0
A0A8B6CY00	A0A8W8HLX2	2-oxoglutarate dehydrogenase, mitochondrial	0	G1020.16	0
A0A6J8EWE8	K1R8C6	40S ribosomal protein S12	7.88E-86	G2853.3	6.16E-86
A0A0L8FIY8	A0A8W8KBR0	Small ribosomal subunit protein eS28	5.56E-37	G2293.1	4.34E-37
A0A2L1TGZ7	Q70MM6	40S ribosomal protein S3 (Fragment)	9.19E-115	G27592.12	5.77E-114
A0A8S3QVR3	A0A8W8MQU1	Small ribosomal subunit protein uS2	1.03E-143	G3387.29	8.05E-144
A0A6J8B1B1	A0A8W8NW87	ATP-dependent 6-phosphofructokinase	0	G7024.1	0
A0A8S3RZZ6	K1QWX2	60S acidic ribosomal protein P0	4.33E-169	G5077.17	3.38E-169
A0A8S3V934	K1RDM2	60S ribosomal protein L18a	1.84E-105	G28274.6	1.43E-105
A0A077GYU2	A0A8W8N1A1	Large ribosomal subunit protein uL14	2.45E-92	G3885.13	1.91E-92
A0A077GZK7	A0A8W8LHY8	Large ribosomal subunit protein eL28	4.43E-62	G27586.1	3.46E-62
A0A077H0N7	A0A8W8L109	60S ribosomal protein L7a	1.38E-155	G25420.3	1.08E-155
A0A8B6D5A0	A0A8W8L4N8	Large ribosomal subunit protein uL6	2.10E-100	G26435.12	1.64E-100

Supplementary Table S4. Functional analysis of DEPs identified in response to 2'-deoxyadenosine (2). Gene Ontology (GO) enrichment was performed using the g:GOST tool in g:Profiler. The resulting GO terms are categorized into Molecular Functions (MF), Biological Processes (BP), and Cellular Components (CC). Both GO term names, and their corresponding IDs are listed.

Category	GO Name	GO ID	Adjusted <i>p</i> -value
GO:MF	structural molecule activity	GO:0005198	6.19E-19
GO:MF	structural constituent of ribosome	GO:0003735	3.49E-10
GO:MF	unfolded protein binding	GO:0051082	6.50E-05
GO:MF	actin binding	GO:0003779	0.001
GO:MF	structural constituent of chromatin	GO:0030527	0.001
GO:MF	ATP-dependent protein folding chaperone	GO:0140662	0.018
GO:MF	protein folding chaperone	GO:0044183	0.018
GO:MF	actin filament binding	GO:0051015	0.021
GO:MF	calcium ion binding	GO:0005509	0.023
GO:MF	protein disulfide isomerase activity	GO:0003756	0.030
GO:MF	intramolecular oxidoreductase activity, transposing S-S bonds	GO:0016864	0.030
GO:MF	cytoskeletal protein binding	GO:0008092	0.030
GO:MF	extracellular matrix structural constituent	GO:0005201	0.031
GO:MF	anion binding	GO:0043168	0.031
GO:MF	hydrolase activity, acting on carbon-sulfur bonds	GO:0046508	0.044
GO:MF	ATP binding	GO:0005524	0.044
GO:MF	5S rRNA binding	GO:0008097	0.044
GO:MF	G protein activity	GO:0003925	0.044
GO:MF	P-type potassium transmembrane transporter activity	GO:0008556	0.044
GO:MF	porin activity	GO:0015288	0.044
GO:MF	adenosylhomocysteinase activity	GO:0004013	0.044
GO:MF	transketolase or transaldolase activity	GO:0016744	0.044
GO:MF	S-succinyltransferase activity	GO:0016751	0.044
GO:MF	glutamate dehydrogenase (NAD ⁺) activity	GO:0004352	0.044
GO:MF	heterocyclic compound binding	GO:1901363	0.044
GO:MF	citrate (Si)-synthase activity	GO:0004108	0.044
GO:MF	transaldolase activity	GO:0004801	0.044
GO:MF	glutamate dehydrogenase (NADP ⁺) activity	GO:0004354	0.044
GO:MF	wide pore channel activity	GO:0022829	0.044
GO:MF	clathrin light chain binding	GO:0032051	0.044
GO:MF	ribonucleotide binding	GO:0032553	0.044
GO:MF	dihydrolipoyllysine-residue succinyltransferase activity	GO:0004149	0.044
GO:MF	purine ribonucleotide binding	GO:0032555	0.044
GO:MF	protein heterodimerization activity	GO:0046982	0.044
GO:MF	purine ribonucleoside triphosphate binding	GO:0035639	0.044
GO:MF	citrate synthase activity	GO:0036440	0.044
GO:MF	Rab GDP-dissociation inhibitor activity	GO:0005093	0.044
GO:MF	adenyl ribonucleotide binding	GO:0032559	0.044
GO:MF	RNA binding	GO:0003723	0.044
GO:MF	nucleotide binding	GO:0000166	0.044
GO:MF	nucleoside phosphate binding	GO:1901265	0.044
GO:MF	ribonucleoside triphosphate phosphatase activity	GO:0017111	0.045
GO:MF	protein-containing complex binding	GO:0044877	0.045
GO:MF	rRNA binding	GO:0019843	0.048
GO:MF	RNA helicase activity	GO:0003724	0.048
GO:MF	ATP-dependent activity, acting on RNA	GO:0008186	0.048
GO:MF	carbohydrate derivative binding	GO:0097367	0.049
GO:MF	purine nucleotide binding	GO:0017076	0.049
GO:BP	translation	GO:0006412	0
GO:BP	protein folding	GO:0006457	0.001

GO:BP	actin filament capping	GO:0051693	0.002
GO:BP	negative regulation of actin filament depolymerization	GO:0030835	0.002
GO:BP	regulation of protein depolymerization	GO:1901879	0.002
GO:BP	negative regulation of protein depolymerization	GO:1901880	0.002
GO:BP	regulation of actin filament depolymerization	GO:0030834	0.002
GO:BP	cilium movement involved in cell motility	GO:0060294	0.002
GO:BP	negative regulation of protein-containing complex disassembly	GO:0043242	0.002
GO:BP	generation of precursor metabolites and energy	GO:0006091	0.002
GO:BP	protein maturation	GO:0051604	0.002
GO:BP	tricarboxylic acid cycle	GO:0006099	0.002
GO:BP	cilium or flagellum-dependent cell motility	GO:0001539	0.002
GO:BP	cilium-dependent cell motility	GO:0060285	0.002
GO:BP	regulation of protein-containing complex disassembly	GO:0043244	0.002
GO:BP	negative regulation of protein polymerization	GO:0032272	0.004
GO:BP	negative regulation of protein-containing complex assembly	GO:0031333	0.004
GO:BP	negative regulation of actin filament polymerization	GO:0030837	0.004
GO:BP	protein depolymerization	GO:0051261	0.005
GO:BP	negative regulation of supramolecular fiber organization	GO:1902904	0.005
GO:BP	actin filament depolymerization	GO:0030042	0.005
GO:BP	gene expression	GO:0010467	0.005
GO:BP	negative regulation of cytoskeleton organization	GO:0051494	0.005
GO:BP	macromolecule biosynthetic process	GO:0009059	0.006
GO:BP	biosynthetic process	GO:0009058	0.006
GO:BP	cilium movement	GO:0003341	0.007
GO:BP	actin filament organization	GO:0007015	0.007
GO:BP	regulation of actin filament length	GO:0030832	0.007
GO:BP	regulation of protein-containing complex assembly	GO:0043254	0.007
GO:BP	regulation of cellular component size	GO:0032535	0.007
GO:BP	nicotinamide nucleotide metabolic process	GO:0046496	0.007
GO:BP	regulation of protein polymerization	GO:0032271	0.007
GO:BP	regulation of actin filament polymerization	GO:0030833	0.007
GO:BP	regulation of anatomical structure size	GO:0090066	0.007
GO:BP	pyridine nucleotide metabolic process	GO:0019362	0.007
GO:BP	aerobic respiration	GO:0009060	0.007
GO:BP	regulation of actin polymerization or depolymerization	GO:0008064	0.007
GO:BP	actin filament polymerization	GO:0030041	0.008
GO:BP	regulation of actin filament organization	GO:0110053	0.008
GO:BP	negative regulation of organelle organization	GO:0010639	0.009
GO:BP	negative regulation of cellular component organization	GO:0051129	0.009
GO:BP	cell motility	GO:0048870	0.009
GO:BP	regulation of actin cytoskeleton organization	GO:0032956	0.009
GO:BP	regulation of actin filament-based process	GO:0032970	0.009
GO:BP	cellular respiration	GO:0045333	0.010
GO:BP	pyridine-containing compound metabolic process	GO:0072524	0.011
GO:BP	regulation of supramolecular fiber organization	GO:1902903	0.011
GO:BP	protein-containing complex disassembly	GO:0032984	0.011
GO:BP	regulation of cellular component organization	GO:0051128	0.011
GO:BP	actin polymerization or depolymerization	GO:0008154	0.012
GO:BP	regulation of cellular component biogenesis	GO:0044087	0.013
GO:BP	protein metabolic process	GO:0019538	0.013
GO:BP	supramolecular fiber organization	GO:0097435	0.014
GO:BP	cellular component disassembly	GO:0022411	0.015
GO:BP	energy derivation by oxidation of organic compounds	GO:0015980	0.017
GO:BP	cell-matrix adhesion	GO:0007160	0.018
GO:BP	protein polymerization	GO:0051258	0.018
GO:BP	cell-substrate adhesion	GO:0031589	0.019
GO:BP	regulation of cytoskeleton organization	GO:0051493	0.023
GO:BP	cellular component biogenesis	GO:0044085	0.026

GO:BP	actin cytoskeleton organization	GO:0030036	0.027
GO:BP	purine-containing compound metabolic process	GO:0072521	0.028
GO:BP	actin filament-based process	GO:0030029	0.028
GO:BP	ATP metabolic process	GO:0046034	0.029
GO:BP	one-carbon metabolic process	GO:0006730	0.032
GO:BP	purine nucleotide metabolic process	GO:0006163	0.042
GO:BP	purine ribonucleoside triphosphate metabolic process	GO:0009205	0.045
GO:BP	purine nucleoside triphosphate metabolic process	GO:0009144	0.045
GO:BP	regulation of organelle organization	GO:0033043	0.047
GO:BP	protein localization to endoplasmic reticulum exit site	GO:0070973	0.047
GO:BP	ribonucleoside triphosphate metabolic process	GO:0009199	0.047
GO:BP	ribosomal small subunit assembly	GO:0000028	0.047
GO:BP	cellular component assembly	GO:0022607	0.048
GO:CC	ribosome	GO:0005840	1.02E-10
GO:CC	protein-containing complex	GO:0032991	3.73E-10
GO:CC	membraneless organelle	GO:0043228	1.82E-09
GO:CC	intracellular membraneless organelle	GO:0043232	1.82E-09
GO:CC	ribonucleoprotein complex	GO:1990904	1.82E-09
GO:CC	intracellular anatomical structure	GO:0005622	1.18E-06
GO:CC	organelle	GO:0043226	1.53E-06
GO:CC	cytosolic ribosome	GO:0022626	5.93E-06
GO:CC	intracellular organelle	GO:0043229	1.55E-05
GO:CC	cytoplasm	GO:0005737	2.58E-05
GO:CC	endoplasmic reticulum lumen	GO:0005788	0
GO:CC	ribosomal subunit	GO:0044391	0.001
GO:CC	cytosol	GO:0005829	0.001
GO:CC	nucleosome	GO:0000786	0.002
GO:CC	oligosaccharyltransferase complex	GO:0008250	0.006
GO:CC	protein-DNA complex	GO:0032993	0.006
GO:CC	small ribosomal subunit	GO:0015935	0.018
GO:CC	actin cytoskeleton	GO:0015629	0.018
GO:CC	cytoskeleton	GO:0005856	0.022
GO:CC	basement membrane	GO:0005604	0.036

Supplementary Material of Chapter 4

Supplementary Table S5. List of differentially expressed proteins (DEPs) in *Mytilus galloprovincialis* plantigrades exposed to hypoxanthine arabinoside (1'). DEPs were obtained from pairwise comparisons between the control group (0.1% DMSO) and exposed group at 12.5 μ M, using a threshold of $\log_2$ (1.5). Represented are the relative expression of DEPs on the control group (Ctr-) and the exposed group (12.5 μ M), and the obtained fold change of each DEP.

Reference sequence	Protein name	Relative expression		Fold Change
		Ctr-	12.5 μ M	
A0A8B6C5G7	Acetyl-CoA acyltransferase 2 (EC 2.3.1.16)	1.56	-1.56	-3.12
A0A8B6F498	Acyl-coenzyme A oxidase	0.64	-0.64	-1.28
A0A8B6DSL8	Adenosylhomocysteinase (EC 3.13.2.1)	0.61	-0.61	-1.21
A0A8S3SJS5	CAP1_2	1.24	-1.24	-2.47
A0A8S3S1H7	Adk (EC 2.7.4.3)	0.50	-0.50	-1.00
A0A8S3TZ88	Adk (EC 2.7.4.3)	0.87	-0.87	-1.73
A0A8B6D0Q7	ADP-ribosylation factor 1	0.35	-0.35	-0.70
A0A8B6C281	Advillin	1.26	-1.26	-2.53
A0A8B6EIW3	alanine transaminase (EC 2.6.1.2)	1.13	-1.13	-2.26
A0A8B6BGG4	Alpha-aminoadipic semialdehyde synthase (EC 1.5.1.8)	0.64	-0.64	-1.28
A0A8B6D196	Aminopeptidase (EC 3.4.11.-)	1.05	-1.05	-2.10
A0A8S3QGC9	Annexin	0.44	-0.44	-0.87
A0A8S3VH93	Antistasin-like domain-containing protein	2.05	-2.05	-4.09
A0A8S3QFL2	AP complex subunit beta	0.34	-0.34	-0.69
A0A8S3S184	Apextrin C-terminal domain-containing protein	1.07	-1.07	-2.14
A0A8B6BZ74	Apoptosis inhibitor 5	0.47	-0.47	-0.94
A0A8B6D0T5	Apple domain-containing protein	0.81	-0.81	-1.63
A0A8S3TG22	AQP4	1.37	-1.37	-2.74
A0A8S3VBZ6	ARHGDI	0.48	-0.48	-0.96
A0A8B6H8N6	Asparaginyl-tRNA synthetase (EC 6.1.1.22)	1.15	-1.15	-2.29
A0A8S3SXM2	ATP-dependent 6-phosphofructokinase (ATP-PFK) (Phosphofructokinase) (EC 2.7.1.11) (Phosphohexokinase)	-0.34	0.34	0.68
A0A8B6GBE6	ATP synthase-coupling factor 6, mitochondrial	1.19	-1.19	-2.37
A0A8B6H7T7	ATP synthase subunit beta (EC 7.1.2.2)	1.39	-1.39	-2.78
A0A077GYU5	ATP synthase subunit d, mitochondrial	1.10	-1.10	-2.19
A0A8B6GU32	ATP synthase subunit gamma	0.55	-0.55	-1.09
A0A6J8F3Y7	ATP synthase subunit O, mitochondrial (Oligomycin sensitivity conferral protein)	-0.71	0.71	1.41

A0A6J8C7Z5	ATPeF0F	2.34	-2.34	-4.67
A0A8S3Q2E4	ATPeF1D	1.74	-1.74	-3.47
A0A6J8DY85	ATPeV1E	1.29	-1.29	-2.57
A0A8B6BPI1	B box-type domain-containing protein	1.40	-1.40	-2.79
A0A8B6F754	BPTI/Kunitz inhibitor domain-containing protein	1.40	-1.40	-2.81
A0A8B6DQ42	C-type lectin domain-containing protein	1.46	-1.46	-2.91
A0A8B6GW86	C1q domain-containing protein	1.78	-1.78	-3.55
A0A6J8E3N6	C1q domain-containing protein	1.72	-1.72	-3.44
A0A8B6HA48	C2 domain-containing protein	-0.54	0.54	1.08
A0A8S3SGA7	Cadherin domain-containing protein	-1.03	1.03	2.06
A0A8B6FBT9	Calmodulin	1.01	-1.01	-2.03
A0A8S3R7D4	Calreticulin	0.74	-0.74	-1.48
A0A8B6DWN7	cAMP-dependent protein kinase regulator	0.93	-0.93	-1.85
A0A8B6D440	Cathepsin D (EC 3.4.23.5)	0.50	-0.50	-1.01
A0A8B6GVE1	CD80-like immunoglobulin C2-set domain-containing protein	0.61	-0.61	-1.22
A0A8S3URK5	CDY (EC 2.3.1.48)	1.76	-1.76	-3.53
A0A8B6FIN1	Chitin-binding type-2 domain-containing protein	1.46	-1.46	-2.92
A0A8B6DBL3	Chitin-binding type-2 domain-containing protein	1.38	-1.38	-2.77
A0A8S3T4F4	Choline transporter-like protein	0.51	-0.51	-1.02
A0A8B6F3H7	Cilia- and flagella-associated protein 161	1.31	-1.31	-2.62
A0A8B6GX94	Clathrin heavy chain	0.73	-0.73	-1.45
A0A6J8ESY4	Small ribosomal subunit protein uS15 (40S ribosomal protein S13)	1.34	-1.34	-2.67
A0A6J8EHM6	Small ribosomal subunit protein uS5 (40S ribosomal protein S2)	0.98	-0.98	-1.95
A0A8B6G7J5	Large ribosomal subunit protein uL13 (60S ribosomal protein L13a)	1.54	-1.54	-3.08
A0A077H0N7	60S ribosomal protein L7a	1.08	-1.08	-2.16
Q9Y0D6	Actin	-0.52	0.52	1.04
A0A8S3Q5J1	ACTN1_4	1.05	-1.05	-2.10
A0A8B6GV47	ADP/ATP translocase (ADP,ATP carrier protein)	1.56	-1.56	-3.12
A0A8B6D2T4	ADP-ribosylation factor	2.09	-2.09	-4.19
A0A8B6GKE0	Aldehyde dehydrogenase (NAD+) (EC 1.2.1.3)	0.87	-0.87	-1.73
A0A8S3T4E0	ALDH (EC 1.2.1.3)	1.03	-1.03	-2.07
A0A8B6CW94	Alpha-1,4 glucan phosphorylase (EC 2.4.1.1)	0.34	-0.34	-0.68
A0A8B6DXX6	Apextrin C-terminal domain-containing protein	1.17	-1.17	-2.34
A0A8B6HT02	Armadillo repeat-containing protein 4	-0.34	0.34	0.67
A0A8B6CRC1	ATP synthase subunit alpha	1.56	-1.56	-3.12
A0A6J8EBJ6	Band 7 domain-containing protein	1.37	-1.37	-2.73
A0A8B6GBK5	C-1-tetrahydrofolate synthase, cytoplasmic (EC 1.5.1.5) (EC 3.5.4.9) (EC 6.3.4.3)	0.48	-0.48	-0.95

A0A8B6FV13	C1q domain-containing protein	0.81	-0.81	-1.61
A0A8B6G4E0	Calcium-transporting ATPase (EC 7.2.2.10)	0.69	-0.69	-1.38
A0A8B6E763	Calmodulin	0.82	-0.82	-1.63
A0A8S3QR75	CAPNN (EC 3.4.22.-)	1.00	-1.00	-2.00
A0A8B6DHE3	Cathepsin B (EC 3.4.22.1)	0.53	-0.53	-1.06
A0A6J8A856	CEP135	0.61	-0.61	-1.22
A0A8B6EZW6	Cilia- and flagella-associated protein 45	1.16	-1.16	-2.32
A0A8B6C153	Citrate synthase	0.95	-0.95	-1.90
A0A8B6CP95	Collagen, type IV, alpha	0.34	-0.34	-0.68
A0A8B6FHM0	Collagen, type XIII, alpha	-0.68	0.68	1.35
A0A8S3UY90	CROCC	0.64	-0.64	-1.28
A0A8B6C6M2	Crystallin, alpha B	0.90	-0.90	-1.81
A0A6J8D0X9	Fibropellin-1	-0.93	0.93	1.86
A0A8S3QII9	CTSL (EC 3.4.22.15)	0.93	-0.93	-1.86
A0A8B6D685	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial (EC 2.3.1.61) (2-oxoglutarate dehydrogenase complex component E2) (E2K)	0.51	-0.51	-1.01
A0A8S3RHZ5	DLG1	0.54	-0.54	-1.08
A0A8B6DX16	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2 (Ribophorin-2)	0.47	-0.47	-0.95
A0A8B6FGA8	Dynein heavy chain, axonemal	-1.52	1.52	3.04
A0A8S3RF13	Transketolase (EC 2.2.1.1)	1.43	-1.43	-2.86
A0A8S3R8F7	E2.7.3.3 (EC 2.7.3.3)	1.15	-1.15	-2.31
A0A6J8A1B7	EEF1A	1.02	-1.02	-2.04
A0A8B6EQ50	EF-hand domain-containing protein	0.62	-0.62	-1.24
A0A8B6CPN1	EGF-like domain-containing protein	1.08	-1.08	-2.15
A0A8B6FH66	Elongation factor 1-gamma	2.40	-2.40	-4.79
A0A8B6EYD8	Elongation factor 2	0.98	-0.98	-1.97
A0A8B6FQU1	Erythrocyte membrane protein band 4.1	-0.85	0.85	1.69
A0A8B6FTA2	Far upstream element-binding protein	1.48	-1.48	-2.96
A0A8B6GX54	Fibulin-1	0.44	-0.44	-0.89
A0A8B6H907	Filamin	-1.98	1.98	3.96
A0A8B6DL75	Filamin	0.59	-0.59	-1.17
A0A8B6E885	Gelsolin-like domain-containing protein	1.40	-1.40	-2.80
A0A8B6BF20	Glutathione S-transferase (EC 2.5.1.18)	1.52	-1.52	-3.04
A0A8B6DZ81	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	0.79	-0.79	-1.57
A0A8B6DYX8	Heat shock 70kDa protein 5	0.43	-0.43	-0.86
C0Z203	Heat shock protein 90	1.07	-1.07	-2.13
A0A8B6BR27	Heat shock protein 90kDa beta	0.77	-0.77	-1.53

A0A8B6GHQ6	Hemicentin	0.88	-0.88	-1.75
A0A8B6H9V2	Hemicentin	0.39	-0.39	-0.79
A0A0K0YAY3	Heparan sulfate proteoglycan-like protein-1	0.58	-0.58	-1.15
A0A8B6EPK5	Heterogeneous nuclear ribonucleoprotein R	1.51	-1.51	-3.02
A0A0L8GYE0	Histone H4	1.37	-1.37	-2.73
A0A8S3VDI8	HSD17B4 (EC 1.1.1.-, EC 4.2.1.107, EC 4.2.1.119)	0.55	-0.55	-1.11
A0A8B6CAW3	K Homology domain-containing protein	0.63	-0.63	-1.25
A0A8B6BT14	Large subunit ribosomal protein L4e	1.38	-1.38	-2.76
A0A8S3QSD1	LDB3	1.23	-1.23	-2.46
A0A8B6EFR5	Leucyl aminopeptidase (EC 3.4.11.1)	0.99	-0.99	-1.98
A0A8B6GXC9	Uncharacterized protein	0.74	-0.74	-1.47
A0A8S3VFX6	LIM zinc-binding domain-containing protein	0.66	-0.66	-1.32
A0A8S3PRL2	LLGL	0.45	-0.45	-0.90
A0A8S3Q6R0	LMNB	1.20	-1.20	-2.40
A0A8S3TFK4	Malate dehydrogenase (EC 1.1.1.37)	0.85	-0.85	-1.71
A0A8B6C2Y2	Malate dehydrogenase (EC 1.1.1.37)	1.24	-1.24	-2.48
A0A8B6DBF4	Metalloendopeptidase (EC 3.4.24.-)	0.93	-0.93	-1.86
A0A8B6FW38	Molecular chaperone DnaK	0.96	-0.96	-1.92
A0A8B6F2T3	Nesprin-1	-1.21	1.21	2.41
A0A8B6EEA4	Neural cell adhesion molecule	-0.31	0.31	0.62
A0A8B6HFZ0	non-specific serine/threonine protein kinase (EC 2.7.11.1)	0.37	-0.37	-0.75
A0A8B6ETB5	Nucleolin	1.33	-1.33	-2.66
A0A8B6DGA0	Outer dense fiber protein 3	0.99	-0.99	-1.98
A0A8B6D9K2	P-type domain-containing protein	1.54	-1.54	-3.08
A0A8S3RZQ4	Parkin coregulated gene protein homolog, Parkin coregulated gene protein	2.29	-2.29	-4.58
A0A8B6DTM2	PDZ domain-containing protein	0.66	-0.66	-1.32
A0A6J8B435	peptidylglycine monooxygenase (EC 1.14.17.3)	-0.47	0.47	0.95
A0A8B6F8H6	Peroxiredoxin 6, 1-Cys peroxiredoxin (EC 1.11.1.7)	1.13	-1.13	-2.25
A0A8B6FIW7	Solute carrier family 25 (Mitochondrial phosphate transporter), member 3	1.98	-1.98	-3.96
A0A8B6CG53	phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32)	0.60	-0.60	-1.19
A0A8B6HP01	phosphoenolpyruvate mutase (EC 5.4.2.9)	1.31	-1.31	-2.61
A0A8B6C763	Plastin-3	0.81	-0.81	-1.62
A0A8B6CYA7	Poly [ADP-ribose] polymerase (PARP) (EC 2.4.2.-)	-0.73	0.73	1.47
A0A8S3QR29	thioredoxin-dependent peroxiredoxin (EC 1.11.1.24)	1.35	-1.35	-2.70
A0A8B6BJW5	Prohibitin	1.12	-1.12	-2.25
A0A8B6DIX4	Prostaglandin-H2 D-isomerase / glutathione transferase (EC 5.3.99.2)	1.48	-1.48	-2.96
A0A8B6EJ02	Protein disulfide-isomerase (EC 5.3.4.1)	0.67	-0.67	-1.33

A0A8B6G1S8	Protein disulfide-isomerase (EC 5.3.4.1)	-0.40	0.40	0.80
A0A6J8BIG8	Small ribosomal subunit protein RACK1	0.79	-0.79	-1.58
A0A8B6D4L6	Radixin	1.30	-1.30	-2.60
A0A3G1CJN6	Ribosomal protein S3a	0.74	-0.74	-1.49
A0A8B6C4T1	RNA helicase (EC 3.6.4.13)	1.36	-1.36	-2.71
A0A8B6DTX0	Rootletin	0.85	-0.85	-1.71
A0A8B6BF70	SHSP domain-containing protein	0.90	-0.90	-1.80
A0A8B6DCE2	40S ribosomal protein S3	-0.39	0.39	0.78
A0A8S3QQF6	Sodium/potassium-transporting ATPase subunit alpha	1.26	-1.26	-2.52
A0A126Q9D5	Staphylococcal nuclease domain-containing protein 1	0.93	-0.93	-1.86
A0A6J8B610	Stress-induced-phosphoprotein 1	0.61	-0.61	-1.21
A0A6J8DUP3	STOM	0.73	-0.73	-1.45
A0A8S3TEG9	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial (EC 1.3.5.1)	1.92	-1.92	-3.84
A0A6J8ESE0	TAGLN	1.07	-1.07	-2.14
A0A8B6GHP0	Tektin	0.84	-0.84	-1.68
A0A8B6DFA7	Thymosin beta	0.41	-0.41	-0.83
Q966V3	Transgelin	0.52	-0.52	-1.03
A0A8B6H439	Transglutaminase-like domain-containing protein	0.83	-0.83	-1.65
A0A6J8BFQ0	Trichohyalin-plectin-homology domain-containing protein	0.30	-0.30	-0.60
A0A8B6BTY3	Triosephosphate isomerase (EC 5.3.1.1)	2.12	-2.12	-4.25
A0A8B6F8B1	Tropomyosin	0.79	-0.79	-1.57
A0A3S1A1H2	Tubulin alpha chain	0.67	-0.67	-1.33
A0A8B6BWE2	Tubulin beta chain	0.44	-0.44	-0.87
A0A6J8CNX6	Uncharacterized protein	1.33	-1.33	-2.66
A0A8B6BL03	Ubiquinol-cytochrome c reductase core subunit 2	0.81	-0.81	-1.63
A0A8B6HIV7	Uncharacterized protein	1.02	-1.02	-2.04
A0A8B6EIG0	Uncharacterized protein	1.25	-1.25	-2.49
A0A8B6DFD7	Uncharacterized protein	0.60	-0.60	-1.20
A0A8B6G096	Uncharacterized protein	0.52	-0.52	-1.04
A0A8B6E104	Uncharacterized protein	2.01	-2.01	-4.01
A0A8B6EM75	Uncharacterized protein	1.52	-1.52	-3.04
A0A8B6C479	Uncharacterized protein	0.32	-0.32	-0.63
A0A6J8CV26	Uncharacterized protein	0.43	-0.43	-0.86
A0A8B6E647	Uncharacterized protein	-1.44	1.44	2.89
A0A8B6GX65	Uncharacterized protein	-0.62	0.62	1.24
A0A6J8AGA3	Uncharacterized protein	0.96	-0.96	-1.91
A0A8S3SZC2	Choice-of-anchor I domain-containing protein	0.43	-0.43	-0.86

A0A8B6DPQ0	SCO-spondin	0.33	-0.33	-0.67
A0A8B6DC97	MAM domain-containing protein	0.70	-0.70	-1.39
A0A6J8BUN8	Uncharacterized protein	0.79	-0.79	-1.57
A0A8B6GIV6	Mammalian ependymin-related protein 1	0.75	-0.75	-1.50
A0A8B6G9E5	Uncharacterized protein	0.48	-0.48	-0.96
A0A8B6CB58	Uncharacterized protein	1.25	-1.25	-2.50
A0A8B6BI55	Vinculin	1.94	-1.94	-3.88
A0A8B6BU43	VWFA domain-containing protein	-1.69	1.69	3.38
A0A8S3TBN4	YBX1	2.18	-2.18	-4.36
A0A8S3PR18	YWHAB_Q_Z	0.75	-0.75	-1.49
A0A8B6EBM4	Coatomer subunit alpha	0.43	-0.43	-0.87
A0A8S3VPZ7	Coatomer subunit delta	-0.56	0.56	1.12
A0A8B6CVU1	Cofilin	1.04	-1.04	-2.07
A0A8B6BSQ3	Coiled-coil domain-containing protein 170	-0.33	0.33	0.66
A0A8B6FD83	Collagen, type II, alpha	-0.40	0.40	0.79
A0A8B6EZ83	Collagen, type IV, alpha	0.56	-0.56	-1.12
A0A8B6CQJ1	Copper acquisition factor BIM1-like domain-containing protein	0.87	-0.87	-1.74
A0A8B6CAW5	Copper acquisition factor BIM1-like domain-containing protein	0.40	-0.40	-0.81
A0A8S3Q3L3	CRISPLD	0.71	-0.71	-1.42
A0A6J8BMS7	CTTN	0.51	-0.51	-1.02
A0A8S3RLA1	Uncharacterized protein	1.00	-1.00	-1.99
A0A8S3RUL4	cystathionine gamma-lyase (EC 4.4.1.1) (Gamma-cystathionase)	0.64	-0.64	-1.29
A0A8B6HEV7	Cysteine and glycine-rich protein	2.29	-2.29	-4.57
A0A6J8AEC3	Cysteine-rich with EGF-like domain protein 2-B,Cysteine-rich with EGF-like domain protein 2-A,Cysteine-rich with EGF-like domain protein 2,Cysteine-rich with EGF-like domain protein 1	1.08	-1.08	-2.17
A0A8B6F031	Cytochrome b-c1 complex subunit 6	0.48	-0.48	-0.97
A0A8B6G281	Cytochrome c oxidase subunit 5b	2.29	-2.29	-4.58
A0A8B6EWU5	Cytosolic fatty-acid binding proteins domain-containing protein	0.79	-0.79	-1.57
A0A8B6H151	Death-associated protein 1	1.52	-1.52	-3.03
A0A077H0R0	Deltamethrin resistance protein prag01 domain-containing protein	0.78	-0.78	-1.56
A0A8B6GTY8	Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase (EC 1.1.1.179) (EC 1.3.1.20) (D-xylose 1-dehydrogenase) (D-xylose-NADP dehydrogenase) (Dimeric dihydrodiol dehydrogenase)	1.46	-1.46	-2.93
A0A8B6DQH5	Dihydrolipoyl dehydrogenase (EC 1.8.1.4)	0.76	-0.76	-1.52
A0A6J8BTF0	DNALI	0.94	-0.94	-1.88
A0A8B6BL12	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1 (Oligosaccharyl transferase subunit DAD1)	2.33	-2.33	-4.65
A0A8B6H0X9	Drebrin-like protein	0.87	-0.87	-1.74
A0A8B6FMV8	Secreted protein	0.51	-0.51	-1.01
A0A6J8DUW4	Uncharacterized protein	0.83	-0.83	-1.67

A0A6J8BZ13	Dynein light intermediate chain	0.89	-0.89	-1.77
A0A8B6G9Z7	E1 ubiquitin-activating enzyme (EC 6.2.1.45) (Ubiquitin-activating enzyme E1)	0.77	-0.77	-1.53
A0A8B6EYR0	EF-hand domain-containing family member C2	1.58	-1.58	-3.17
A0A8B6CHC6	EF-hand domain-containing protein	0.45	-0.45	-0.90
A0A3G1CJM7	EF-hand domain-containing protein	0.85	-0.85	-1.70
A0A3R5Q145	EF-hand domain-containing protein	1.49	-1.49	-2.98
A0A8B6GTM1	EF-hand domain-containing protein	0.70	-0.70	-1.39
A0A8B6BHU6	EF-hand domain-containing protein	1.13	-1.13	-2.26
A0A8B6EJE1	EF-hand domain-containing protein	1.46	-1.46	-2.91
A0A8B6DVI4	EGF-like domain-containing protein	0.94	-0.94	-1.87
A0A8S3RV51	EGF-like domain-containing protein	0.87	-0.87	-1.74
A0A6J8CPT2	Electron transfer flavoprotein subunit alpha (Alpha-ETF)	1.09	-1.09	-2.18
A0A8S3V871	EML1_2	1.06	-1.06	-2.13
A0A8B6C964	Endonuclease/exonuclease/phosphatase domain-containing protein	0.98	-0.98	-1.95
A0A8B6H228	Endoplasmic reticulum resident protein 44	0.79	-0.79	-1.59
A0A8S3VIN2	Enolase	3.32	-3.32	-6.64
A0A8B6CMG1	enoyl-CoA hydratase (EC 4.2.1.17)	0.79	-0.79	-1.57
A0A8B6GL66	Epidermal growth factor receptor kinase substrate 8	0.33	-0.33	-0.66
A0A6J8D3C7	ERLIN	0.61	-0.61	-1.22
A0A6J8EG86	Eukaryotic translation initiation factor 3 subunit B (eIF3b) (Eukaryotic translation initiation factor 3 subunit 9)	0.62	-0.62	-1.25
A0A8S3RJH3	Eukaryotic translation initiation factor 3 subunit I (eIF3i)	1.23	-1.23	-2.46
A0A8S3PS95	Eukaryotic translation initiation factor 5A (eIF-5A)	1.50	-1.50	-3.00
A0A8S3VLQ5	F-actin-capping protein subunit alpha	2.22	-2.22	-4.43
A0A8B6DPU0	Cytosolic fatty-acid binding proteins domain-containing protein	1.75	-1.75	-3.51
A0A8B6DQX4	Lipocalin/cytosolic fatty-acid binding domain-containing protein	1.65	-1.65	-3.31
A0A8B6BMA1	Uncharacterized protein	1.23	-1.23	-2.45
A0A8B6E270	FAS1 domain-containing protein	1.64	-1.64	-3.28
A0A8B6F6T7	FAS1 domain-containing protein	1.18	-1.18	-2.36
A0A6J8D3U3	Fascin	1.23	-1.23	-2.47
A0A8B6G0F8	Fibronectin type-III domain-containing protein	-0.72	0.72	1.44
A0A8B6EE15	Formyltetrahydrofolate dehydrogenase (EC 1.5.1.6)	1.97	-1.97	-3.94
A0A8S3UCU2	FrmA (EC 1.1.1.1, EC 1.1.1.284)	0.79	-0.79	-1.58
A0A8S3TR72	Fructose-bisphosphate aldolase (EC 4.1.2.13)	1.25	-1.25	-2.51
A0A8B6BVI8	Fructose-bisphosphate aldolase (EC 4.1.2.13)	1.29	-1.29	-2.58
A0A8S3Q6H6	Fumarylacetoacetase (EC 3.7.1.2) (Fumarylacetoacetate hydrolase)	1.62	-1.62	-3.25
A0A0C5Q4G0	Galectin	0.83	-0.83	-1.65
A0A8B6CKU6	Gasdermin pore forming domain-containing protein	0.62	-0.62	-1.23

A0A8B6D0L8	Gelsolin	1.03	-1.03	-2.05
A0A8S3QNI2	Glucose-6-phosphate isomerase (EC 5.3.1.9)	0.94	-0.94	-1.87
A0A8B6BHJ8	Glucosidase 2 subunit beta	0.39	-0.39	-0.78
A0A8B6DQP9	glutamate dehydrogenase [NAD(P)(+)] (EC 1.4.1.3)	0.87	-0.87	-1.74
A0A8S3Q5G9	Glutathione S-transferase omega (GSTO) (EC 1.20.4.2) (EC 1.8.5.1) (EC 2.5.1.18) (Glutathione-dependent dehydroascorbate reductase) (Monomethylarsonic acid reductase)	1.65	-1.65	-3.30
A0A6J8BZR7	Glyoxalase 1,Glyoxalase domain-containing protein 4	1.19	-1.19	-2.37
A0A8S3VGU6	GMP reductase (GMPR) (EC 1.7.1.7) (Guanosine 5'-monophosphate oxidoreductase) (Guanosine monophosphate reductase)	1.39	-1.39	-2.79
A0A6J8ENV8	GNB1	0.68	-0.68	-1.35
A0A8S3R8I4	Golgi reassembly-stacking protein 2,Golgi reassembly-stacking protein 1	0.38	-0.38	-0.77
A0A6J8F5H8	aspartate transaminase (EC 2.6.1.1)	0.66	-0.66	-1.32
A0A2H4WCW9	Guanine nucleotide-binding protein subunit alpha	1.43	-1.43	-2.86
A0A6J8CA81	GSK3B (EC 2.7.11.26)	0.99	-0.99	-1.98
A0A0L8I0K2	small monomeric GTPase (EC 3.6.5.2)	1.09	-1.09	-2.18
A0A8B6HGH7	Heat shock 70kDa protein 4	0.55	-0.55	-1.11
A0A411G5T5	Hemicentin-like protein	0.31	-0.31	-0.62
A0A8B6D225	Heterogeneous nuclear ribonucleoprotein A1/A3	0.62	-0.62	-1.24
A0A8B6ETA9	Histone H1/5	0.84	-0.84	-1.68
Q6WV85	Histone H2B	0.37	-0.37	-0.74
A0A8S3RBA2	HNRNPA1_3	1.74	-1.74	-3.48
A0A6J8ESL8	HNRNPABD	1.07	-1.07	-2.14
A0A8S3U136	hydroxyacid-oxoacid transhydrogenase (EC 1.1.99.24)	0.54	-0.54	-1.09
A0A8B6GQQ8	Ig-like domain-containing protein	1.60	-1.60	-3.19
A0A8B6DGI1	Insulin-like growth factor 2 mRNA-binding protein 1	0.59	-0.59	-1.17
A0A8B6CIE6	Isocitrate dehydrogenase [NAD] subunit, mitochondrial	0.71	-0.71	-1.42
A0A8B6ET27	Kazal-like domain-containing protein	1.29	-1.29	-2.58
A0A6J8A1Y0	KCTD8_12_16	0.70	-0.70	-1.40
A0A8B6FVU2	Kinesin-like protein	1.42	-1.42	-2.84
A0A8B6G9Q8	Laminin, alpha 3/5	0.43	-0.43	-0.86
A0A8B6CXT1	Laminin, beta 1	0.77	-0.77	-1.53
A0A8B6CGB4	Large subunit ribosomal protein L10Ae	1.35	-1.35	-2.69
A0A8B6F9E7	Large ribosomal subunit protein eL22 (60S ribosomal protein L22)	-0.52	0.52	1.04
A0A8B6BGD5	Large subunit ribosomal protein L37e	1.73	-1.73	-3.46
A0A8B6GGC2	Large subunit ribosomal protein L5e	0.83	-0.83	-1.67
A0A8B6E6I0	Large subunit ribosomal protein L7e	0.68	-0.68	-1.36
A0A8B6BTE6	Large ribosomal subunit protein P1 (60S acidic ribosomal protein P1)	0.63	-0.63	-1.25
A0A6J8B8C6	LHFPL	1.14	-1.14	-2.28

A0A8B6GHC2	Lipocalin/cytosolic fatty-acid binding domain-containing protein	2.17	-2.17	-4.34
A0A0K0YB29	LKD-rich protein-1	0.35	-0.35	-0.70
A0A8S3U3J8	LRP2	1.62	-1.62	-3.23
A0A8S3UA31	LRP2	1.54	-1.54	-3.08
A0A8B6GPG7	Uncharacterized protein	0.81	-0.81	-1.62
A0A8B6G660	Lupus La protein	1.28	-1.28	-2.55
A0A8B6DCW7	MAGUK p55 subfamily member 6	1.15	-1.15	-2.30
A0A6J8CXM9	Malectin-B, Malectin-A, Malectin	1.93	-1.93	-3.85
A0A8B6D8H6	Mammalian ependymin-related protein 1	0.69	-0.69	-1.37
A0A6J8EFL5	Mammalian ependymin-related protein 1	1.50	-1.50	-3.00
A0A8S3RY54	MAPRE	1.28	-1.28	-2.57
A0A8B6BF41	MARVEL domain-containing protein	1.62	-1.62	-3.23
A0A8B6CZ83	Choice-of-anchor I domain-containing protein	1.45	-1.45	-2.89
Q697L7	Metallothionein 10-III	2.04	-2.04	-4.09
A0A8S3QX71	methyilmalonate-semialdehyde dehydrogenase (CoA acylating) (EC 1.2.1.27)	1.38	-1.38	-2.76
A0A6J8F1I0	MTCH	1.06	-1.06	-2.12
A0A8B6H0Z6	Myosin VI	-0.66	0.66	1.31
A0A8B6EU84	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	1.14	-1.14	-2.28
A0A8S3U4W4	NADP-dependent oxidoreductase domain-containing protein	0.53	-0.53	-1.06
A0A6J8BDN2	NADP-dependent oxidoreductase domain-containing protein	0.47	-0.47	-0.94
A0A6J8CRB1	NHP2	1.49	-1.49	-2.97
A0A8S3QGN5	NME8	0.64	-0.64	-1.27
A0A8S3PMK0	Nuclear migration protein nudC (Nuclear distribution protein C homolog)	0.94	-0.94	-1.89
A0A8B6C4N8	protein-disulfide reductase (EC 1.8.1.8)	0.43	-0.43	-0.85
A0A8S3ULS9	Nucleoside diphosphate kinase (EC 2.7.4.6)	1.12	-1.12	-2.24
A0A8S3UGN8	Obg-like ATPase 1	0.42	-0.42	-0.83
A0A8B6EUV0	OTU domain-containing protein	-0.29	0.29	0.59
A0A8B6BMW1	Pancreatic elastase II (EC 3.4.21.71)	1.46	-1.46	-2.92
A0A6J8DW13	PDIA5 (EC 5.3.4.1)	0.65	-0.65	-1.29
A0A8S3TBS5	PDZ domain-containing protein	1.34	-1.34	-2.68
A0A8B6DFV3	Peptidase M24 domain-containing protein	1.52	-1.52	-3.03
A0A8B6D8S4	Peptidase metallopeptidase domain-containing protein	0.36	-0.36	-0.72
A0A8B6BIH5	Peptidyl-prolyl cis-trans isomerase (PPIase) (EC 5.2.1.8)	0.89	-0.89	-1.78
A0A161I1V8	Peptidyl-prolyl cis-trans isomerase (PPIase) (EC 5.2.1.8)	0.35	-0.35	-0.69
A0A6J8DKH3	Peptidyl-prolyl cis-trans isomerase (PPIase) (EC 5.2.1.8)	0.92	-0.92	-1.83
A0A8S3SEX5	Peptidyl-prolyl cis-trans isomerase (PPIase) (EC 5.2.1.8)	1.89	-1.89	-3.78
A0A8B6EBJ6	peptidylprolyl isomerase (EC 5.2.1.8)	1.51	-1.51	-3.01

A0A8S3RID5	peptidylprolyl isomerase (EC 5.2.1.8)	0.65	-0.65	-1.30
A0A8B6D5H2	Uncharacterized protein	0.40	-0.40	-0.80
A0A8B6GKK6	Peroxiredoxin-5 (EC 1.11.1.24)	1.11	-1.11	-2.22
A0A8S3R7F6	phosphoglycerate mutase (2,3-diphosphoglycerate-dependent) (EC 5.4.2.11)	0.43	-0.43	-0.86
A0A8S3S8I1	phosphoglucomutase (alpha-D-glucose-1,6-bisphosphate-dependent) (EC 5.4.2.2)	0.32	-0.32	-0.64
A0A6J7ZU18	Phosphoglycerate kinase (EC 2.7.2.3)	0.87	-0.87	-1.75
A0A6J8AQ45	Phospholipid scramblase	0.68	-0.68	-1.36
A0A6J8EPJ2	Phospholipid scramblase	1.42	-1.42	-2.84
A0A8B6DQL6	Enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (2-phosphoglycerate dehydratase)	0.80	-0.80	-1.60
A0A8S3SL70	PMPCB (EC 3.4.24.64)	0.42	-0.42	-0.84
A0A6J8E914	PRKAR	1.08	-1.08	-2.15
A0A8B6FT25	Profilin	1.28	-1.28	-2.57
A0A8B6CHE6	Profilin	1.73	-1.73	-3.46
A0A8B6D5R1	Programmed cell death 6-interacting protein	-0.41	0.41	0.82
A0A8B6G5S6	Prohibitin	2.03	-2.03	-4.06
A0A8B6CYK1	Proteasome subunit alpha type	1.66	-1.66	-3.33
A0A8S3UDK6	Proteasome subunit alpha type	1.09	-1.09	-2.18
A0A8B6DC08	Protein DEK	1.02	-1.02	-2.04
A0A8B6FXU3	Protein disulfide-isomerase A6 (EC 5.3.4.1)	0.66	-0.66	-1.32
A0A6J8DCE2	Protein RCC2 homolog,Protein RCC2	1.30	-1.30	-2.60
A0A8B6EIQ6	Protein sleepless	2.52	-2.52	-5.04
A0A8S3S9K8	Uncharacterized protein	1.33	-1.33	-2.65
A0A8B6CNR6	Protocadherin Fat 4	-0.88	0.88	1.76
A0A8S3UA94	PSAP	-0.77	0.77	1.54
A0A0G2YLG5	PTPRF (EC 3.1.3.48) (Shell protein-6)	0.76	-0.76	-1.52
A0A6J8BU32	PURA	0.31	-0.31	-0.61
A0A8B6BR62	Pyruvate dehydrogenase E1 component subunit alpha (EC 1.2.4.1)	1.47	-1.47	-2.95
A0A8S3QRM1	QDPR (EC 1.5.1.34)	0.60	-0.60	-1.19
A0A8S3RSD5	Queuosine 5'-phosphate N-glycosylase/hydrolase (EC 3.2.2.-) (Queuosine-nucleotide N-glycosylase/hydrolase)	-0.40	0.40	0.81
A0A6J8CI59	RAB1A	1.39	-1.39	-2.79
A0A0B7AP29	Ras-related protein Rab-2A	0.32	-0.32	-0.64
A0A8B6FT60	Radial spoke head protein 4A	0.75	-0.75	-1.50
A0A8B6GLW0	Radial spoke head protein 9 homolog	0.40	-0.40	-0.81
A0A2C9JE50	Ras-related protein Rab-11B-like	1.72	-1.72	-3.44
A0A6J8CUS4	Ras-related protein Rab-14 (EC 3.6.5.2)	0.66	-0.66	-1.33
A0A8B6G941	Ras-related protein Rab-35	0.87	-0.87	-1.73
A0A6J8F240	RBM4	1.43	-1.43	-2.86

A0A8B6C295	Receptor expression-enhancing protein	1.44	-1.44	-2.89
A0A6J8DY17	RIB43A-like with coiled-coils protein 2	1.16	-1.16	-2.32
A0A077H3K4	Ribosomal protein L23a	1.78	-1.78	-3.56
A0A077H3J9	Small ribosomal subunit protein uS9 (40S ribosomal protein S16)	1.55	-1.55	-3.10
A0A8S3QYE0	Uncharacterized protein	0.84	-0.84	-1.68
A0A8B6FIE8	RNA-binding protein 4	1.36	-1.36	-2.73
A0A6J8A0F1	Large ribosomal subunit protein eL24 (60S ribosomal protein L24)	0.44	-0.44	-0.88
A0A8S3UK82	RP-L26e	1.98	-1.98	-3.97
A0A8B6EXY4	RRM domain-containing protein	0.92	-0.92	-1.83
A0A8B6HNM7	S-(Hydroxymethyl)glutathione dehydrogenase / alcohol dehydrogenase (EC 1.1.1.284)	1.10	-1.10	-2.19
A0A8B6CEB0	Saposin	0.55	-0.55	-1.11
A0A6J8DVF9	SEC61A	0.45	-0.45	-0.91
A0A8B6G4G0	Selenoprotein F/M domain-containing protein	2.15	-2.15	-4.30
A0A8S3RKC5	Serine hydroxymethyltransferase (EC 2.1.2.1)	1.00	-1.00	-2.00
A0A8B6H826	SH3 domain-containing protein	0.69	-0.69	-1.38
A0A8B6GPC3	SHSP domain-containing protein	1.06	-1.06	-2.13
A0A8S3RCR9	SLC25A23S	0.59	-0.59	-1.17
A0A8B6F648	Small nuclear ribonucleoprotein Sm D2 (Sm-D2) (snRNP core protein D2)	2.22	-2.22	-4.44
A0A8B6CSI5	Small subunit ribosomal protein S17e	2.16	-2.16	-4.33
A0A8B6HL62	Small subunit ribosomal protein S19e	-0.42	0.42	0.83
A0A6J8BWT3	SNX1_2	1.24	-1.24	-2.49
A0A8S3VFI8	SPATA6	0.93	-0.93	-1.86
A0A6J8D7K5	SQOR (EC 1.8.5.8)	1.01	-1.01	-2.02
A0A6J8E460	Succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial (EC 6.2.1.5) (Succinyl-CoA synthetase beta chain) (SCS-beta)	1.09	-1.09	-2.18
A0A8S3R089	Succinate--CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial (EC 6.2.1.4) (EC 6.2.1.5) (Succinyl-CoA synthetase subunit alpha) (SCS-alpha)	0.90	-0.90	-1.80
A0A8B6CGG6	Suppressor of G2 allele of SKP1	0.66	-0.66	-1.31
A0A8B6EYT5	Synaptotagmin-1	0.84	-0.84	-1.67
A0A8B6DIG2	T-complex protein 1 subunit alpha (CCT-alpha)	0.42	-0.42	-0.84
A0A8B6E6D8	T-complex protein 1 subunit gamma	2.11	-2.11	-4.21
A0A8B6F9J1	T-complex protein 1 subunit zeta	0.65	-0.65	-1.30
A0A6J8BSI9	Tektin	1.34	-1.34	-2.67
A0A6J8BBL7	Tektin	1.02	-1.02	-2.04
A0A8S3SXV7	Tetraspanin	0.83	-0.83	-1.66
A0A8B6CJW7	Tetraspanin	1.54	-1.54	-3.09
A0A8B6DN29	Tetraspanin	1.29	-1.29	-2.59
A0A6J8AMT5	Tetratricopeptide repeat protein 29	0.67	-0.67	-1.35

A0A8B6H5W0	Thioredoxin	1.97	-1.97	-3.95
A0A8S3QJ24	Thioredoxin-like protein 1	0.57	-0.57	-1.15
A0A8B6FN66	Thyroglobulin type-1 domain-containing protein	1.77	-1.77	-3.55
A0A8B6C4T2	Thyroglobulin type-1 domain-containing protein	1.43	-1.43	-2.87
A0A8S3R738	TIMP4	0.81	-0.81	-1.61
A0A8S3R9Y2	TIMP4	1.59	-1.59	-3.17
A0A8B6FQW6	Transaldolase (EC 2.2.1.2)	1.22	-1.22	-2.44
A0A8B6FLR4	Transcription elongation factor	0.68	-0.68	-1.36
A0A8B6CWL2	Transgelin	1.54	-1.54	-3.08
A0A6J7ZVG6	Transgelin	1.05	-1.05	-2.11
A0A8B6H8C4	Tripeptidyl-peptidase 2 (EC 3.4.14.10) (Tripeptidyl aminopeptidase)	-0.84	0.84	1.67
A0A8B6F7C4	Tropomyosin	0.80	-0.80	-1.59
A0A6J8DNP0	TROVE2	0.58	-0.58	-1.16
A0A0K2D7M8	TSP_1 domain containing protein-1	1.12	-1.12	-2.24
A0A0B6Z2B0	Tubulin beta chain	0.47	-0.47	-0.94
A0A8B6DWZ2	Tumor necrosis factor ligand superfamily member 10	1.20	-1.20	-2.39
A0A8S3TTF4	Tumor protein D54	1.77	-1.77	-3.53
A0A8B6FHC2	Tyrosinase (EC 1.14.18.1)	1.31	-1.31	-2.62
A0A8B6FCM7	Tyrosine-protein kinase ephrin type A/B receptor-like domain-containing protein	0.97	-0.97	-1.95
A0A8S3SPC1	UBA domain-containing protein	-0.53	0.53	1.05
A0A8S3QZQ4	UBE2N (EC 2.3.2.23)	2.09	-2.09	-4.18
A0A8B6CGQ6	Ubiquinol-cytochrome c reductase iron-sulfur subunit (EC 1.10.2.2)	1.10	-1.10	-2.19
A0A0B6Y8N5	Ubiquitin-ribosomal protein eL40 fusion protein	1.92	-1.92	-3.84
A0A6J8CSZ8	Uncharacterized oxidoreductase C513.07	1.58	-1.58	-3.15
A0A8B6FG82	Uncharacterized protein	-0.70	0.70	1.39
A0A3L5TR65	Uncharacterized protein	1.28	-1.28	-2.55
A0A8B6FNN6	Uncharacterized protein	1.67	-1.67	-3.35
A0A8B6G8E3	SCO-spondin	0.91	-0.91	-1.82
A0A8B6GHG6	Uncharacterized protein	2.00	-2.00	-4.01
A0A8B6CU91	Uncharacterized protein	1.47	-1.47	-2.95
A0A8B6CEC3	Metallothionein	0.47	-0.47	-0.95
A0A8B6HNQ5	Uncharacterized protein	1.23	-1.23	-2.45
A0A8B6FSV1	Uncharacterized protein	0.57	-0.57	-1.13
A0A8B6CTG8	Uncharacterized protein	1.69	-1.69	-3.38
A0A8B6ENM5	Uncharacterized protein	1.61	-1.61	-3.21
A0A8B6CCA0	Uncharacterized protein	1.45	-1.45	-2.90
A0A8B6DTT6	Uncharacterized protein	1.49	-1.49	-2.98

A0A6J8CD73	Uncharacterized protein	0.81	-0.81	-1.61
A0A8S3T9B8	Uncharacterized protein	1.55	-1.55	-3.10
A0A8S3RZM2	Kyphoscoliosis peptidase	2.43	-2.43	-4.86
A0A8B6F5H6	Uncharacterized protein	1.85	-1.85	-3.70
A0A8B6H837	Uncharacterized protein	0.99	-0.99	-1.97
A0A8B6FQ26	Uncharacterized protein	2.14	-2.14	-4.28
A0A8B6DR40	EF-hand domain-containing protein 1	0.55	-0.55	-1.10
A0A8B6BSD4	Counting factor associated protein D	-1.26	1.26	2.52
A0A8B6CRI3	Uncharacterized protein	0.85	-0.85	-1.70
A0A8B6CM77	Uncharacterized protein	0.33	-0.33	-0.65
A0A8B6DHU3	Uncharacterized protein	0.45	-0.45	-0.90
A0A8B6CG35	Uncharacterized protein	0.46	-0.46	-0.92
A0A8S3RSI4	Uncharacterized protein	2.29	-2.29	-4.58
A0A8B6DI09	Uncharacterized protein	1.45	-1.45	-2.89
A0A8B6CVX7	Uncharacterized protein	1.03	-1.03	-2.06
A0A8B6HFX8	Uncharacterized protein	2.13	-2.13	-4.26
A0A8B6F299	Mucin-2-like	0.44	-0.44	-0.89
A0A8B6EZW5	Uncharacterized protein	1.64	-1.64	-3.28
A0A8B6C5E2	Uncharacterized protein	2.59	-2.59	-5.18
A0A6J8CCF5	NodB homology domain-containing protein	-0.30	0.30	0.61
A0A8S3U5X7	Uncharacterized protein	0.81	-0.81	-1.62
A0A8B6C9P4	Uncharacterized protein	0.80	-0.80	-1.61
A0A8B6H7C0	Uncharacterized protein	1.07	-1.07	-2.13
A0A8B6G6F1	VWFD domain-containing protein	-7.11	7.11	14.20
A0A8B6FPE6	Uncharacterized protein	1.66	-1.66	-3.33
A0A8S3S991	Uncharacterized protein	2.28	-2.28	-4.57
A0A8B6BE54	Rlla domain-containing protein	0.47	-0.47	-0.95
A0A8B6GK87	Uncharacterized protein	-1.11	1.11	2.22
A0A8S3SVU1	Sperm microtubule inner protein 1 C-terminal domain-containing protein	1.44	-1.44	-2.88
A0A8B6D1E0	Uncharacterized protein	1.25	-1.25	-2.50
A0A8B6CXZ8	Ciliary microtubule inner protein 2C	1.25	-1.25	-2.50
A0A8B6HI74	Uncharacterized protein	0.93	-0.93	-1.86
A0A6J8EM21	Phosphatidylinositol transfer protein N-terminal domain-containing protein	0.83	-0.83	-1.66
A0A8S3UJ52	Uncharacterized protein	1.25	-1.25	-2.49
A0A8B6D1P6	Uncharacterized protein	1.61	-1.61	-3.23
A0A8B6EZY7	CTCK domain-containing protein	1.45	-1.45	-2.91
A0A8S3QP16	Uncharacterized protein	0.47	-0.47	-0.93

A0A8S3UR85	Uncharacterized protein	1.11	-1.11	-2.23
A0A8S3SPV4	Uncharacterized protein	1.20	-1.20	-2.40
A0A8S3TKR9	Uncharacterized protein	1.04	-1.04	-2.07
A0A8B6FV73	Uncharacterized protein	1.19	-1.19	-2.38
A0A8S3S0N2	Uncharacterized protein	-0.62	0.62	1.24
A0A8S3QG66	Uncharacterized protein	1.29	-1.29	-2.59
A0A8B6GYD1	Uncharacterized protein	0.80	-0.80	-1.60
A0A8S3U726	Uncharacterized protein	0.46	-0.46	-0.92
A0A8S3QIJ9	UspA domain-containing protein	0.64	-0.64	-1.27
A0A8B6G9T0	UspA domain-containing protein	0.77	-0.77	-1.54
A0A8B6ESB0	UTP--glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)	0.97	-0.97	-1.93
A0A8S3QEM3	Vacuolar proton pump subunit B (V-ATPase subunit B) (Vacuolar proton pump subunit B)	0.57	-0.57	-1.15
A0A6J8DNW3	VDAC2	1.11	-1.11	-2.22
A0A8B6E1P9	Vitelline membrane outer layer 1-like protein	2.29	-2.29	-4.58
A0A8B6DLG6	Vitellogenic carboxypeptidase-like protein (EC 3.4.16.-)	0.72	-0.72	-1.44
A0A8B6C9L4	VWFA domain-containing protein	0.58	-0.58	-1.17
A0A8B6HDB4	Actin-interacting protein 1	1.11	-1.11	-2.21
A0A8B6F1U6	Cilia- and flagella-associated protein 52	1.22	-1.22	-2.43
A0A8B6CY00	2-oxoglutarate dehydrogenase E1 component (EC 1.2.4.2)	0.66	-0.66	-1.31
A0A210Q521	26S protease regulatory subunit 6B	0.82	-0.82	-1.64
A0A8B6GS06	26S proteasome regulatory subunit N2	1.17	-1.17	-2.34
A0A8B6GHX0	26S proteasome regulatory subunit N3	0.62	-0.62	-1.23
A0A8S3VGU8	4-hydroxyphenylpyruvate dioxygenase	1.20	-1.20	-2.39
A0A8S3QVD1	Small ribosomal subunit protein uS17 (40S ribosomal protein S11)	1.09	-1.09	-2.17
A0A6J8EWE8	40S ribosomal protein S12	0.84	-0.84	-1.69
A0A2C9JRX0	Small ribosomal subunit protein uS19 (40S ribosomal protein S15)	1.11	-1.11	-2.22
A0A8B6G0N3	Small ribosomal subunit protein uS13 (40S ribosomal protein S18)	1.13	-1.13	-2.26
V4AS57	40S ribosomal protein S25	1.67	-1.67	-3.34
A0A8B6EK07	40S ribosomal protein S26	0.56	-0.56	-1.13
A0A8B6CVW2	40S ribosomal protein S27	1.29	-1.29	-2.57
A0A210PU23	40S ribosomal protein S5	1.13	-1.13	-2.27
A0A3G1CJL1	40S ribosomal protein S8	1.20	-1.20	-2.41
A0A8B6BQ82	5'-AMP-activated protein kinase subunit beta-1	1.64	-1.64	-3.28
A0A8S3RZZ6	60S acidic ribosomal protein P0	1.64	-1.64	-3.29
B6ZCB1	Large ribosomal subunit protein P2 (60S acidic ribosomal protein P2)	0.31	-0.31	-0.62
A0A8S3V934	60S ribosomal protein L18a	0.83	-0.83	-1.67
A0A077GYU2	Large ribosomal subunit protein uL14 (60S ribosomal protein L23)	1.22	-1.22	-2.44

A0A077H3N0	60S ribosomal protein L27	1.07	-1.07	-2.15
A0A077GZK7	Large ribosomal subunit protein eL28 (60S ribosomal protein L28)	1.28	-1.28	-2.55
A0A8B6G8D8	Large ribosomal subunit protein eL31 (60S ribosomal protein L31)	2.26	-2.26	-4.51
A0A8B6DEA0	Large ribosomal subunit protein eL34 (60S ribosomal protein L34)	2.18	-2.18	-4.36
A0A8S3R313	Large ribosomal subunit protein eL36 (60S ribosomal protein L36)	0.58	-0.58	-1.16
A0A8B6EI40	Large ribosomal subunit protein eL6 (60S ribosomal protein L6)	0.55	-0.55	-1.09
A0A6J8A5K2	Large ribosomal subunit protein uL2 (60S ribosomal protein L8)	1.59	-1.59	-3.18
A0A8B6FK68	Xylose isomerase (EC 5.3.1.5)	1.37	-1.37	-2.73
A0A8S3UF93	YWHAE	1.02	-1.02	-2.04

Supplementary Table S6. List of differentially expressed proteins (DEPs) in *Mytilus galloprovincialis* plantigrades exposed to 2'-deoxyinosine (2'). DEPs were obtained from pairwise comparisons between the control group (0.1% DMSO) and exposed group at 12.5 μ M, using a threshold of $\text{lfc} = \log_2(1.5)$. Represented are the relative expression of DEPs on the control group (Ctr-) and the exposed group (12.5 μ M), and the obtained fold change of each DEP.

Reference Sequence	Protein Name	Relative Expression		Fold Change
		Ctr-	12.5 μ M	
A0A8B6DSL8	Adenosylhomocysteinase (EC 3.13.2.1)	0.70	-0.70	-1.40
A0A8B6GV47	ADP/ATP translocase (ADP,ATP carrier protein)	-0.63	0.63	1.26
A0A8B6GU32	ATP synthase subunit gamma	0.98	-0.98	-1.96
A0A8S3Q2E4	ATPeF1D	-1.44	1.44	2.89
A0A6J8EBJ6	Band 7 domain-containing protein	0.33	-0.33	-0.66
A0A8B6GW86	C1q domain-containing protein	1.34	-1.34	-2.68
A0A8B6G4E0	Calcium-transporting ATPase (EC 7.2.2.10)	-0.61	0.61	1.23
A0A8B6FIN1	Chitin-binding type-2 domain-containing protein	-1.02	1.02	2.03
A0A8B6C153	Citrate synthase	-0.54	0.54	1.09
A0A8B6ENY2	Cellular nucleic acid-binding protein	-0.30	0.30	0.59
A0A8S3R8F7	E2.7.3.3 (EC 2.7.3.3)	0.59	-0.59	-1.18
D9IWS1	Elongation factor 1-alpha	0.71	-0.71	-1.42
A0A8B6EYD8	Elongation factor 2	-0.58	0.58	1.16
A0A8S3QB23	H3	-0.37	0.37	0.74
A0A8B6DBF4	Metalloendopeptidase (EC 3.4.24.-)	1.04	-1.04	-2.09
A0A8B6G1S8	Protein disulfide-isomerase (EC 5.3.4.1)	0.31	-0.31	-0.61
A0A8S3Q9N6	SHSP domain-containing protein	0.82	-0.82	-1.64
A0A8B6D810	Spectrin alpha	-0.41	0.41	0.81
Q966V3	Transgelin	-0.77	0.77	1.54
A0A076FIR9	Tubulin beta chain	0.52	-0.52	-1.03
A0A8B6CEW4	Uncharacterized protein	-0.80	0.80	1.60
A0A8B6DC97	MAM domain-containing protein	-0.88	0.88	1.75
A0A8B6BV40	VWFA domain-containing protein	-1.25	1.25	2.51
A0A8B6FD83	Collagen. type II. alpha	-0.49	0.49	0.98
A0A8B6G747	Collagen. type V/XI/XXIV/XXVII. alpha	-0.60	0.60	1.19
A0A8S3QII9	CTSL (EC 3.4.22.15)	0.51	-0.51	-1.02
A0A8B6HEV7	Cysteine and glycine-rich protein	-0.52	0.52	1.03
A0A8B6F031	Cytochrome b-c1 complex subunit 6	1.12	-1.12	-2.25

A0A8B6D685	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex. mitochondrial (EC 2.3.1.61) (2-oxoglutarate dehydrogenase complex component E2) (E2K)	0.96	-0.96	-1.92
A0A409V740	Dna-binding a protein	-1.35	1.35	2.69
A0A3L5TQZ0	Fc-e cd23 receptor	0.66	-0.66	-1.32
A0A6J8ECB1	Glucose-6-phosphate isomerase (EC 5.3.1.9)	0.41	-0.41	-0.82
A0A8B6DQP9	glutamate dehydrogenase [NAD(P)(+)] (EC 1.4.1.3)	0.96	-0.96	-1.93
A0A8B6H497	Granulins domain-containing protein	-0.38	0.38	0.76
A0A411G5T5	Hemicentin-like protein	1.43	-1.43	-2.86
K1Q324	Heterogeneous nuclear ribonucleoprotein K	0.30	-0.30	-0.60
A0A0B7AUT8	Histone H2A	1.08	-1.08	-2.16
A0A8B6DFJ0	Insulin-like growth factor 2 mRNA-binding protein 1	-0.59	0.59	1.19
A0A8B6BTE6	Large ribosomal subunit protein P1 (60S acidic ribosomal protein P1)	0.38	-0.38	-0.76
A0A8S3Q6R0	LMNB	-0.37	0.37	0.75
A0A8B6C2Y2	Malate dehydrogenase (EC 1.1.1.37)	-0.78	0.78	1.56
A0A8B6FW38	Molecular chaperone DnaK	0.30	-0.30	-0.60
A0A6J8APW2	NID	0.42	-0.42	-0.84
A0A8S3ULS9	Nucleoside diphosphate kinase (EC 2.7.4.6)	0.88	-0.88	-1.76
A0A8B6D9K2	P-type domain-containing protein	0.96	-0.96	-1.91
A0A6J8DKH3	Peptidyl-prolyl cis-trans isomerase (PPIase) (EC 5.2.1.8)	0.84	-0.84	-1.67
A0A8S3TQI9	Sarcoplasmic calcium-binding protein	-1.49	1.49	2.97
A0A6J8EGE4	TRAF4	-0.57	0.57	1.14
A0A8S3QSQ2	Ubiquitin-ribosomal protein eL40 fusion protein	-0.32	0.32	0.64
A0A3L5TR65	Uncharacterized protein	-0.92	0.92	1.84
A0A8B6E104	Uncharacterized protein	-0.92	0.92	1.84
A0A8B6GUE8	WAP domain-containing protein	1.22	-1.22	-2.44
A0A8B6CB58	Uncharacterized protein	0.84	-0.84	-1.68
A0A8B6DFD7	Uncharacterized protein	-0.51	0.51	1.02
A0A8B6BNI7	Uncharacterized protein	0.98	-0.98	-1.96
A0A8B6DPQ0	SCO-spondin	0.51	-0.51	-1.01
A0A8B6FV73	Uncharacterized protein	0.48	-0.48	-0.97
A0A8B6CCQ9	Uncharacterized protein	-0.37	0.37	0.74
A0A8B6H3D0	Vesicle-associated membrane protein-associated protein A	-0.97	0.97	1.94
A0A8B6GXU1	Vitelline membrane outer layer 1-like protein	1.63	-1.63	-3.25
A0A8B6EK07	40S ribosomal protein S26	0.30	-0.30	-0.61
A0A3G1CJL1	40S ribosomal protein S8	0.78	-0.78	-1.56
A0A8S3QVR3	Small ribosomal subunit protein uS2	-1.00	1.00	2.00
A0A6J8B1B1	6-phosphofructokinase (EC 2.7.1.11) (Phosphohexokinase)	0.78	-0.78	-1.56

A0A8S3RZZ6	60S acidic ribosomal protein P0	-0.90	0.90	1.81
A0A6J8DYJ1	Large ribosomal subunit protein uL11 (60S ribosomal protein L12)	0.38	-0.38	-0.76
A0A8S3V934	60S ribosomal protein L18a	0.66	-0.66	-1.32
A0A077H0N7	60S ribosomal protein L7a	-0.59	0.59	1.19

Supplementary Table S7. Results of BLASTp analysis for DEPs identified in response to hypoxanthine arabinoside (1'). The table lists homologous proteins from *Magallana gigas*, along with their corresponding accession numbers retrieved from the UniProt and Ensembl Metazoa databases.

Reference Sequence	UniProt			Ensembl	
	Accession number	Protein name	e-Value	Accession number	e-Value
A0A8B6C5G7	A0A8W8N8B2	3-ketoacyl-CoA thiolase, mitochondrial	0	G4782.3	0
A0A8B6F498	A0A8W8KG37	Acyl-coenzyme A oxidase	7.45E-75	G23748.2	5.82E-75
A0A8B6DSL8	K1RW85	Adenosylhomocysteinase	0	G22300.4	0
A0A8S3SJS5	A0A8W8NE65	Adenylyl cyclase-associated protein	1.88E-178	G5795.12	1.47E-178
A0A8S3S1H7	A0A8W8KME6	Adenylate kinase isoenzyme 5	0	G24232.1	0
A0A8S3TZ88	K1PRS8	Adenylate kinase 8	0	G25200.4	0
A0A8B6D0Q7	A0A8W8LYD4	ADP-ribosylation factor	6.42E-98	G29686.9	5.02E-98
A0A8B6C281	K1RGK4	Villin-1	0	G27734.1	1.32E-114
A0A8B6EIW3	K1RGF4	alanine transaminase	0	G30434.4	0
A0A8B6BGG4	A0A8W8LL21	Saccharopine dehydrogenase (NAD(+), L-glutamate-forming)	0	G28495.1	0
A0A8B6D196	A0A8W8IJS1	Aminopeptidase	0	G14427.1	0
A0A8S3QGC9	K1RTF1	Annexin	0	G26210.8	0
A0A8S3VH93	A0A8W8I0T9	Cysteine-rich motor neuron 1 protein	1.72E-10	G11972.1	1.34E-10
A0A8S3QFL2	K1QQ16	AP complex subunit beta	0	G35149.7	0
A0A8S3S184	A0A8W8LLL1	Apextrin C-terminal domain-containing protein	2.78E-84	G28650.5	2.17E-84
A0A8B6BZ74	A0A8W8N9W6	Apoptosis inhibitor 5	0	G4455.1	0
A0A8B6D0T5	A0A8W8N549	Apple domain-containing protein	0	G432.1	0
A0A8S3TG22	A0A8W8JWW8	Aquaporin-4	1.34E-143	G20854.9	1.05E-143
A0A8S3VBZ6	A0A8W8LUT3	Rho GDP-dissociation inhibitor 1	2.68E-76	G297.6	2.09E-76
A0A8B6H8N6	A0A8W8IFX6	Asparagine--tRNA ligase, cytoplasmic	0	G13717.6	0
A0A8S3SXM2	A0A8W8NW87	ATP-dependent 6-phosphofructokinase	0	G7024.1	0
A0A8B6GBE6	A0A8W8L2N3	ATP synthase-coupling factor 6, mitochondrial	1.28E-05	G26087.10	1.00E-05
A0A8B6H7T7	A0A8W8MB85	ATP synthase subunit beta	0	G31998.34	0
A0A077GYU5	A0A8W8MGJ9	ATP synthase subunit d, mitochondrial	3.25E-12	G3298.5	2.54E-12
A0A8B6GU32	A0A8W8LM86	ATP synthase subunit gamma, mitochondrial	4.16E-124	G28739.12	3.25E-124
A0A6J8F3Y7	K1R9E2	EF-hand calcium-binding domain-containing protein 6	0	G16976.1	0
A0A6J8C7Z5	A0A8W8NDX9	ATP synthase subunit f, mitochondrial	1.49E-13	G650.2	1.17E-13
A0A8S3Q2E4	K1PQJ9	ATP synthase subunit delta, mitochondrial	2.28E-45	G269.2	1.78E-45
A0A6J8DY85	K1PIH2	V-type proton ATPase subunit E	3.90E-114	G19302.3	3.05E-114
A0A8B6BPI1	K1REC7	Tripartite motif-containing protein 56	3.30E-25	G23372.1	4.95E-25

A0A8B6F754	K1PKN2	Papilin	1.75E-49	G11186.1	9.91E-49
A0A8B6DQ42	A0A8W8HZ39	C-type lectin domain-containing protein	4.20E-23	G10490.1	3.28E-23
A0A8B6GW86	A0A8W8J8Q2	C1q domain-containing protein	1.57E-20	G17851.1	1.23E-20
A0A6J8E3N6	A0A8W8M3J3	C1q domain-containing protein	5.55E-06	G31159.1	4.34E-06
A0A8B6HA48	A0A8W8K425	C2 domain-containing protein	0	G22340.8	0
A0A8S3SGA7	A0A8W8LX26	Cadherin domain-containing protein	0	G2999.15	0
A0A8B6FBT9	K1Q3D9	Calmodulin	1.89E-23	G32596.6	3.67E-23
A0A8S3R7D4	A0A8W8KPL5	Calreticulin	0	G2451.27	0
A0A8B6DWN7	A0A8W8MDX4	Cyclic nucleotide-binding domain-containing protein	0	G3254.14	0
A0A8B6D440	A0A8W8NL52	Peptidase A1 domain-containing protein	0	G5738.16	0
A0A8B6GVE1	A0A8W8ME56	Ig-like domain-containing protein	2.35E-19	G32348.2	1.84E-19
A0A8S3URK5	A0A8W8N0M4	Chromo domain-containing protein	8.72E-21	G35434.7	6.81E-21
A0A8B6FIN1	K1QY92	Chitin-binding type-2 domain-containing protein	5.43E-49	G22619.1	4.24E-49
A0A8B6DBL3	A0A8W8KZ55	Chitin-binding type-2 domain-containing protein	1.18E-48	G25920.6	9.25E-49
A0A8S3T4F4	A0A8W8L7P8	Choline transporter-like protein	0	G2630.12	0
A0A8B6F3H7	K1RQ81	Uncharacterized protein C15orf26-like protein	8.94E-155	G11651.1	6.98E-155
A0A8B6GX94	A0A8W8KCV2	Clathrin heavy chain	0	G23360.1	0
A0A6J8ESY4	A0A8W8N791	Small ribosomal subunit protein uS15	6.61E-106	G4635.1	5.16E-106
A0A6J8EHM6	A0A8W8HYQ8	Small ribosomal subunit protein uS5	1.77E-160	G1172.7	1.39E-160
A0A8B6G7J5	A0A8W8J403	Large ribosomal subunit protein uL13	4.84E-119	G16773.14	3.78E-119
A0A077H0N7	A0A8W8L109	60S ribosomal protein L7a	1.38E-155	G25420.3	1.08E-155
Q9Y0D6	Q8TA69	Actin 2	0	G27522.32	0
A0A8S3Q5J1	K1RH58	Alpha-actinin, sarcomeric	0	G13016.1	2.24E-112
A0A8B6GV47	K1R0Y9	ADP/ATP translocase	0	G730.11	0
A0A8B6D2T4	A0A8W8MRV1	ADP-ribosylation factor	5.31E-124	G34817.29	4.15E-124
A0A8B6GKE0	K1QNT7	Aldedh domain-containing protein	0	G12763.28	0
A0A8S3T4E0	K1QNT7	Aldedh domain-containing protein	0	G12763.28	0
A0A8B6CW94	A0A8W8I5S2	Alpha-1,4 glucan phosphorylase	0	G12639.2	0
A0A8B6DXX6	A0A8W8LM76	Apextrin C-terminal domain-containing protein	1.68E-95	G28654.4	1.31E-95
A0A8B6HT02	A0A8W8L239	Armadillo repeat-containing protein 4	0	G26323.3	0
A0A8B6CRC1	K1R6Z7	ATP synthase subunit alpha	0	G11926.23	0
A0A6J8EBJ6	A0A8W8L8X3	Flotillin-1	0	G26928.23	0
A0A8B6GBK5	A0A8W8I7B5	C-1-tetrahydrofolate synthase, cytoplasmic	0	G12935.2	0
A0A8B6FVI3	A0A8W8JHD6	C1q domain-containing protein	6.20E-27	G18610.6	4.84E-27
A0A8B6G4E0	A0A8W8KCX1	Calcium-transporting ATPase	0	G23365.9	0
A0A8B6E763	K1PMY9	Calmodulin	0	G11730.2	2.12E-126
A0A8S3QR75	A0A8W8HQW9	Calpain catalytic domain-containing protein	0	G10641.20	0

A0A8B6DHE3	A0A8W8NDX0	Peptidase C1A papain C-terminal domain-containing protein	1.20E-176	G5397.18	9.41E-177
A0A6J8A856	K1PGZ1	Testis-specific gene 10 protein	0	G35361.3	0
A0A8B6EZW6	A0A8W8KMV0	Cilia- and flagella-associated protein 45	0	G24574.5	0
A0A8B6C153	A0A8W8MWR9	Citrate synthase	0	G35409.40	0
A0A8B6CP95	K1R1A5	Collagen alpha-1(IV) chain	0	G15188.2	9.46E-120
A0A8B6FHM0	K1RH30	Collagen alpha-1(IV) chain	1.65E-39	G9790.1	1.61E-37
A0A8S3UY90	A0A8W8K042	Rootletin	0	G21075.1	0
A0A8B6C6M2	K1R702	Body wall muscle protein HR-29	1.07E-48	G26545.1	9.92E-49
A0A6J8D0X9	A0A8W8JHD7	Fibropellin-1	0	G1940.1	0
A0A8S3QII9	K1Q7M2	Cathepsin L	0	G9706.23	0
A0A8B6D685	A0A8W8I7C9	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	1.36E-159	G12941.5	1.06E-159
A0A8S3RHZ5	A0A8W8IR75	Disks large-like protein 1	0	G15080.9	0
A0A8B6DX16	A0A8W8N7H8	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	0	G4985.2	0
A0A8B6FGA8	K1PAG1	Dynein beta chain, ciliary	0	G761.1	0
A0A8S3RF13	A0A8W8LWE6	Transketolase	0	G30122.32	0
A0A8S3R8F7	A0A8W8JPQ6	Arginine kinase	1.14E-178	G19705.1	8.89E-179
A0A6J8A1B7	A0A8W8N948	Elongation factor 1-alpha	0	G4818.20	0
A0A8B6EQ50	K1PY28	Sarcoplasmic calcium-binding protein	1.97E-39	G32504.6	8.87E-39
A0A8B6CPN1	K1R0G7	Fibropellin-1 (Fragment)	7.73E-83	G5363.1	4.06E-80
A0A8B6FH66	A0A8W8I0T1	Elongation factor 1-gamma	0	G11860.32	0
A0A8B6EYD8	A0A8W8HXZ3	Elongation factor 2	0	G11483.21	0
A0A8B6FQU1	A0A8W8N1K3	FERM domain-containing protein	1.09E-163	G3572.64	8.55E-164
A0A8B6FTA2	K1RKC1	Far upstream element-binding protein 3	2.43E-169	G12312.1	2.51E-12
A0A8B6GX54	A0A8W8M883	Fibulin-1	0	G31035.19	0
A0A8B6H907	A0A8W8J8A2	Filamin-A	0	G1715.4	0
A0A8B6DL75	A0A8W8IQJ7	Calponin-homology (CH) domain-containing protein	0	G15398.3	0
A0A8B6E885	A0A8W8LLG1	Gelsolin-like domain-containing protein	2.41E-160	G28864.12	1.88E-160
A0A8B6BF20	A0A8W8NBH2	glutathione transferase	4.33E-86	G5106.4	3.38E-86
A0A8B6DZ81	K1Q350	Glyceraldehyde-3-phosphate dehydrogenase	0	G4037.3	0
A0A8B6DYX8	K1QIR8	78 kDa glucose-regulated protein	0	G29360.3	0
C0Z203	A0A8W8HTN3	Histidine kinase/HSP90-like ATPase domain-containing protein	0	G10994.4	0
A0A8B6BR27	A5LGG7	Glucose-regulated protein 94	0	G30100.1	0
A0A8B6GHQ6	A0A8W8KZ99	Ig-like domain-containing protein	0	G25939.1	0
A0A8B6H9V2	A0A8W8KZ99	Ig-like domain-containing protein	0	G25939.1	0
A0A0K0YAY3	A0A8W8K0M3	Basement membrane-specific heparan sulfate proteoglycan core protein	0	G21617.9	0
A0A8B6EPK5	A0A8W8J5R5	RRM domain-containing protein	0	G16953.11	0

A0A0L8GYE0	A0A8W8K8G0	Histone H4	6.13E-49	G22554.1	4.79E-49
A0A8S3VDI8	A0A8W8HNN7	Peroxisomal multifunctional enzyme type 2	0	G10373.3	0
A0A8B6CAW3	A0A8W8MGF9	K Homology domain-containing protein	0	G32958.1	0
A0A8B6BT14	A0A8W8I0U1	Large ribosomal subunit protein uL4 C-terminal domain-containing protein	0	G11965.6	0
A0A8S3QSD1	K1PQ23	PDZ and LIM domain protein 5	1.24E-172	G2996.3	8.11E-110
A0A8B6EFR5	A0A8W8IRY8	Cytosol aminopeptidase domain-containing protein	0	G15308.19	0
A0A8B6GXC9	K1QNP7	LIM and SH3 domain protein Lasp	1.01E-109	G3888.9	5.23E-52
A0A8S3VFX6	K1QT91	LIM domain and actin-binding protein 1	0	G26782.9	4.98E-13
A0A8S3PRL2	A0A8W8IU92	Lethal(2) giant larvae protein-like protein 1	0	G1588.7	0
A0A8S3Q6R0	K1RFA3	Lamin Dm0	0	G11465.2	0
A0A8S3TFK4	K1PU26	Malate dehydrogenase (Fragment)	0	G13589.13	1.84E-62
A0A8B6C2Y2	A0A8W8K8K1	Malate dehydrogenase, mitochondrial	2.45E-162	G22856.16	1.91E-162
A0A8B6DBF4	A0A8W8NN09	Metalloendopeptidase	8.22E-113	G6474.29	6.42E-113
A0A8B6FW38	A0A8W8J1A5	Stress-70 protein, mitochondrial	0	G16610.7	0
A0A8B6F2T3	A0A8W8HZQ8	Calponin-homology (CH) domain-containing protein	0	G11852.2	0
A0A8B6EEA4	A0A8W8L2H7	Neural cell adhesion molecule 1	1.40E-144	G26071.5	1.09E-144
A0A8B6HFZ0	A0A8W8LQI9	non-specific serine/threonine protein kinase	0	G29219.1	0
A0A8B6ETB5	K1PQP2	Serine/arginine-rich splicing factor 2	7.07E-41	G23736.5	2.45E-14
A0A8B6DGA0	K1PSE7	Outer dense fiber protein 3	2.64E-159	G18379.13	2.06E-159
A0A8B6D9K2	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8S3RZQ4	A0A8W8J283	Parkin coregulated gene protein	2.25E-154	G16920.1	1.76E-154
A0A8B6DTM2	A0A8W8IGZ9	PDZ domain-containing protein	3.91E-59	G1399.61	3.05E-59
A0A6J8B435	A0A8W8HZM2	Fibrillin-2	0	G11837.1	0
A0A8B6F8H6	A0A8W8LA78	Thioredoxin domain-containing protein	2.77E-115	G27091.1	2.17E-115
A0A8B6FIW7	K1QYT5	Phosphate carrier protein, mitochondrial	0	G30148.5	0
A0A8B6CG53	K1QEA6	phosphoenolpyruvate carboxykinase (GTP)	0	G23781.36	0
A0A8B6HP01	A0A8W8KGB3	phosphoenolpyruvate mutase	0	G23366.8	0
A0A8B6C763	A0A8W8NS22	Plastin-2	0	G6498.41	0
A0A8B6CYA7	K1Q639	Receptor-type tyrosine-protein phosphatase T	0	G12173.1	0
A0A8S3QR29	A0A8W8JX77	thioredoxin-dependent peroxiredoxin	1.32E-124	G2146.6	1.03E-124
A0A8B6BJW5	A0A8W8K0N9	Prohibitin	1.81E-141	G21620.10	1.42E-141
A0A8B6DIX4	A0A8W8LQN5	Glutathione S-transferase 1	1.40E-55	G29525.1	1.09E-55
A0A8B6EJ02	K1Q6X5	Protein disulfide-isomerase	0	G1339.29	0
A0A8B6G1S8	A0A8W8I1E8	Protein disulfide-isomerase	0	G11932.5	0
A0A6J8BIG8	K1RV41	Small ribosomal subunit protein RACK1	0	G3645.44	0
A0A8B6D4L6	K1PUJ1	Moesin/ezrin/radixin homolog 1	0	G17750.31	0
A0A3G1CJN6	A0A8W8MQ51	Small ribosomal subunit protein eS1	6.02E-169	G34618.1	4.71E-169

A0A8B6C4T1	A0A8W8MU34	RNA helicase	0	G35350.2	0
A0A8B6DTX0	A0A8W8K042	Rootletin	0	G21075.1	0
A0A8B6BF70	K1Q8F7	Alpha-crystallin B chain	1.40E-56	G34560.2	1.67E-55
A0A8B6DCE2	A0A8W8LEA1	40S ribosomal protein S3	3.11E-161	G27592.12	2.43E-161
A0A8S3QQF6	K1R0L4	Sodium/potassium-transporting ATPase subunit alpha	0	G25027.37	0
A0A126Q9D5	A0A8W8JHY1	Staphylococcal nuclease domain-containing protein 1	0	G18611.7	0
A0A6J8B610	A0A8W8I6N1	Stress-induced-phosphoprotein 1	0	G12646.14	0
A0A6J8DUP3	A0A8W8KYL1	Band 7 domain-containing protein	6.16E-142	G25137.36	4.81E-142
A0A8S3TEG9	A0A8W8N2K5	Succinate dehydrogenase	0	G5034.22	0
A0A6J8ESE0	A0A8W8N942	Calponin-homology (CH) domain-containing protein	1.11E-84	G4815.29	8.66E-85
A0A8B6GHP0	A0A8W8IGH4	Tektin	0	G1416.3	0
A0A8B6DFA7	A0A8W8KM53	Thymosin beta-4	1.19E-62	G24500.65	9.33E-63
Q966V3	A0A8W8LCL2	Transgelin	0	G27652.16	0
A0A8B6H439	A0A8W8ILL9	Transglutaminase-like domain-containing protein	0	G14594.1	0
A0A6J8BFQ0	A0A8W8MB90	Trichohyalin-plectin-homology domain-containing protein	0	G31463.4	0
A0A8B6BTY3	A0A8W8JZQ8	Triosephosphate isomerase	4.27E-150	G21794.2	3.34E-150
A0A8B6F8B1	A0A8W8HL87	Tropomyosin	3.39E-125	G10110.138	2.65E-125
A0A3S1A1H2	A0A8W8MF40	Tubulin alpha chain	0	G32475.58	0
A0A8B6BWE2	K1R278	Tubulin beta chain	0	G24506.8	0
A0A6J8CNX6	A0A8W8MKL6	Tubulin polymerization-promoting protein family member 3	4.05E-39	G3391.16	3.17E-39
A0A8B6BL03	K1QLS3	Cytochrome b-c1 complex subunit 2, mitochondrial	2.36E-45	G331.9	2.51E-42
A0A8B6HIV7	K1PBC0	Non-neuronal cytoplasmic intermediate filament protein	0	G24263.8	2.20E-168
A0A8B6EIG0	K1QUX9	Prolow-density lipoprotein receptor-related protein 1	5.24E-26	G27135.2	2.22E-25
A0A8B6DFD7	A0A8W8HVV8	Papilin	3.74E-21	G11186.1	2.92E-21
A0A8B6G096	A0A8W8KRU1	Uncharacterized protein	7.14E-16	G2504.5	5.58E-16
A0A8B6E104	K1PSR5	Fatty acid-binding protein, heart	3.26E-34	G21524.4	2.90E-34
A0A8B6EM75	K1QPB7	Uncharacterized protein	2.03E-50	G23239.2	4.28E-21
A0A8B6C479	A0A8W8LS81	Ependymin-related protein 1	5.49E-24	G294.3	4.29E-24
A0A6J8CV26	A0A8W8NFT4	Extended synaptotagmin-2	0	G6772.4	0
A0A8B6E647	A0A8W8J1Z0	Kielin/chordin-like protein	0	G16891.13	0
A0A8B6GX65	K1QTV9	Collagen alpha-4(VI) chain	0	G21248.1	0
A0A6J8AGA3	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8S3SZC2	A0A8W8IAW2	Mesenchyme-specific cell surface glycoprotein	8.33E-160	G13376.4	6.51E-160
A0A8B6DPQ0	K1R459	SCO-spondin	0	G35504.1	1.30E-133
A0A8B6DC97	A0A8W8NN09	Metalloendopeptidase	6.29E-128	G6474.29	4.91E-128
A0A6J8BUN8	A0A8W8LRT0	Tenascin-X	2.74E-06	G29424.4	2.14E-06
A0A8B6GIV6	K1QJ28	Mammalian ependymin-related protein 1	1.09E-89	G12752.5	7.87E-63

A0A8B6G9E5	K1QT65	Collagen alpha-3(VI) chain	1.36E-49	G21249.19	3.83E-49
A0A8B6CB58	A0A8W8JRD3	Apextrin C-terminal domain-containing protein	2.36E-08	G20570.1	1.84E-08
A0A8B6BI55	A0A8W8MX28	Vinculin	0	G35429.17	0
A0A8B6BU43	K1PKC1	Collagen alpha-6(VI) chain	2.30E-88	G4540.1	2.12E-79
A0A8S3TBN4	A0A8W8JCM3	CSD domain-containing protein	3.06E-15	G18521.3	2.39E-15
A0A8S3PR18	K1P9N7	14-3-3 protein zeta	7.96E-117	G23866.50	3.06E-101
A0A8B6EBM4	K1QET2	Coatomer subunit alpha	0	G3968.1	0
A0A8S3VPZ7	A0A8W8KS23	Coatomer subunit delta	0	G25063.1	0
A0A8B6CVU1	D7EZG8	Actin-depolymerizing factor 6	3.47E-24	G23042.5	2.71E-24
A0A8B6BSQ3	A0A8W8I947	Coiled-coil domain-containing protein 170	0	G13159.9	0
A0A8B6FD83	K1PDS7	Collagen alpha-2(I) chain	0	G5252.2	5.45E-67
A0A8B6EZ83	K1RSS9	Collagen alpha-5(VI) chain	7.16E-50	G3584.8	5.59E-50
A0A8B6CQJ1	K1QYS9	Reelin domain-containing protein	1.23E-43	G21989.2	9.60E-44
A0A8B6CAW5	K1QYS9	Reelin domain-containing protein	1.75E-24	G21989.2	1.37E-24
A0A8S3Q3L3	K1RAD3	Cysteine-rich secretory protein LCCL domain-containing 2	2.05E-92	G1476.1	8.71E-80
A0A6J8BMS7	A0A8W8J450	SH3 domain-containing protein	0	G17159.4	0
A0A8S3RLA1	A0A8W8M1D6	CUB domain-containing protein	7.88E-28	G31121.1	4.47E-26
A0A8S3RUL4	A0A8W8LT11	cystathionine gamma-lyase	0	G2948.1	0
A0A8B6HEV7	A0A8W8NFU0	LIM zinc-binding domain-containing protein	3.22E-96	G5979.9	2.51E-96
A0A6J8AEC3	A0A8W8LZS3	EGF-like domain-containing protein	1.39E-108	G30352.4	1.09E-108
A0A8B6F031	K1QKV1	Cytochrome b-c1 complex subunit 6, mitochondrial	4.13E-12	G2226.1	3.23E-12
A0A8B6G281	K1PZT2	Cytochrome c oxidase subunit 5B, mitochondrial	1.34E-14	G2133.2	1.05E-14
A0A8B6EWU5	K1PSR5	Fatty acid-binding protein, heart	2.37E-60	G21524.4	1.85E-60
A0A8B6H151	K1R406	Death-associated protein 1	8.13E-30	G25521.2	6.35E-30
A0A077H0R0	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8B6GTY8	K1QYD8	Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase	1.65E-103	G15600.7	1.77E-103
A0A8B6DQH5	A0A8W8LVQ0	Dihydrolipoyl dehydrogenase	0	G29337.12	0
A0A6J8BTF0	A0A8W8NK98	33 kDa inner dynein arm light chain, axonemal	4.58E-170	G6194.23	3.58E-170
A0A8B6BL12	A0A8W8K2R5	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1	3.21E-63	G22173.1	2.50E-63
A0A8B6H0X9	K1R3G2	Drebrin-like protein	5.52E-151	G16042.3	4.16E-69
A0A8B6FMV8	---NA---	---NA---	---NA---	---NA---	---NA---
A0A6J8DUW4	K1PHB6	DUF4476 domain-containing protein	2.03E-123	G22182.3	1.59E-123
A0A6J8BZ13	K1QRD0	Dynein light intermediate chain	0	G21392.2	0
A0A8B6G9Z7	A0A8W8LSU0	E1 ubiquitin-activating enzyme	0	G29533.5	0
A0A8B6EYR0	A0A8W8ISY1	EF-hand domain-containing family member C2	0	G15272.5	0
A0A8B6CHC6	A0A8W8MAQ3	EF-hand domain-containing protein	4.15E-22	G32594.10	3.24E-22
A0A3G1CJM7	Q70MN9	Myosin essential light chain (Fragment)	2.92E-90	G14845.4	1.19E-89

A0A3R5Q145	A0A8W8LLN7	EF-hand domain-containing protein	3.35E-78	G2857.8	2.61E-78
A0A8B6GTM1	A0A8W8NHG0	EF-hand domain-containing protein	2.79E-163	G6194.19	2.18E-163
A0A8B6BHU6	A0A8W8L145	EF-hand domain-containing protein	7.35E-84	G26141.4	5.74E-84
A0A8B6EJE1	K1QAD2	EF-hand domain-containing protein	1.54E-68	G6522.1	1.20E-68
A0A8B6DVI4	A0A8W8K6F9	Fibropellin-1	1.46E-99	G22152.4	1.14E-99
A0A8S3RV51	A0A8W8JQE5	EGF-like domain-containing protein	1.64E-168	G2048.1	1.28E-168
A0A6J8CPT2	A0A8W8HS69	Electron transfer flavoprotein subunit alpha	7.16E-180	G10765.1	5.59E-180
A0A8S3V871	K1PWP8	Echinoderm microtubule-associated protein-like 1	0	G22103.1	4.60E-60
A0A8B6C964	K1RU07	Glucose-repressible alcohol dehydrogenase transcriptional effector	1.71E-149	G18106.3	3.01E-54
A0A8B6H228	A0A8W8KVI2	Thioredoxin domain-containing protein	0	G25203.7	0
A0A8S3VIN2	A0A8W8KCF7	Enolase	1.10E-70	G22912.4	8.59E-71
A0A8B6CMG1	K1QSX8	enoyl-CoA hydratase	0	G245.6	0
A0A8B6GL66	A0A8W8NDW1	SH3 domain-containing protein	0	G6496.3	0
A0A6J8D3C7	A0A8W8J6N8	Band 7 domain-containing protein	0	G1706.5	0
A0A6J8EG86	A0A8W8HZ31	Eukaryotic translation initiation factor 3 subunit B	0	G1175.2	0
A0A8S3RJH3	A0A8W8MYR0	Eukaryotic translation initiation factor 3 subunit I	0	G3659.10	0
A0A8S3PS95	A0A8W8KDB3	Eukaryotic translation initiation factor 5A	1.18E-77	G23412.4	9.25E-78
A0A8S3VLQ5	K1PLV6	F-actin-capping protein subunit alpha	1.48E-172	G29303.22	1.16E-172
A0A8B6DPU0	A0A8W8N171	FABP domain-containing protein	1.58E-20	G35511.6	1.24E-20
A0A8B6DQX4	K1QM68	Cytochrome P450 3A29	4.19E-09	G35510.1	3.28E-09
A0A8B6BMA1	K1QM68	Cytochrome P450 3A29	2.73E-15	G35510.1	2.13E-15
A0A8B6E270	K1PXW8	Transforming growth factor-beta-induced protein ig-h3	5.92E-125	G26746.4	4.62E-125
A0A8B6F6T7	K1R2K2	Periostin	2.17E-104	G29366.1	1.69E-104
A0A6J8D3U3	K1QEZ3	Fascin	0	G718.5	0
A0A8B6G0F8	A0A8W8LV63	Fibronectin type-III domain-containing protein	0	G29757.2	0
A0A8B6EE15	A0A8W8LWV5	10-formyltetrahydrofolate dehydrogenase	0	G29489.1	0
A0A8S3UCU2	A0A8W8MM64	Alcohol dehydrogenase class-3	3.07E-103	G3500.19	2.39E-103
A0A8S3TR72	K1RTQ6	Fructose-bisphosphate aldolase	0	G34645.19	0
A0A8B6BVI8	A0A8W8MQD3	Fructose-bisphosphate aldolase	0	G34649.12	0
A0A8S3Q6H6	A0A8W8P139	Fumarylacetoacetase	0	G8930.1	0
A0A0C5Q4G0	A0A8W8I5I4	Galectin	1.06E-130	G12471.17	8.26E-131
A0A8B6CKU6	A0A8W8KLR8	Gasdermin pore forming domain-containing protein	7.54E-68	G24166.4	5.89E-68
A0A8B6D0L8	A0A8W8M5G4	Gelsolin-like domain-containing protein	0	G31264.12	0
A0A8S3QNI2	K1PTI6	Glucose-6-phosphate isomerase	0	G20982.5	0
A0A8B6BHJ8	A0A8W8L0L3	Glucosidase 2 subunit beta	0	G2609.11	0
A0A8B6DQP9	K1QRQ2	glutamate dehydrogenase	0	G3271.17	0
A0A8S3Q5G9	A0A8W8K6J4	Glutathione S-transferase omega	1.09E-81	G21877.3	8.48E-82

A0A6J8BZR7	K1P979	Glyoxalase domain-containing protein 4	1.54E-170	G21694.2	1.20E-170
A0A8S3VUG6	A0A8W8KIR2	GMP reductase	0	G23328.11	0
A0A6J8ENV8	K1PWZ3	Guanine nucleotide-binding protein subunit beta	0	G22114.37	0
A0A8S3R8I4	A0A8W8KX36	PDZ GRASP-type domain-containing protein	6.98E-137	G24970.8	5.46E-137
A0A6J8F5H8	K1RAL0	aspartate transaminase	0	G16087.4	0
A0A2H4WCW9	K1QAV0	Guanine nucleotide-binding protein subunit alpha	0	G9338.1	0
A0A6J8CA81	K1QVX4	Glycogen synthase kinase-3 beta	0	G25031.10	3.29E-87
A0A0L8I0K2	A0A8W8IC92	GTP-binding protein SAR1b	1.45E-126	G1334.14	1.13E-126
A0A8B6HQH7	A0A8W8KS31	Heat shock 70 kDa protein 4L	0	G24810.12	0
A0A411G5T5	A0A8W8KZI1	Ig-like domain-containing protein	2.17E-10	G25938.1	1.70E-10
A0A8B6D225	A0A8W8NBT7	RRM domain-containing protein	3.24E-89	G5522.42	2.53E-89
A0A8B6ETA9	A0A8W8MJ95	H15 domain-containing protein	8.63E-43	G34589.4	6.74E-43
Q6WV85	K1R2L4	Histone H2B	2.04E-68	G22555.2	1.01E-64
A0A8S3RBA2	A0A8W8NEU6	RRM domain-containing protein	1.01E-71	G5522.28	7.86E-72
A0A6J8ESL8	A0A8W8HSK9	RRM domain-containing protein	4.20E-82	G1087.10	3.28E-82
A0A8S3U136	A0A8W8N6S3	hydroxyacid-oxoacid transhydrogenase	0	G5569.6	0
A0A8B6GQQ8	A0A8W8HSI3	Ig-like domain-containing protein	2.49E-13	G10784.34	1.95E-13
A0A8B6DGI1	A0A8W8M908	RRM domain-containing protein	0	G31955.1	0
A0A8B6CIE6	A0A8W8HL81	Isocitrate dehydrogenase	0	G10081.7	0
A0A8B6ET27	K1QAG0	Serine-threonine kinase receptor-associated protein	6.66E-60	G17845.1	8.50E-57
A0A6J8A1Y0	K1QJV9	BTB/POZ domain-containing protein KCTD16	1.25E-153	G26898.18	9.73E-154
A0A8B6FVU2	K1QUC7	Kinesin-related protein 1	0	G4885.1	0
A0A8B6G9Q8	A0A8W8JJV6	Laminin subunit alpha	0	G189.5	0
A0A8B6CXT1	A0A8W8MMB2	Laminin subunit beta-1	0	G35028.4	0
A0A8B6CGB4	A0A8W8MU73	Large ribosomal subunit protein uL1	1.15E-107	G34615.1	9.01E-108
A0A8B6F9E7	A0A8W8JVI9	Large ribosomal subunit protein eL22	4.85E-62	G20685.3	3.79E-62
A0A8B6BGD5	A0A8W8IQU1	Ribosomal protein L37	4.46E-37	G1543.3	3.48E-37
A0A8B6GGC2	A0A8W8KZ78	Large ribosomal subunit protein uL18 C-terminal eukaryotes domain-containing protein	8.25E-166	G25620.1	6.44E-166
A0A8B6E6I0	A0A8W8NCD2	60S ribosomal protein L7	9.51E-144	G5571.10	7.43E-144
A0A8B6BTE6	A0A8W8N7H9	Large ribosomal subunit protein P1	1.62E-32	G5649.7	1.26E-32
A0A6J8B8C6	A0A8W8LLZ8	Lipoma HMGIC fusion partner-like 3 protein	1.87E-134	G28860.1	1.46E-134
A0A8B6GHC2	K1P2H1	Fatty acid-binding protein, adipocyte	8.48E-57	G21693.2	6.63E-57
A0A0K0YB29	A0A8W8IPH2	Kyphoscoliosis peptidase	6.56E-94	G15010.18	5.12E-94
A0A8S3U3J8	K1R6E6	Basement membrane-specific heparan sulfate proteoglycan core protein	9.29E-29	G21617.9	7.39E-29
A0A8S3UA31	A0A8W8HVV8	Papilin	1.42E-13	G11186.1	1.11E-13
A0A8B6GPG7	A0A8W8J9G8	Membrane glycoprotein lig-1	5.47E-116	G17951.7	4.27E-116
A0A8B6G660	K1PG07	Lupus La-like protein	2.01E-127	G25932.1	1.57E-127

A0A8B6DCW7	A0A8W8KU66	MAGUK p55 subfamily member 6	0	G24992.5	0
A0A6J8CXM9	A0A8W8KCS5	Malectin domain-containing protein	1.06E-124	G23356.6	8.24E-125
A0A8B6D8H6	A0A8W8I653	Mammalian ependymin-related protein 1	1.13E-74	G12752.5	8.79E-75
A0A6J8EFL5	A0A8W8I304	Mammalian ependymin-related protein 1	7.23E-74	G12308.3	5.65E-74
A0A8S3RY54	A0A8W8J1M9	Microtubule-associated protein RP/EB family member 1	2.57E-114	G1666.6	2.01E-114
A0A8B6BF41	K1PR60	CKLF-like MARVEL transmembrane domain-containing protein 4	1.64E-37	G21838.6	6.59E-36
A0A8B6CZ83	K1PQP7	Mesenchyme-specific cell surface glycoprotein	3.48E-128	G13376.4	6.68E-128
Q697L7	Q9BIV4	Metallothionein	1.43E-16	G30623.10	4.47E-15
A0A8S3QX71	K1R252	methylmalonate-semialdehyde dehydrogenase (CoA acylating)	0	G13035.21	0
A0A6J8F1I0	A0A8W8MVH8	Mitochondrial carrier-like protein 2	1.19E-86	G35527.2	9.27E-87
A0A8B6H0Z6	K1Q1S3	Myosin-VI	0	G19685.4	0
A0A8B6EU84	A0A8W8MQE5	PDZ domain-containing protein	1.25E-109	G35457.24	9.80E-110
A0A8S3U4W4	A0A8W8MK96	NADP-dependent oxidoreductase domain-containing protein	3.54E-175	G32718.3	2.76E-175
A0A6J8BDN2	A0A8W8N229	Aldo-keto reductase family 1 member B1	2.84E-170	G4317.3	2.22E-170
A0A6J8CRB1	A0A8W8IK45	H/ACA ribonucleoprotein complex subunit 2	1.63E-50	G14693.2	1.28E-50
A0A8S3QGN5	K1QZ84	Thioredoxin domain-containing protein 3-like protein	0	G26290.2	0
A0A8S3PMK0	A0A8W8JP01	Nuclear migration protein nudC	6.71E-125	G19615.4	5.24E-125
A0A8B6C4N8	A0A8W8KLV1	Nucleoredoxin	6.96E-57	G24033.3	5.44E-57
A0A8S3ULS9	K1RHA5	Nucleoside diphosphate kinase	3.22E-88	G27377.8	1.12E-72
A0A8S3UGN8	A0A8W8KM88	Obg-like ATPase 1	0	G24214.1	0
A0A8B6EUV0	A0A8W8KI25	FHA domain-containing protein	2.56E-137	G23561.1	2.00E-137
A0A8B6BMW1	A0A8W8KKU1	Peptidase S1 domain-containing protein	1.61E-65	G24325.2	1.26E-65
A0A6J8DW13	A0A8W8NDY3	Thioredoxin domain-containing protein	0	G6502.1	0
A0A8S3TBS5	A0A8W8K954	PDZ domain-containing protein	7.71E-62	G22516.2	6.03E-62
A0A8B6DFV3	K1RHP3	Peptidase	0	G6069.10	0
A0A8B6D8S4	A0A8W8KK61	Peptidase metalloproteinase domain-containing protein	5.70E-159	G23496.3	4.45E-159
A0A8B6BIH5	A0A8W8N8J6	Peptidyl-prolyl cis-trans isomerase	3.77E-90	G5794.10	2.94E-90
A0A161I1V8	A0A8W8HZN8	Peptidyl-prolyl cis-trans isomerase	4.97E-107	G11826.2	3.88E-107
A0A6J8DKH3	A0A8W8IB78	Peptidyl-prolyl cis-trans isomerase	9.28E-140	G13223.1	7.25E-140
A0A8S3SEX5	A0A8W8HZN8	Peptidyl-prolyl cis-trans isomerase	8.07E-121	G11826.2	6.30E-121
A0A8B6EBJ6	K1QXK0	peptidylprolyl isomerase	4.43E-44	G2434.3	3.90E-44
A0A8S3RID5	A0A8W8N2J5	peptidylprolyl isomerase	6.84E-171	G4369.3	5.34E-171
A0A8B6D5H2	A0A8W8J0I5	Peroxidase	1.24E-119	G16328.2	9.67E-120
A0A8B6GKK6	K1R7F8	Peroxioredoxin-5	1.93E-86	G11001.1	9.73E-51
A0A8S3R7F6	A0A8W8IHJ9	phosphoglycerate mutase (2,3-diphosphoglycerate-dependent)	4.17E-160	G14096.8	3.26E-160
A0A8S3S8I1	K1PQD4	phosphoglucomutase (alpha-D-glucose-1,6-bisphosphate-dependent)	0	G1842.9	0
A0A6J7ZU18	A0A8W8L3H8	Phosphoglycerate kinase	0	G26194.4	0

A0A6J8AQ45	A0A8W8J208	Phospholipid scramblase	5.25E-142	G16506.13	4.10E-142
A0A6J8EPJ2	A0A8W8LD13	Phospholipid scramblase	3.96E-123	G27559.1	3.09E-123
A0A8B6DQL6	A0A8W8KBH8	Enolase	0	G22912.3	0
A0A8S3SL70	K1Q9P5	Mitochondrial-processing peptidase subunit beta	1.12E-125	G28532.5	1.27E-125
A0A6J8E914	A0A8W8LLK7	Cyclic nucleotide-binding domain-containing protein	0	G28586.3	0
A0A8B6FT25	---NA---	---NA---	---NA---	G1982.6	8.10E-04
A0A8B6CHE6	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8B6D5R1	A0A8W8NKX3	BRO1 domain-containing protein	0	G6211.5	0
A0A8B6G5S6	A0A8W8L3N4	Prohibitin	5.25E-155	G26216.2	4.10E-155
A0A8B6CYK1	A0A8W8I619	Proteasome subunit alpha type	1.38E-161	G12565.1	1.08E-161
A0A8S3UDK6	A0A8W8J5Q6	Proteasome subunit alpha type	1.22E-172	G17351.2	9.55E-173
A0A8B6DC08	A0A8W8NEZ2	DEK-C domain-containing protein	2.20E-28	G553.4	1.58E-29
A0A8B6FXU3	A0A8W8MXV3	Thioredoxin domain-containing protein	0	G4424.4	0
A0A6J8DCE2	A0A8W8JZC3	Protein RCC2	0	G21740.3	0
A0A8B6EIQ6	K1PQY0	Protein sleepless	4.44E-34	G2186.6	3.47E-34
A0A8S3S9K8	A0A8W8LRP1	Protein SSUH2-like protein	5.01E-158	G28720.3	3.91E-158
A0A8B6CNR6	K1QB61	Protocadherin Fat 4	0	G2955.1	0
A0A8S3UA94	A0A8W8LP70	Proactivator polypeptide	9.39E-164	G2909.4	7.33E-164
A0A0G2YLG5	A0A8W8LGM6	Fibronectin type-III domain-containing protein	0	G27955.1	0
A0A6J8BU32	K1RWS2	Transcriptional activator protein Pur-alpha	2.25E-152	G6097.5	1.76E-152
A0A8B6BR62	A0A8W8JG17	Pyruvate dehydrogenase E1 component subunit alpha	0	G19054.7	0
A0A8S3QRM1	K1PFL3	Dihydropteridine reductase	1.85E-122	G14476.1	1.45E-122
A0A8S3RSD5	A0A8W8LN62	Queuosine 5'-phosphate N-glycosylase/hydrolase	7.15E-139	G28796.1	5.59E-139
A0A6J8CI59	A0A8W8HWK6	Ras-related protein Rab-1A	4.79E-139	G11385.6	3.74E-139
A0A0B7AP29	K1QBM3	Ras-related protein Rab-2	1.18E-140	G21850.11	9.24E-141
A0A8B6FT60	A0A8W8IBB4	Radial spoke head protein 4-like protein A	0	G13256.3	0
A0A8B6GLW0	A0A8W8ICW5	Radial spoke head protein 9 homolog	1.14E-152	G13591.5	8.87E-153
A0A2C9JE50	A0A8W8P296	Ras-related protein Rab-11A	1.43E-148	G8381.5	1.12E-148
A0A6J8CUS4	K1QC78	Ras-related protein Rab-14	3.22E-160	G27922.1	2.52E-160
A0A8B6G941	A0A8W8KBX5	Ras-related protein Rab-35	2.84E-134	G22846.2	2.22E-134
A0A6J8F240	A0A8W8HY86	RNA-binding protein 4	1.01E-104	G11518.6	7.90E-105
A0A8B6C295	A0A8W8HL97	Receptor expression-enhancing protein	1.73E-89	G10083.4	1.35E-89
A0A6J8DY17	K1PYT4	RIB43A-like with coiled-coils protein 2	0	G30050.2	0
A0A077H3K4	A0A8W8KKZ1	Large ribosomal subunit protein uL23 N-terminal domain-containing protein	5.58E-75	G24036.6	4.36E-75
A0A077H3J9	K1PM50	40S ribosomal protein S16	2.53E-94	G5555.1	5.35E-94
A0A8S3QYE0	A0A8W8L0E1	Profilin	1.73E-42	G26073.12	1.35E-42
A0A8B6FIE8	A0A8W8HY86	RNA-binding protein 4	5.43E-21	G11518.6	4.24E-21

A0A6J8A0F1	Q70MN8	Large ribosomal subunit protein eL24 (Fragment)	3.74E-69	G18702.4	3.41E-69
A0A8S3UK82	A0A8W8IP93	Large ribosomal subunit protein uL24	3.84E-89	G15236.1	3.00E-89
A0A8B6EXY4	A0A8W8MB64	RRM domain-containing protein	0	G32666.2	0
A0A8B6HNM7	A0A8W8MRK6	S-(hydroxymethyl)glutathione dehydrogenase	4.68E-159	G3500.16	3.65E-159
A0A8B6CEB0	A0A8W8KAL0	Saposin B-type domain-containing protein	2.33E-136	G2310.4	1.82E-136
A0A6J8DVF9	A0A8W8M8E6	Translocon Sec61/SecY plug domain-containing protein	0	G31051.31	0
A0A8B6G4G0	A0A8W8JX33	Selenoprotein M	1.46E-18	G20897.10	1.14E-18
A0A8S3RKC5	K1RD83	Serine hydroxymethyltransferase	0	G3195.5	0
A0A8B6H826	A0A8W8IW86	SH3 domain-containing protein	0	G15848.1	0
A0A8B6GPC3	A0A8W8JB64	SHSP domain-containing protein	2.01E-33	G17984.2	1.57E-33
A0A8S3RCR9	K1QR48	Calcium-binding mitochondrial carrier protein SCaMC-2	0	G24895.1	0
A0A8B6F648	A0A8W8IE99	Small nuclear ribonucleoprotein Sm D2	7.72E-80	G13575.2	6.03E-80
A0A8B6CSI5	A0A8W8I406	40S ribosomal protein S17	2.69E-88	G12279.5	2.10E-88
A0A8B6HL62	K1QC22	40S ribosomal protein S19	7.29E-81	G26391.10	5.70E-81
A0A6J8BWT3	K1Q9D7	Sorting nexin-2	0	G10437.1	0
A0A8S3VFI8	A0A8W8MZ41	Spermatogenesis-associated protein 6 N-terminal domain-containing protein	4.92E-110	G3665.4	3.84E-110
A0A6J8D7K5	K1QTY5	Sulfide:quinone oxidoreductase, mitochondrial	3.59E-171	G7982.1	5.17E-171
A0A6J8E460	K1QCQ5	Succinate--CoA ligase	0	G16455.1	0
A0A8S3R089	A0A8W8INR7	Succinate--CoA ligase	0	G15171.5	0
A0A8B6CGG6	A0A8W8L0F8	Suppressor of G2 allele of SKP1-like protein	7.21E-134	G25356.4	5.63E-134
A0A8B6EYT5	A0A8W8JCM1	C2 domain-containing protein	1.50E-170	G17776.3	1.17E-170
A0A8B6DIG2	A0A8W8LAX1	T-complex protein 1 subunit alpha	0	G27437.1	0
A0A8B6E6D8	A0A8W8I1T4	T-complex protein 1 subunit gamma	0	G12090.2	0
A0A8B6F9J1	K1PXN5	T-complex protein 1 subunit zeta	0	G22682.5	0
A0A6J8BSI9	A0A8W8KQ90	Peptidase S54 rhomboid domain-containing protein	0	G2486.6	0
A0A6J8BBL7	A0A8W8N7D1	Tektin	0	G4594.6	0
A0A8S3SXV7	A0A8W8NSM9	Tetraspanin	3.52E-73	G6559.4	2.75E-73
A0A8B6CJW7	A0A8W8KN44	Tetraspanin	7.89E-15	G24614.1	6.16E-15
A0A8B6DN29	A0A8W8NIE7	Tetraspanin	1.86E-60	G5949.1	1.45E-60
A0A6J8AMT5	K1QIL7	Tetratricopeptide repeat protein 29	0	G25787.8	0
A0A8B6H5W0	A0A8W8L209	Thioredoxin	2.59E-37	G26313.13	2.03E-37
A0A8S3QJ24	A0A8W8P6T5	Thioredoxin-like protein 1	2.81E-153	G9681.1	2.20E-153
A0A8B6FN66	A0A8W8NVV8	Thyroglobulin type-1 domain-containing protein	6.64E-89	G6992.7	5.19E-89
A0A8B6C4T2	A0A8W8NHK5	Thyroglobulin type-1 domain-containing protein	2.78E-31	G6992.4	2.17E-31
A0A8S3R738	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8S3R9Y2	A0A8W8MM55	NTR domain-containing protein	2.74E-14	G34317.1	2.14E-14
A0A8B6FQW6	A0A8W8J501	Transaldolase	0	G17273.18	0

A0A8B6FLR4	A0A8W8JW85	Transcription elongation factor	3.77E-122	G2126.2	2.95E-122
A0A8B6CWL2	K1PFT9	Transgelin	1.80E-62	G19321.16	8.05E-62
A0A6J7ZVG6	K1QB11	Transgelin	2.51E-94	G28066.5	2.00E-94
A0A8B6H8C4	A0A8W8IXX2	Tripeptidyl-peptidase 2	0	G16345.6	0
A0A8B6F7C4	B7XC66	Tropomyosin	8.32E-106	G10110.176	8.40E-106
A0A6J8DNP0	K1QRQ0	60 kDa SS-A/Ro ribonucleoprotein	0	G3264.20	0
A0A0K2D7M8	A0A8W8HUR9	Protocadherin Fat 4	2.52E-29	G11053.1	1.97E-29
A0A0B6Z2B0	A0A8W8LF37	Tubulin beta chain	2.70E-44	G27780.7	2.11E-44
A0A8B6DWZ2	A0A8W8IRA6	THD domain-containing protein	4.36E-41	G15086.1	3.40E-41
A0A8S3TTF4	A0A8W8N7F2	Tumor protein D54	1.04E-72	G4980.28	8.11E-73
A0A8B6FHC2	U5U0P0	Tyrosinase	3.93E-91	G20964.3	1.06E-90
A0A8B6FCM7	A0A8W8JQE5	EGF-like domain-containing protein	9.21E-60	G2048.1	7.20E-60
A0A8S3SPC1	A0A8W8HU54	Ubiquitin-associated protein 2	8.01E-156	G11062.3	6.25E-156
A0A8S3QZQ4	A0A8W8LWR9	E2 ubiquitin-conjugating enzyme	2.01E-63	G30479.2	1.57E-63
A0A8B6CGQ6	A0A8W8MQ70	Cytochrome b-c1 complex subunit Rieske, mitochondrial	9.01E-73	G35433.9	7.04E-73
A0A0B6Y8N5	K1PD36	Ubiquitin-ribosomal protein eL40 fusion protein	7.62E-91	G26976.2	5.95E-91
A0A6J8CSZ8	A0A8W8IFN0	NAD-dependent epimerase/dehydratase domain-containing protein	0	G14021.8	0
A0A8B6FG82	K1R1A5	Collagen alpha-1(IV) chain	4.72E-38	G9790.1	8.19E-36
A0A3L5TR65	A0A8W8HUR9	Protocadherin Fat 4	1.68E-16	G11053.1	1.31E-16
A0A8B6FNN6	K1S3Q2	Macrophage asialoglycoprotein-binding protein	7.62E-05	G15878.8	1.10E-04
A0A8B6G8E3	A0A8W8MB95	SCO-spondin	4.99E-125	G32173.6	3.89E-125
A0A8B6GHG6	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8B6CU91	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8B6CEC3	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8B6HNNQ5	A0A8W8P090	Uncharacterized protein	3.62E-07	G9683.12	2.82E-07
A0A8B6FSV1	A0A8W8J417	Ndr family protein	0.00	G16645.30	0.00
A0A8B6CTG8	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8B6ENM5	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8B6CCA0	K1PRV7	Profilin	2.31E-05	G16496.5	1.80E-05
A0A8B6DTT6	K1QFX2	Low-density lipoprotein receptor-related protein 1B	2.50E-25	G26996.2	4.16E-25
A0A6J8CD73	K1PYZ9	SCO-spondin	1.36E-27	G1987.2	4.76E-27
A0A8S3T9B8	A0A8W8I8Z4	UPAR/Ly6 domain-containing protein	1.13E-25	G15797.1	8.86E-26
A0A8S3RZM2	A0A8W8IPH2	Kyphoscoliosis peptidase	6.89E-93	G15010.18	5.38E-93
A0A8B6F5H6	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8B6H837	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8B6FQ26	K1PJB8	Guanylate cyclase	2.31E-13	G5735.2	1.81E-13
A0A8B6DR40	K1Q894	EF-hand domain-containing protein 1	0	G4319.2	0

A0A8B6BSD4	A0A8W8NWQ5	Counting factor associated protein D	0.00	G78.3	0.00
A0A8B6CRI3	K1R6E6	Basement membrane-specific heparan sulfate proteoglycan core protein	1.19E-25	G21617.9	1.40E-25
A0A8B6CM77	A0A8W8LS81	Ependymin-related protein 1	2.65E-21	G294.3	2.07E-21
A0A8B6DHU3	A0A8W8LRT0	Tenascin-X	2.00E-26	G29424.4	1.57E-26
A0A8B6CG35	K1Q8V3	Ependymin-related protein 1	1.66E-09	G21093.10	1.30E-09
A0A8S3RSI4	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8B6DI09	K1RN59	Laminin-like protein epi-1	0	G3871.5	0
A0A8B6CVX7	K1PBN9	Importin subunit alpha-2	6.21E-08	G13492.1	1.83E-06
A0A8B6HFY8	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8B6F299	A0A167GGF3	9BACLWIAG-tail domain	3.60E-07	---NA---	---NA---
A0A8B6EZW5	K1RSS9	Collagen alpha-5(VI) chain	1.60E-37	G25577.1	4.30E-37
A0A8B6C5E2	K1PX59	Monocarboxylate transporter 12	1.66E-21	G13874.1	1.30E-21
A0A6J8CCF5	A0A8W8KT22	NodB homology domain-containing protein	7.72E-120	G24491.7	6.03E-120
A0A8S3U5X7	A0A8W8J3I6	Kielin/chordin-like protein	5.07E-41	G16891.3	8.25E-37
A0A8B6C9P4	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8B6H7C0	A0A8W8I820	VWFD domain-containing protein	4.19E-92	G12919.1	3.27E-92
A0A8B6G6F1	K1RSH8	von Willebrand factor D and EGF domain-containing protein	0	G16100.1	0
A0A8B6FPE6	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8S3S991	A0A8W8LRI1	Mammalian ependymin-related protein 1	5.94E-22	G287.2	4.64E-22
A0A8B6BE54	A0A8W8I3X0	PLAT domain-containing protein	5.27E-07	G12270.1	4.11E-07
A0A8B6GK87	A0A8W8MXH1	SCO-spondin	0	G35504.1	0
A0A8S3SVU1	A0A8W8LYZ9	Sperm microtubule inner protein 1 C-terminal domain-containing protein	1.22E-102	G30500.1	9.50E-103
A0A8B6D1E0	K1PWD9	Uncharacterized protein	2.36E-06	G5080.3	1.84E-06
A0A8B6CXZ8	A0A8W8JKX2	Ciliary microtubule inner protein 2C	1.32E-124	G1996.1	1.03E-124
A0A8B6HI74	A0A8W8ID54	Uncharacterized protein	1.08E-75	G13413.1	8.43E-76
A0A6J8EM21	A0A8W8KIJ1	Phosphatidylinositol transfer protein beta	9.11E-155	G24041.8	7.11E-155
A0A8S3UJ52	A0A8W8MPX9	Ig-like domain-containing protein	1.11E-36	G34792.1	8.65E-37
A0A8B6D1P6	A0A8W8KIA0	Beta-microseminoprotein	8.57E-05	G24018.2	6.69E-05
A0A8B6EZY7	A0A8W8MB95	SCO-spondin	0	G32173.6	0
A0A8S3QP16	A0A8W8J2V0	Uncharacterized protein	9.17E-106	G16810.7	7.16E-106
A0A8S3UR85	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8S3SPV4	A0A8W8KQP5	IgGfC-binding protein N-terminal domain-containing protein	3.12E-35	G24885.2	2.44E-35
A0A8S3TKR9	K1QAZ7	Coiled-coil domain-containing protein 55	2.86E-11	G22155.2	3.14E-11
A0A8B6FV73	A0A8W8J7T4	LIM zinc-binding domain-containing protein	0	G17454.23	0
A0A8S3S0N2	A0A8W8JQB4	Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	0	G2047.1	0
A0A8S3QG66	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8B6GYD1	K1QW43	Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit B	2.21E-76	G32429.1	1.73E-76

A0A8S3U726	K1QJ28	Mammalian ependymin-related protein 1	4.49E-73	G12308.3	1.04E-58
A0A8S3QIJ9	A0A8W8MWU0	UspA domain-containing protein	2.61E-87	G34954.1	2.04E-87
A0A8B6G9T0	A0A8W8LQ90	UspA domain-containing protein	1.66E-43	G29172.5	1.29E-43
A0A8B6ESB0	A0A8W8ICU3	UTP--glucose-1-phosphate uridylyltransferase	0	G13586.6	0
A0A8S3QEM3	K1QI28	Vacuolar proton pump subunit B	0	G15131.8	0
A0A6J8DNW3	A5LGH1	Voltage-dependent anion channel	1.73E-137	G1931.16	1.13E-48
A0A8B6E1P9	K1QVB4	Vitelline membrane outer layer protein 1	4.00E-46	G19499.2	1.87E-45
A0A8B6DLG6	A0A8W8P1D5	Serine carboxypeptidase CPVL	0	G8154.4	0.00
A0A8B6C9L4	A0A8W8KJ84	VWFA domain-containing protein	8.69E-106	G23385.8	6.79E-106
A0A8B6HDB4	K1Q662	Actin-interacting protein 1	0	G15632.1	0
A0A8B6F1U6	K1RDT2	Cilia- and flagella-associated protein 52	0	G3365.1	0
A0A8B6CY00	A0A8W8HLX2	2-oxoglutarate dehydrogenase, mitochondrial	0	G1020.16	0
A0A210Q521	A0A8W8IDA4	AAA domain-containing protein	0	G13469.5	0
A0A8B6GS06	A0A8W8IJT2	26S proteasome regulatory subunit RPN2 C-terminal domain-containing protein	3.12E-162	G14316.3	2.44E-162
A0A8B6GHX0	A0A8W8MJN0	PCI domain-containing protein	0	G32628.3	0
A0A8S3VGU8	K1QX22	4-hydroxyphenylpyruvate dioxygenase	0	G23380.1	0
A0A8S3QVD1	A0A8W8MBV1	Small ribosomal subunit protein uS17	4.13E-79	G4085.2	3.22E-79
A0A6J8EWE8	K1R8C6	40S ribosomal protein S12	7.88E-86	G2853.3	6.16E-86
A0A2C9JRX0	A0A8W8I248	Small ribosomal subunit protein uS19	7.48E-86	G11948.8	5.84E-86
A0A8B6G0N3	Q4H450	Small ribosomal subunit protein uS13	3.05E-103	G24712.5	2.38E-103
V4AS57	A0A8W8LCY1	40S ribosomal protein S25	8.88E-36	G26985.2	6.93E-36
A0A8B6EK07	A0A8W8LH25	40S ribosomal protein S26	2.40E-69	G28190.5	1.87E-69
A0A8B6CVW2	A0A8W8MXY1	Signal peptide peptidase-like 3	9.71E-94	G35094.6	7.59E-94
A0A210PU23	Q4H451	Ribosomal protein S5	1.14E-145	G25192.2	8.87E-146
A0A3G1CJL1	A0A8W8NHW3	40S ribosomal protein S8	1.90E-129	G5375.11	1.48E-129
A0A8B6BQ82	A0A8W8HTG6	AMP-activated protein kinase glycogen-binding domain-containing protein	2.31E-33	G10958.1	1.80E-33
A0A8S3RZZ6	K1QWX2	60S acidic ribosomal protein P0	4.33E-169	G5077.17	3.38E-169
B6ZCB1	K1Q358	Large ribosomal subunit protein P2	1.66E-33	G23358.6	4.56E-32
A0A8S3V934	K1RDM2	60S ribosomal protein L18a	1.84E-105	G28274.6	1.43E-105
A0A077GYU2	A0A8W8N1A1	Large ribosomal subunit protein uL14	2.45E-92	G3885.13	1.91E-92
A0A077H3N0	A0A8W8M7W6	60S ribosomal protein L27	8.59E-85	G32202.11	6.71E-85
A0A077GZK7	A0A8W8LHY8	Large ribosomal subunit protein eL28	4.43E-62	G27586.1	3.46E-62
A0A8B6G8D8	A0A8W8L6C3	Large ribosomal subunit protein eL31	2.33E-72	G26894.6	1.82E-72
A0A8B6DEA0	A0A8W8NT75	Large ribosomal subunit protein eL34	1.17E-66	G732.3	9.10E-67
A0A8S3R313	A0A8W8I5V2	Large ribosomal subunit protein eL36	3.25E-50	G12711.1	2.54E-50
A0A8B6EI40	A0A8W8KDU6	Large ribosomal subunit protein eL6	1.80E-116	G23471.12	1.41E-116
A0A6J8A5K2	A0A8W8MW71	Large ribosomal subunit protein uL2	1.64E-167	G35349.1	1.28E-167

A0A8B6FK68	A0A8W8LFS8	Xylose isomerase	0.00	G2787.2	0.00
A0A8S3UF93	K1R5F2	14-3-3 protein epsilon	2.04E-152	G23866.50	1.59E-152

Supplementary Table S8. Results of BLASTp analysis for DEPs identified in response to 2'-deoxyinosine (2'). The table lists homologous proteins from *Magallana gigas*, along with their corresponding accession numbers retrieved from the UniProt and Ensembl Metazoa databases.

Reference Sequence	UniProt			Ensembl	
	Accession number	Protein Name	e-Value	Accession number	e-Value
A0A8B6DSL8	K1RW85	Adenosylhomocysteinase	0	G22300.4	0
A0A8B6GV47	K1R0Y9	ADP/ATP translocase	0	G730.11	0
A0A8B6GU32	A0A8W8LM86	ATP synthase subunit gamma, mitochondrial	4.16E-124	G28739.12	3.25E-124
A0A8S3Q2E4	K1PQJ9	ATP synthase subunit delta, mitochondrial	2.28E-45	G269.2	1.78E-45
A0A6J8EBJ6	A0A8W8L8X3	Flotillin-1	0	G26928.23	0
A0A8B6GW86	A0A8W8J8Q2	C1q domain-containing protein	1.57E-20	G17851.1	1.23E-20
A0A8B6G4E0	A0A8W8KCX1	Calcium-transporting ATPase	0	G23365.9	0
A0A8B6FIN1	K1QY92	Chitin-binding type-2 domain-containing protein	5.43E-49	G22619.1	4.24E-49
A0A8B6C153	A0A8W8MWR9	Citrate synthase	0	G35409.40	0
A0A8B6ENY2	A0A8W8M8M9	CCHC-type domain-containing protein	2.64E-28	G31077.33	2.06E-28
A0A8S3R8F7	A0A8W8JPQ6	Arginine kinase	1.14E-178	G19705.1	8.89E-179
D9IWS1	A0A8W8N948	Elongation factor 1-alpha	0	G4818.20	0
A0A8B6EYD8	A0A8W8HXZ3	Elongation factor 2	0	G11483.21	0
A0A8S3QB23	K1R2L4	Histone H2B	2.28E-66	G22555.2	1.03E-62
A0A8B6DBF4	A0A8W8NN09	Metalloendopeptidase	8.22E-113	G6474.29	6.42E-113
A0A8B6G1S8	A0A8W8I1E8	Protein disulfide-isomerase	0.00	G11932.5	0
A0A8S3Q9N6	K1QZZ5	Stress-induced protein 1	1.00E-33	G17984.2	1.30E-33
A0A8B6D810	K1R401	Spectrin alpha chain	0	G25515.3	0
Q966V3	A0A8W8LCL2	Transgelin	0	G27652.16	0
A0A076FIR9	K1R7V7	Tubulin beta chain	0	G15286.68	0
A0A8B6CEW4	A0A8W8KET0	Uncharacterized protein	9.04E-33	G23190.43	7.06E-33
A0A8B6DC97	A0A8W8NN09	Metalloendopeptidase	6.29E-128	G6474.29	4.91E-128
A0A8B6BV40	K1PKC1	Collagen alpha-6(VI) chain	1.16E-89	G4540.1	7.57E-80
A0A8B6FD83	K1PDS7	Collagen alpha-2(I) chain	0	G5252.2	5.45E-67
A0A8B6G747	A0A8W8KPZ6	Fibrillar collagen NC1 domain-containing protein	0	G24458.2	0
A0A8S3QI19	K1Q7M2	Cathepsin L	0	G9706.23	0
A0A8B6HEV7	A0A8W8NFU0	LIM zinc-binding domain-containing protein	3.22E-96	G5979.9	2.51E-96
A0A8B6F031	K1QKV1	Cytochrome b-c1 complex subunit 6, mitochondrial	4.13E-12	G2226.1	3.23E-12
A0A8B6D685	A0A8W8I7C9	Dihydropyridyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	1.36E-159	G12941.5	1.06E-159

A0A409V740	---NA---	---NA---	---NA---	---NA---	---NA---
A0A3L5TQZ0	A0A8W8HZA6	C-type lectin domain-containing protein	3.96E-44	G11780.1	3.09E-44
A0A6J8ECB1	K1PTI6	Glucose-6-phosphate isomerase	5.75E-177	G20982.5	1.04E-176
A0A8B6DQP9	K1QRQ2	glutamate dehydrogenase	0	G3271.17	0
A0A8B6H497	A0A8W8MQI2	Granulins domain-containing protein	0	G34862.1	0
A0A411G5T5	A0A8W8KZI1	Ig-like domain-containing protein	2.17E-10	G25938.1	1.70E-10
K1Q324	K1Q324	Heterogeneous nuclear ribonucleoprotein K	0	G10728.1	1.06E-101
A0A0B7AUT8	A0A8W8NPB5	Histone H2A	1.79E-84	G661.4	1.40E-84
A0A8B6DFJ0	A0A8W8M908	RRM domain-containing protein	0	G31955.2	0
A0A8B6BTE6	A0A8W8N7H9	Large ribosomal subunit protein P1	1.62E-32	G5649.7	1.26E-32
A0A8S3Q6R0	K1RFA3	Lamin Dm0	0	G11465.2	0.00
A0A8B6C2Y2	A0A8W8K8K1	Malate dehydrogenase, mitochondrial	2.45E-162	G22856.16	1.91E-162
A0A8B6FW38	A0A8W8J1A5	Stress-70 protein, mitochondrial	0	G16610.7	0
A0A6J8APW2	A0A8W8IDE4	Nidogen-1	0	G13651.1	0
A0A8S3ULS9	K1RHA5	Nucleoside diphosphate kinase	3.22E-88	G27377.8	1.12E-72
A0A8B6D9K2	---NA---	---NA---	---NA---	---NA---	---NA---
A0A6J8DKH3	A0A8W8IB78	Peptidyl-prolyl cis-trans isomerase	9.28E-140	G13223.1	7.25E-140
A0A8S3TQI9	A0A8W8MDY5	EF-hand domain-containing protein	2.18E-82	G32504.8	1.71E-82
A0A6J8EGE4	A0A8W8JWQ7	TNF receptor-associated factor 4	0	G21164.1	0
A0A8S3QSQ2	K1PD36	Ubiquitin-ribosomal protein eL40 fusion protein	2.89E-92	G26976.2	2.26E-92
A0A3L5TR65	A0A8W8HUR9	Protocadherin Fat 4	1.68E-16	G11053.1	1.31E-16
A0A8B6E104	K1PSR5	Fatty acid-binding protein, heart	3.26E-34	G21524.4	2.90E-34
A0A8B6GUE8	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8B6CB58	A0A8W8JRD3	Apexrin C-terminal domain-containing protein	2.36E-08	G20570.1	1.84E-08
A0A8B6DFD7	A0A8W8HVV8	Papilin	3.74E-21	G11186.1	2.92E-21
A0A8B6BNI7	---NA---	---NA---	---NA---	---NA---	---NA---
A0A8B6DPQ0	K1R459	SCO-spondin	0	G35504.1	1.30E-133
A0A8B6FV73	A0A8W8J7T4	LIM zinc-binding domain-containing protein	0	G17454.23	0
A0A8B6CCQ9	K1PRV7	Profilin	6.77E-06	G16496.5	5.29E-06
A0A8B6H3D0	A0A8W8KJT0	MSP domain-containing protein	2.61E-105	G23892.18	2.04E-105
A0A8B6GXU1	A0A8W8JME6	Vitelline membrane outer layer protein 1	2.26E-48	G19499.2	1.76E-48
A0A8B6EK07	A0A8W8LH25	40S ribosomal protein S26	2.40E-69	G28190.5	1.87E-69
A0A3G1CJL1	A0A8W8NHW3	40S ribosomal protein S8	1.90E-129	G5375.11	1.48E-129
A0A8S3QVR3	A0A8W8MQU1	Small ribosomal subunit protein uS2	1.03E-143	G3387.29	8.05E-144
A0A6J8B1B1	A0A8W8NW87	ATP-dependent 6-phosphofructokinase	0	G7024.1	0
A0A8S3RZZ6	K1QWX2	60S acidic ribosomal protein P0	4.33E-169	G5077.17	3.38E-169
A0A6J8DYJ1	A0A8W8N1Z8	Large ribosomal subunit protein uL11	1.10E-94	G4972.15	8.61E-95

A0A8S3V934	K1RDM2	60S ribosomal protein L18a	1.84E-105	G28274.6	1.43E-105
A0A077H0N7	A0A8W8L109	60S ribosomal protein L7a	1.38E-155	G25420.3	1.08E-155

Supplementary Table S9. Functional analysis of DEPs identified in response to hypoxanthine arabinoside (1'). Gene Ontology (GO) enrichment was performed using the g:GOST tool in g:Profiler. The resulting GO terms are categorized into Molecular Functions (MF), Biological Processes (BP), and Cellular Components (CC). Both GO term names, and their corresponding IDs are listed.

Category	GO Name	GO ID	Adjusted <i>p</i> -value
GO:MF	structural molecule activity	GO:0005198	2.20E-31
GO:MF	structural constituent of ribosome	GO:0003735	3.90E-25
GO:MF	actin binding	GO:0003779	2.75E-09
GO:MF	cytoskeletal protein binding	GO:0008092	4.46E-07
GO:MF	intramolecular oxidoreductase activity	GO:0016860	5.06E-05
GO:MF	protein-containing complex binding	GO:0044877	6.73E-05
GO:MF	isomerase activity	GO:0016853	6.73E-05
GO:MF	calcium ion binding	GO:0005509	1.69E-04
GO:MF	actin filament binding	GO:0051015	2.00E-04
GO:MF	ribonucleoside triphosphate phosphatase activity	GO:0017111	5.89E-04
GO:MF	intramolecular oxidoreductase activity, transposing S-S bonds	GO:0016864	6.05E-04
GO:MF	proton-transporting ATP synthase activity, rotational mechanism	GO:0046933	6.05E-04
GO:MF	protein disulfide isomerase activity	GO:0003756	6.05E-04
GO:MF	oxidoreductase activity, acting on the aldehyde or oxo group of donors	GO:0016903	6.97E-04
GO:MF	ATPase-coupled monoatomic cation transmembrane transporter activity	GO:0019829	1.02E-03
GO:MF	pyrophosphatase activity	GO:0016462	1.06E-03
GO:MF	hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	GO:0016818	1.32E-03
GO:MF	hydrolase activity, acting on acid anhydrides	GO:0016817	1.32E-03
GO:MF	unfolded protein binding	GO:0051082	1.37E-03
GO:MF	proton transmembrane transporter activity	GO:0015078	2.90E-03
GO:MF	peroxiredoxin activity	GO:0051920	4.79E-03
GO:MF	oxidoreductase activity	GO:0016491	0.01
GO:MF	heterocyclic compound binding	GO:1901363	0.01
GO:MF	RNA binding	GO:0003723	0.01
GO:MF	alcohol dehydrogenase (NAD ⁺) activity	GO:0004022	0.01
GO:MF	fructose-bisphosphate aldolase activity	GO:0004332	0.01
GO:MF	alcohol dehydrogenase [NAD(P) ⁺] activity	GO:0018455	0.01
GO:MF	L-malate dehydrogenase (NAD ⁺) activity	GO:0030060	0.01
GO:MF	translation elongation factor activity	GO:0003746	0.01
GO:MF	anion binding	GO:0043168	0.01
GO:MF	active monoatomic ion transmembrane transporter activity	GO:0022853	0.01
GO:MF	extracellular matrix structural constituent	GO:0005201	0.01
GO:MF	P-type ion transporter activity	GO:0015662	0.01
GO:MF	intramolecular oxidoreductase activity, interconverting aldoses and ketoses	GO:0016861	0.01
GO:MF	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	GO:0016620	0.01
GO:MF	phosphotransferase activity, phosphate group as acceptor	GO:0016776	0.01
GO:MF	P-type transmembrane transporter activity	GO:0140358	0.02
GO:MF	P-type calcium transporter activity	GO:0005388	0.02
GO:MF	aldehyde-lyase activity	GO:0016832	0.02
GO:MF	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	GO:0016616	0.02
GO:MF	GTPase activity	GO:0003924	0.02
GO:MF	vitamin binding	GO:0019842	0.02
GO:MF	pyridoxal phosphate binding	GO:0030170	0.02
GO:MF	vitamin B6 binding	GO:0070279	0.02
GO:MF	ATP hydrolysis activity	GO:0016887	0.02
GO:MF	nucleotide binding	GO:0000166	0.02
GO:MF	intramolecular phosphotransferase activity	GO:0016868	0.02
GO:MF	nucleoside phosphate binding	GO:1901265	0.02

GO:MF	cAMP-dependent protein kinase regulator activity	GO:0008603	0.03
GO:MF	ribosome binding	GO:0043022	0.03
GO:MF	peptidyl-prolyl cis-trans isomerase activity	GO:0003755	0.03
GO:MF	cis-trans isomerase activity	GO:0016859	0.04
GO:MF	oxidoreductase activity, acting on the CH-NH group of donors, NAD or NADP as acceptor	GO:0016646	0.04
GO:MF	malate dehydrogenase activity	GO:0016615	0.04
GO:MF	cAMP binding	GO:0030552	0.04
GO:MF	oxidoreductase activity, acting on the aldehyde or oxo group of donors, disulfide as acceptor	GO:0016624	0.04
GO:MF	antioxidant activity	GO:0016209	0.04
GO:MF	nucleobase-containing compound kinase activity	GO:0019205	0.05
GO:MF	phospholipid transporter activity	GO:0005548	0.05
GO:MF	purine ribonucleotide binding	GO:0032555	0.05
GO:MF	purine nucleotide binding	GO:0017076	0.05
GO:BP	translation	GO:0006412	2.40E-17
GO:BP	purine ribonucleoside triphosphate metabolic process	GO:0009205	6.85E-15
GO:BP	purine nucleoside triphosphate metabolic process	GO:0009144	6.85E-15
GO:BP	ribonucleoside triphosphate metabolic process	GO:0009199	1.21E-14
GO:BP	generation of precursor metabolites and energy	GO:0006091	4.02E-14
GO:BP	ATP metabolic process	GO:0046034	6.13E-14
GO:BP	nucleoside triphosphate metabolic process	GO:0009141	1.02E-13
GO:BP	nucleoside phosphate metabolic process	GO:0006753	9.66E-10
GO:BP	purine-containing compound metabolic process	GO:0072521	1.79E-09
GO:BP	purine nucleotide metabolic process	GO:0006163	3.02E-09
GO:BP	small molecule metabolic process	GO:0044281	3.31E-09
GO:BP	nucleotide metabolic process	GO:0009117	4.35E-09
GO:BP	nucleobase-containing small molecule metabolic process	GO:0055086	4.35E-09
GO:BP	purine ribonucleotide metabolic process	GO:0009150	1.84E-08
GO:BP	ribonucleoside diphosphate catabolic process	GO:0009191	3.69E-08
GO:BP	ADP catabolic process	GO:0046032	3.69E-08
GO:BP	ribonucleotide metabolic process	GO:0009259	3.69E-08
GO:BP	purine nucleoside diphosphate catabolic process	GO:0009137	3.69E-08
GO:BP	ribose phosphate metabolic process	GO:0019693	3.69E-08
GO:BP	pyruvate metabolic process	GO:0006090	3.69E-08
GO:BP	glycolytic process	GO:0006096	3.69E-08
GO:BP	nucleoside diphosphate catabolic process	GO:0009134	3.69E-08
GO:BP	purine ribonucleoside diphosphate catabolic process	GO:0009181	3.69E-08
GO:BP	purine ribonucleotide catabolic process	GO:0009154	4.88E-08
GO:BP	ribonucleotide catabolic process	GO:0009261	4.88E-08
GO:BP	ADP metabolic process	GO:0046031	4.88E-08
GO:BP	pyridine nucleotide catabolic process	GO:0019364	4.88E-08
GO:BP	pyridine-containing compound catabolic process	GO:0072526	4.88E-08
GO:BP	carboxylic acid metabolic process	GO:0019752	6.37E-08
GO:BP	purine nucleotide catabolic process	GO:0006195	7.12E-08
GO:BP	oxoacid metabolic process	GO:0043436	9.36E-08
GO:BP	organic acid metabolic process	GO:0006082	1.31E-07
GO:BP	purine nucleoside diphosphate metabolic process	GO:0009135	1.32E-07
GO:BP	tricarboxylic acid cycle	GO:0006099	1.32E-07
GO:BP	purine ribonucleoside diphosphate metabolic process	GO:0009179	1.32E-07
GO:BP	energy derivation by oxidation of organic compounds	GO:0015980	1.32E-07
GO:BP	ribonucleoside diphosphate metabolic process	GO:0009185	1.32E-07
GO:BP	nicotinamide nucleotide metabolic process	GO:0046496	2.13E-07
GO:BP	nucleoside diphosphate metabolic process	GO:0009132	2.59E-07
GO:BP	aerobic respiration	GO:0009060	2.59E-07
GO:BP	pyridine nucleotide metabolic process	GO:0019362	3.20E-07
GO:BP	purine-containing compound catabolic process	GO:0072523	4.88E-07
GO:BP	purine ribonucleoside triphosphate biosynthetic process	GO:0009206	1.11E-06
GO:BP	purine nucleoside triphosphate biosynthetic process	GO:0009145	1.11E-06

GO:BP	organophosphate metabolic process	GO:0019637	1.29E-06
GO:BP	cellular respiration	GO:0045333	1.29E-06
GO:BP	pyridine-containing compound metabolic process	GO:0072524	1.54E-06
GO:BP	ribonucleoside triphosphate biosynthetic process	GO:0009201	2.06E-06
GO:BP	nucleoside phosphate catabolic process	GO:1901292	2.06E-06
GO:BP	nucleoside triphosphate biosynthetic process	GO:0009142	2.75E-06
GO:BP	nucleotide catabolic process	GO:0009166	4.89E-06
GO:BP	organophosphate catabolic process	GO:0046434	7.97E-06
GO:BP	proton motive force-driven ATP synthesis	GO:0015986	1.23E-05
GO:BP	ATP biosynthetic process	GO:0006754	1.89E-05
GO:BP	cilium movement involved in cell motility	GO:0060294	2.65E-05
GO:BP	carbohydrate catabolic process	GO:0016052	3.47E-05
GO:BP	cilium or flagellum-dependent cell motility	GO:0001539	4.51E-05
GO:BP	cilium-dependent cell motility	GO:0060285	4.51E-05
GO:BP	monocarboxylic acid metabolic process	GO:0032787	6.54E-05
GO:BP	cytoskeleton organization	GO:0007010	1.01E-04
GO:BP	biosynthetic process	GO:0009058	1.65E-04
GO:BP	protein metabolic process	GO:0019538	2.19E-04
GO:BP	actin cytoskeleton organization	GO:0030036	2.45E-04
GO:BP	actin filament-based process	GO:0030029	2.70E-04
GO:BP	cilium movement	GO:0003341	6.46E-04
GO:BP	actin filament organization	GO:0007015	1.01E-03
GO:BP	organic acid catabolic process	GO:0016054	1.08E-03
GO:BP	carboxylic acid catabolic process	GO:0046395	1.08E-03
GO:BP	regulation of protein-containing complex disassembly	GO:0043244	1.08E-03
GO:BP	translational elongation	GO:0006414	1.18E-03
GO:BP	supramolecular fiber organization	GO:0097435	1.38E-03
GO:BP	carbohydrate derivative catabolic process	GO:1901136	1.55E-03
GO:BP	nucleobase-containing compound catabolic process	GO:0034655	1.65E-03
GO:BP	cell motility	GO:0048870	2.01E-03
GO:BP	primary metabolic process	GO:0044238	2.63E-03
GO:BP	glucose metabolic process	GO:0006006	2.81E-03
GO:BP	protein-containing complex disassembly	GO:0032984	3.55E-03
GO:BP	actin filament depolymerization	GO:0030042	3.57E-03
GO:BP	protein depolymerization	GO:0051261	3.57E-03
GO:BP	organelle organization	GO:0006996	3.57E-03
GO:BP	cellular catabolic process	GO:0044248	3.62E-03
GO:BP	small molecule catabolic process	GO:0044282	3.62E-03
GO:BP	lysine catabolic process	GO:0006554	4.80E-03
GO:BP	gene expression	GO:0010467	4.80E-03
GO:BP	L-lysine catabolic process to acetyl-CoA via saccharopine	GO:0033512	4.80E-03
GO:BP	L-lysine catabolic process to acetyl-CoA	GO:0019474	4.80E-03
GO:BP	L-lysine catabolic process	GO:0019477	4.80E-03
GO:BP	L-lysine metabolic process	GO:0046440	4.80E-03
GO:BP	nucleotide biosynthetic process	GO:0009165	4.86E-03
GO:BP	proteinogenic amino acid catabolic process	GO:0170040	0.01
GO:BP	carbohydrate biosynthetic process	GO:0016051	0.01
GO:BP	nucleoside phosphate biosynthetic process	GO:1901293	0.01
GO:BP	carbohydrate derivative metabolic process	GO:1901135	0.01
GO:BP	cellular component disassembly	GO:0022411	0.01
GO:BP	purine-containing compound biosynthetic process	GO:0072522	0.01
GO:BP	carbohydrate metabolic process	GO:0005975	0.01
GO:BP	L-amino acid catabolic process	GO:0170035	0.01
GO:BP	protein folding	GO:0006457	0.01
GO:BP	metabolic process	GO:0008152	0.01
GO:BP	purine nucleotide biosynthetic process	GO:0006164	0.01
GO:BP	catabolic process	GO:0009056	0.01
GO:BP	purine ribonucleotide biosynthetic process	GO:0009152	0.01

GO:BP	hexose biosynthetic process	GO:0019319	0.01
GO:BP	monosaccharide biosynthetic process	GO:0046364	0.01
GO:BP	actin filament capping	GO:0051693	0.01
GO:BP	negative regulation of protein depolymerization	GO:1901880	0.01
GO:BP	regulation of protein depolymerization	GO:1901879	0.01
GO:BP	gluconeogenesis	GO:0006094	0.01
GO:BP	regulation of actin filament depolymerization	GO:0030834	0.01
GO:BP	negative regulation of protein-containing complex disassembly	GO:0043242	0.01
GO:BP	negative regulation of actin filament depolymerization	GO:0030835	0.01
GO:BP	ribonucleotide biosynthetic process	GO:0009260	0.01
GO:BP	ribose phosphate biosynthetic process	GO:0046390	0.01
GO:BP	proton transmembrane transport	GO:1902600	0.01
GO:BP	regulation of protein metabolic process	GO:0051246	0.02
GO:BP	macromolecule biosynthetic process	GO:0009059	0.02
GO:BP	acetyl-CoA metabolic process	GO:0006084	0.02
GO:BP	one-carbon metabolic process	GO:0006730	0.02
GO:BP	aspartate family amino acid catabolic process	GO:0009068	0.02
GO:BP	actin polymerization or depolymerization	GO:0008154	0.02
GO:BP	negative regulation of actin filament polymerization	GO:0030837	0.03
GO:BP	fatty acid beta-oxidation	GO:0006635	0.03
GO:BP	negative regulation of protein polymerization	GO:0032272	0.03
GO:BP	negative regulation of protein-containing complex assembly	GO:0031333	0.03
GO:BP	negative regulation of supramolecular fiber organization	GO:1902904	0.03
GO:BP	lysine metabolic process	GO:0006553	0.03
GO:BP	cell adhesion	GO:0007155	0.03
GO:BP	microtubule-based process	GO:0007017	0.04
GO:BP	fatty acid oxidation	GO:0019395	0.04
GO:BP	alpha-amino acid catabolic process	GO:1901606	0.04
GO:BP	negative regulation of cytoskeleton organization	GO:0051494	0.04
GO:BP	cellular component organization or biogenesis	GO:0071840	0.04
GO:BP	proteinogenic amino acid metabolic process	GO:0170039	0.04
GO:BP	tyrosine catabolic process	GO:0006572	0.04
GO:BP	regulation of protein catabolic process	GO:0042176	0.04
GO:BP	L-amino acid metabolic process	GO:0170033	0.04
GO:BP	protein polymerization	GO:0051258	0.04
GO:BP	fatty acid catabolic process	GO:0009062	0.05
GO:BP	organophosphate biosynthetic process	GO:0090407	0.05
GO:BP	plasma membrane bounded cell projection organization	GO:0120036	0.05
GO:CC	ribosome	GO:0005840	8.34E-26
GO:CC	ribonucleoprotein complex	GO:1990904	1.09E-18
GO:CC	protein-containing complex	GO:0032991	2.29E-16
GO:CC	membraneless organelle	GO:0043228	4.66E-15
GO:CC	intracellular membraneless organelle	GO:0043232	4.66E-15
GO:CC	intracellular anatomical structure	GO:0005622	4.52E-10
GO:CC	cytosolic ribosome	GO:0022626	5.70E-08
GO:CC	ribosomal subunit	GO:0044391	6.15E-08
GO:CC	organelle	GO:0043226	7.97E-08
GO:CC	cytosol	GO:0005829	1.21E-06
GO:CC	cytoplasm	GO:0005737	1.21E-06
GO:CC	intracellular organelle	GO:0043229	1.21E-06
GO:CC	proton-transporting ATP synthase complex, catalytic core F(1)	GO:0045261	9.98E-06
GO:CC	proton-transporting two-sector ATPase complex, catalytic domain	GO:0033178	1.02E-05
GO:CC	large ribosomal subunit	GO:0015934	1.49E-05
GO:CC	proton-transporting ATP synthase complex	GO:0045259	5.03E-05
GO:CC	proton-transporting two-sector ATPase complex	GO:0016469	8.71E-05
GO:CC	extracellular matrix	GO:0031012	1.44E-04
GO:CC	external encapsulating structure	GO:0030312	1.44E-04
GO:CC	respiratory chain complex	GO:0098803	2.91E-04

GO:CC	endoplasmic reticulum lumen	GO:0005788	4.07E-04
GO:CC	basement membrane	GO:0005604	1.08E-03
GO:CC	cytoskeleton	GO:0005856	1.23E-03
GO:CC	collagen-containing extracellular matrix	GO:0062023	2.24E-03
GO:CC	cytosolic large ribosomal subunit	GO:0022625	3.52E-03
GO:CC	small ribosomal subunit	GO:0015935	3.78E-03
GO:CC	membrane protein complex	GO:0098796	3.89E-03
GO:CC	plasma membrane bounded cell projection cytoplasm	GO:0032838	0.01
GO:CC	ciliary plasm	GO:0097014	0.01
GO:CC	axoneme	GO:0005930	0.01
GO:CC	cytoplasmic region	GO:0099568	0.01
GO:CC	mitochondrial inner membrane	GO:0005743	0.01
GO:CC	organelle inner membrane	GO:0019866	0.01
GO:CC	cAMP-dependent protein kinase complex	GO:0005952	0.01
GO:CC	intermediate filament	GO:0005882	0.01
GO:CC	radial spoke	GO:0001534	0.01
GO:CC	intermediate filament cytoskeleton	GO:0045111	0.01
GO:CC	actin cytoskeleton	GO:0015629	0.02
GO:CC	motile cilium	GO:0031514	0.02
GO:CC	collagen trimer	GO:0005581	0.02
GO:CC	oligosaccharyltransferase complex	GO:0008250	0.02
GO:CC	Golgi-associated vesicle membrane	GO:0030660	0.02
GO:CC	Golgi-associated vesicle	GO:0005798	0.02
GO:CC	catalytic complex	GO:1902494	0.02
GO:CC	polymeric cytoskeletal fiber	GO:0099513	0.03
GO:CC	COPI vesicle coat	GO:0030126	0.03
GO:CC	cation channel complex	GO:0034703	0.03
GO:CC	oxidoreductase complex	GO:1990204	0.04
GO:CC	mitochondrial envelope	GO:0005740	0.04
GO:CC	supramolecular fiber	GO:0099512	0.04
GO:CC	coated membrane	GO:0048475	0.04
GO:CC	supramolecular polymer	GO:0099081	0.04
GO:CC	cilium	GO:0005929	0.04
GO:CC	vesicle coat	GO:0030120	0.04
GO:CC	membrane coat	GO:0030117	0.04
GO:CC	plasma membrane bounded cell projection	GO:0120025	0.05

Supplementary Table S10. Functional analysis of DEPs identified in response to 2'-deoxyinosine (2'). Gene Ontology (GO) enrichment was performed using the g:GOST tool in g:Profiler. The resulting GO terms are categorized into Molecular Functions (MF), Biological Processes (BP), and Cellular Components (CC). Both GO term names, and their corresponding IDs are listed.

Category	GO Name	GO ID	Adjusted p-value
GO:MF	structural molecule activity	GO:0005198	7.30E-10
GO:MF	structural constituent of ribosome	GO:0003735	2.77E-05
GO:MF	proton-transporting ATP synthase activity, rotational mechanism	GO:0046933	0.01
GO:MF	extracellular matrix structural constituent	GO:0005201	0.01
GO:MF	structural constituent of chromatin	GO:0030527	0.04
GO:MF	adenosylhomocysteinase activity	GO:0004013	0.04
GO:MF	citrate (Si)-synthase activity	GO:0004108	0.04
GO:MF	dihydrolipoylysine-residue succinyltransferase activity	GO:0004149	0.04
GO:MF	glutamate dehydrogenase (NADP+) activity	GO:0004354	0.04
GO:MF	S-succinyltransferase activity	GO:0016751	0.04
GO:MF	intramolecular oxidoreductase activity	GO:0016860	0.04
GO:MF	hydrolase activity, acting on carbon-sulfur bonds	GO:0046508	0.04
GO:MF	glutamate dehydrogenase (NAD+) activity	GO:0004352	0.04
GO:MF	citrate synthase activity	GO:0036440	0.04
GO:MF	translation elongation factor activity	GO:0003746	0.04
GO:MF	L-malate dehydrogenase (NAD+) activity	GO:0030060	0.05
GO:MF	succinyltransferase activity	GO:0016748	0.05
GO:MF	proton channel activity	GO:0015252	0.05
GO:MF	glutamate dehydrogenase [NAD(P)+] activity	GO:0004353	0.05
GO:MF	glucose-6-phosphate isomerase activity	GO:0004347	0.05
GO:MF	6-phosphofructokinase activity	GO:0003872	4.82E-02
GO:BP	purine nucleoside triphosphate metabolic process	GO:0009144	7.56E-05
GO:BP	ribonucleoside triphosphate metabolic process	GO:0009199	7.56E-05
GO:BP	purine ribonucleoside triphosphate metabolic process	GO:0009205	7.56E-05
GO:BP	translation	GO:0006412	9.53E-05
GO:BP	nucleoside triphosphate metabolic process	GO:0009141	9.53E-05
GO:BP	generation of precursor metabolites and energy	GO:0006091	1.11E-04
GO:BP	ATP metabolic process	GO:0046034	4.59E-04
GO:BP	aerobic respiration	GO:0009060	7.70E-04
GO:BP	cellular respiration	GO:0045333	1.15E-03
GO:BP	purine-containing compound metabolic process	GO:0072521	1.15E-03
GO:BP	purine ribonucleotide metabolic process	GO:0009150	1.22E-03
GO:BP	ribonucleotide metabolic process	GO:0009259	1.37E-03
GO:BP	ribose phosphate metabolic process	GO:0019693	1.37E-03
GO:BP	energy derivation by oxidation of organic compounds	GO:0015980	1.48E-03
GO:BP	purine ribonucleoside triphosphate biosynthetic process	GO:0009206	1.48E-03
GO:BP	purine nucleoside triphosphate biosynthetic process	GO:0009145	1.48E-03
GO:BP	tricarboxylic acid cycle	GO:0006099	1.63E-03
GO:BP	ribonucleoside triphosphate biosynthetic process	GO:0009201	1.63E-03
GO:BP	nucleoside triphosphate biosynthetic process	GO:0009142	1.74E-03
GO:BP	purine nucleotide metabolic process	GO:0006163	1.90E-03
GO:BP	nucleoside phosphate metabolic process	GO:0006753	2.55E-03
GO:BP	small molecule metabolic process	GO:0044281	3.65E-03
GO:BP	nucleobase-containing small molecule metabolic process	GO:0055086	3.65E-03
GO:BP	organophosphate metabolic process	GO:0019637	3.85E-03
GO:BP	nucleotide metabolic process	GO:0009117	6.48E-03
GO:BP	proton motive force-driven ATP synthesis	GO:0015986	0.01
GO:BP	ATP biosynthetic process	GO:0006754	0.02
GO:BP	ADP metabolic process	GO:0046031	0.02

GO:BP	purine ribonucleotide catabolic process	GO:0009154	0.02
GO:BP	ribonucleotide catabolic process	GO:0009261	0.02
GO:BP	nucleoside diphosphate catabolic process	GO:0009134	0.02
GO:BP	purine nucleoside diphosphate catabolic process	GO:0009137	0.02
GO:BP	glycolytic process	GO:0006096	0.02
GO:BP	ribonucleoside diphosphate catabolic process	GO:0009191	0.02
GO:BP	purine ribonucleoside diphosphate catabolic process	GO:0009181	0.02
GO:BP	pyridine nucleotide catabolic process	GO:0019364	0.02
GO:BP	pyridine-containing compound catabolic process	GO:0072526	0.02
GO:BP	ADP catabolic process	GO:0046032	0.02
GO:BP	organic acid metabolic process	GO:0006082	0.02
GO:BP	purine ribonucleotide biosynthetic process	GO:0009152	0.02
GO:BP	ribonucleoside diphosphate metabolic process	GO:0009185	0.02
GO:BP	purine nucleoside diphosphate metabolic process	GO:0009135	0.02
GO:BP	carboxylic acid metabolic process	GO:0019752	0.02
GO:BP	oxoacid metabolic process	GO:0043436	0.02
GO:BP	purine nucleotide catabolic process	GO:0006195	0.02
GO:BP	ribosomal small subunit assembly	GO:0000028	0.02
GO:BP	purine ribonucleoside diphosphate metabolic process	GO:0009179	0.02
GO:BP	ribonucleotide biosynthetic process	GO:0009260	0.03
GO:BP	nucleoside diphosphate metabolic process	GO:0009132	0.03
GO:BP	ribose phosphate biosynthetic process	GO:0046390	0.03
GO:BP	pyruvate metabolic process	GO:0006090	0.03
GO:BP	purine-containing compound catabolic process	GO:0072523	0.03
GO:BP	purine nucleotide biosynthetic process	GO:0006164	0.03
GO:BP	translational elongation	GO:0006414	0.03
GO:BP	ribosome biogenesis	GO:0042254	0.03
GO:BP	glycolytic process through fructose-6-phosphate	GO:0061615	0.03
GO:BP	L-lysine catabolic process	GO:0019477	0.03
GO:BP	L-lysine metabolic process	GO:0046440	0.03
GO:BP	L-lysine catabolic process to acetyl-CoA via saccharopine	GO:0033512	0.03
GO:BP	fructose 6-phosphate metabolic process	GO:0006002	0.03
GO:BP	citrate metabolic process	GO:0006101	0.03
GO:BP	L-lysine catabolic process to acetyl-CoA	GO:0019474	0.03
GO:BP	lysine catabolic process	GO:0006554	0.03
GO:BP	nucleotide catabolic process	GO:0009166	0.04
GO:BP	purine-containing compound biosynthetic process	GO:0072522	0.04
GO:BP	biosynthetic process	GO:0009058	0.04
GO:BP	phosphagen biosynthetic process	GO:0042396	0.04
GO:BP	phosphocreatine metabolic process	GO:0006603	0.04
GO:BP	phosphagen metabolic process	GO:0006599	0.04
GO:BP	phosphocreatine biosynthetic process	GO:0046314	0.04
GO:BP	organophosphate biosynthetic process	GO:0090407	0.05
GO:BP	nucleoside phosphate catabolic process	GO:1901292	0.05
GO:BP	nicotinamide nucleotide metabolic process	GO:0046496	0.05
GO:BP	carbohydrate catabolic process	GO:0016052	0.05
GO:BP	mitochondrial ADP transmembrane transport	GO:0140021	0.05
GO:BP	purine-containing compound transmembrane transport	GO:0072530	0.05
GO:BP	tricarboxylic acid metabolic process	GO:0072350	0.05
GO:BP	adenine nucleotide transport	GO:0051503	0.05
GO:BP	mitochondrial ATP transmembrane transport	GO:1990544	0.05
GO:BP	nucleotide transmembrane transport	GO:1901679	0.05
GO:BP	aspartate family amino acid catabolic process	GO:0009068	0.05
GO:BP	nucleotide biosynthetic process	GO:0009165	0.05
GO:BP	purine nucleotide transport	GO:0015865	0.05
GO:BP	ribonucleoprotein complex biogenesis	GO:0022613	0.05
GO:BP	ADP transport	GO:0015866	0.05
GO:BP	ATP transport	GO:0015867	0.05

GO:BP	purine ribonucleotide transport	GO:0015868	0.05
GO:BP	pyridine nucleotide metabolic process	GO:0019362	0.05
GO:BP	organophosphate catabolic process	GO:0046434	0.05
GO:CC	ribosome	GO:0005840	1.91E-06
GO:CC	ribonucleoprotein complex	GO:1990904	7.31E-06
GO:CC	intracellular membraneless organelle	GO:0043232	1.73E-04
GO:CC	membraneless organelle	GO:0043228	1.73E-04
GO:CC	protein-containing complex	GO:0032991	3.32E-04
GO:CC	cytosolic ribosome	GO:0022626	4.50E-04
GO:CC	proton-transporting ATP synthase complex, catalytic core F(1)	GO:0045261	1.28E-03
GO:CC	proton-transporting ATP synthase complex	GO:0045259	8.26E-03
GO:CC	ribosomal subunit	GO:0044391	8.56E-03
GO:CC	proton-transporting two-sector ATPase complex, catalytic domain	GO:0033178	0.01
GO:CC	respiratory chain complex	GO:0098803	0.01
GO:CC	intracellular organelle	GO:0043229	0.02
GO:CC	cytosol	GO:0005829	0.02
GO:CC	large ribosomal subunit	GO:0015934	0.02
GO:CC	organelle	GO:0043226	0.02
GO:CC	oxoglutarate dehydrogenase complex	GO:0045252	0.02
GO:CC	intracellular anatomical structure	GO:0005622	0.02
GO:CC	proton-transporting two-sector ATPase complex	GO:0016469	0.03
GO:CC	nucleosome	GO:0000786	0.03
GO:CC	mitochondrial inner membrane	GO:0005743	0.03
GO:CC	tricarboxylic acid cycle heteromeric enzyme complex	GO:0045239	0.03
GO:CC	organelle inner membrane	GO:0019866	0.03
GO:CC	sarcoplasmic reticulum membrane	GO:0033017	0.03
GO:CC	protein-DNA complex	GO:0032993	0.04
GO:CC	intermediate filament cytoskeleton	GO:0045111	0.04
GO:CC	alpha-ketoacid dehydrogenase complex	GO:0045240	0.04
GO:CC	intermediate filament	GO:0005882	0.04
GO:CC	cation channel complex	GO:0034703	0.05
GO:CC	cytosolic small ribosomal subunit	GO:0022627	0.05
GO:CC	sarcoplasmic reticulum	GO:0016529	0.05

Supplementary Material of Chapter 5

Supplementary Table S11. Differentially expressed proteins (DEPs) from the adhesive mussel larvae samples after the 1.5-fold change analysis between condition groups (Ctr-, 5 μ M and 10 μ M).

Reference	Protein Name	Relative Expression			Fold Change	
		Ctr-	10 μ M	5 μ M	Ctr- vs 10 μ M	Ctr- vs 5 μ M
KFM61959	Acidic leucine rich nuclear phosphoprotein 32 family member A partial	-0.12	-0.16	0.42	0.04	0.54
AIV99912.1	alpha 2 macroglobulin	-0.18	0.42	-0.35	-0.60	-0.17
XP_022317867	annexin B9 like	0.28	0.20	-0.72	0.08	-1.00
XP_021364765	argininosuccinate lyase like	-0.24	0.38	-0.21	-0.62	0.04
ABO26694	axonemal dynein light chain p33 partial	0.11	0.22	-0.49	-0.11	-0.60
XP_022295017	basic leucine zipper and W2 domain containing protein 1 like	-0.25	-0.01	0.38	-0.24	0.62
XP_021361418	beta 1 3 glucan binding protein like	0.34	-0.34	0.00	0.68	-0.34
XP_021359006	beta galactosidase like	0.31	-0.38	0.10	0.69	-0.21
XP_021375560	calpain 5 like	-0.47	0.23	0.36	-0.70	0.84
K1PJ53	Cdc42 like protein	-0.16	0.40	-0.36	-0.56	-0.20
XP_022293175	chloride intracellular channel protein 2 like	-0.41	0.36	0.06	-0.77	0.47
XP_021350575	Cluster of 2 oxoglutarate dehydrogenase mitochondrial like isoform X2	-0.08	-0.21	0.43	0.14	0.51
XP_021374413	Cluster of 40S ribosomal protein S15Aa	-0.21	-0.06	0.40	-0.15	0.60
XP_022341970	Cluster of 60S ribosomal protein L13 like	-0.32	0.34	-0.03	-0.66	0.29
A0A077GYT9	Cluster of 60S ribosomal protein L27a Fragment	0.00	-0.29	0.44	0.29	0.44
XP_021369794	Cluster of actin related protein 3 isoform X1	0.20	0.09	-0.43	0.12	-0.63
XP_022332900	Cluster of adenylate kinase 7 like	-0.09	0.32	-0.35	-0.41	-0.27
XP_015927503	Cluster of ADP ribosylation factor like protein 3	0.35	-0.07	-0.42	0.42	-0.77
XP_022292522	Cluster of aldehyde dehydrogenase family 16 member A1 like	0.36	-0.52	0.24	0.88	-0.12
XP_021353098	Cluster of AP 2 complex subunit alpha 2 like	-0.37	0.18	0.28	-0.55	0.65
XP_022325524	Cluster of BTB POZ domain containing protein KCTD12 like	-0.21	-0.17	0.58	-0.04	0.79
E1UJ66	Cluster of C1q domain containing protein	-0.24	0.00	0.37	-0.24	0.61
XP_021350043	Cluster of calcium calmodulin dependent protein kinase type II delta chain like isoform X8	0.33	-0.35	0.03	0.67	-0.29
XP_021362141	Cluster of calnexin like isoform X1	0.20	0.05	-0.38	0.15	-0.59
NP_001191582	Cluster of cAMP dependent protein kinase regulatory subunit	-0.35	0.44	-0.14	-0.79	0.21
XP_022305463	Cluster of carboxylesterase 1C like	0.01	-0.34	0.49	0.36	0.48
XP_021364896	Cluster of chromodomain helicase DNA binding protein 5 like isoform X6	0.31	-0.31	0.00	0.62	-0.31

XP_021375961	Cluster of coatomer subunit delta like	0.07	0.35	-0.62	-0.28	-0.69
NA.19	Cluster of comp30487_c0_seq1 aalen 222 92_partial5 clen 726 BESTHIT	0.33	-0.02	-0.47	0.35	-0.80
XP_021370266	Cluster of constitutive coactivator of PPAR gamma like protein 1 homolog isoform X1	0.07	0.20	-0.40	-0.13	-0.47
K1Q5I4	Cluster of COP9 signalosome complex subunit 2	-0.05	-0.21	0.39	0.16	0.44
XP_022345969	Cluster of drebrin like protein B isoform X3	-0.32	0.44	-0.18	-0.77	0.14
XP_022329591	Cluster of dual oxidase 2 like isoform X2	0.29	-0.02	-0.40	0.32	-0.70
AMK01816	Cluster of EIF4G partial	-0.27	0.03	0.35	-0.31	0.63
XP_021368420	Cluster of eukaryotic translation initiation factor 3 subunit A like	-0.27	0.05	0.33	-0.32	0.60
XP_022314527	Cluster of eukaryotic translation initiation factor 3 subunit B like	-0.37	0.18	0.29	-0.56	0.66
XP_021363917	Cluster of exportin 2 like	0.46	-0.45	-0.02	0.91	-0.49
XP_021343957	Cluster of F actin capping protein subunit alpha like	0.08	0.24	-0.49	-0.16	-0.57
XP_022302163	Cluster of glucose 6 phosphate 1 dehydrogenase like isoform X1	-0.09	0.32	-0.35	-0.42	-0.26
XP_022296622	Cluster of glutathione S transferase Mu 3 like	-0.40	0.18	0.34	-0.58	0.75
XP_021375144	Cluster of glycine tRNA ligase like	-0.47	0.33	0.22	-0.79	0.68
XP_021359588	Cluster of glycine dehydrogenase decarboxylating mitochondrial like	-0.03	0.28	-0.38	-0.32	-0.34
Q6Q5Y5	Cluster of GST class pi	-0.62	0.36	0.39	-0.98	1.01
XP_021348928	Cluster of heterogeneous nuclear ribonucleoprotein H2 like	0.55	-0.33	-0.34	0.88	-0.90
XP_021358952	Cluster of heterogeneous nuclear ribonucleoprotein K like isoform X7	-0.36	0.25	0.16	-0.61	0.51
XP_021379021	Cluster of hydroxysteroid dehydrogenase like protein 2	0.27	-0.02	-0.37	0.30	-0.65
OPL33119.1	Cluster of hypothetical protein AM593_4239 partial	-0.45	0.38	0.11	-0.84	0.56
XP_009058775	Cluster of hypothetical protein LOTGIDRAFT_217885	-0.29	0.06	0.34	-0.36	0.64
XP_009063021	Cluster of hypothetical protein LOTGIDRAFT_220774	-0.40	0.23	0.26	-0.63	0.65
XP_022300367	Cluster of inter alpha trypsin inhibitor heavy chain H3 like isoform X1	0.38	-0.24	-0.21	0.62	-0.59
XP_022323505	Cluster of kinesin like protein KIF28P isoform X2	-0.17	0.37	-0.30	-0.54	-0.13
XP_022315354	Cluster of LOW QUALITY PROTEIN Golgi specific brefeldin A resistance guanine nucleotide exchange factor 1 like	-0.17	0.40	-0.36	-0.57	-0.19
XP_022337445	Cluster of methenyltetrahydrofolate synthase domain containing protein like	-0.22	0.43	-0.33	-0.65	-0.11
XP_021373758	Cluster of methylmalonyl CoA mutase mitochondrial like	-0.23	0.38	-0.22	-0.61	0.01
XP_022331248	Cluster of myosin IIIb like isoform X4	-0.03	0.28	-0.38	-0.32	-0.34
XP_021370718	Cluster of non-specific lipid transfer protein like isoform X2	0.18	-0.39	0.32	0.57	0.14
XP_021345331	Cluster of phosphoglucomutase 1 like	-0.25	0.02	0.35	-0.27	0.60
OPL33132	Cluster of poly endoribonuclease c like specific partial	-0.57	0.33	0.36	-0.90	0.93
Q8MW55	Cluster of Precollagen NG	0.10	0.18	-0.42	-0.08	-0.52
XP_011434053	Cluster of PREDICTED 26S proteasome non ATPase regulatory subunit 6	0.28	0.00	-0.43	0.28	-0.72
XP_013402087	Cluster of PREDICTED 60S ribosomal protein L26 like isoform X1	0.40	-0.41	0.01	0.81	-0.39
XP_011434532	Cluster of PREDICTED allene oxide synthase lipxygenase protein	-0.04	0.29	-0.37	-0.33	-0.33
XP_011430094	Cluster of PREDICTED aspartate aminotransferase	0.26	0.05	-0.47	0.21	-0.73
XP_011439128	Cluster of PREDICTED bifunctional 3 phosphoadenosine 5 phosphosulfate synthase isoform X4	-0.30	-0.22	0.79	-0.08	1.09

XP_011444095	Cluster of PREDICTED cilia and flagella associated protein 70 isoform X12	0.32	-0.34	0.02	0.66	-0.31
XP_019930280	Cluster of PREDICTED disks large homolog 1	0.45	-0.39	-0.10	0.84	-0.55
XP_005089259	Cluster of PREDICTED erlin 1 like	0.23	0.02	-0.38	0.21	-0.61
XP_011436159	Cluster of PREDICTED mitogen activated protein kinase 1	-0.27	0.35	-0.12	-0.62	0.15
XP_011439173	Cluster of PREDICTED neurobeachin isoform X1	0.22	0.06	-0.42	0.17	-0.64
XP_011427688	Cluster of PREDICTED neutral alpha glucosidase AB isoform X3	-0.35	0.09	0.39	-0.44	0.74
XP_011426298	Cluster of PREDICTED poly ADP ribose polymerase 1	-0.26	-0.07	0.49	-0.19	0.74
XP_011454471	Cluster of PREDICTED ras GTPase activating protein binding protein 2	0.13	-0.40	0.41	0.53	0.28
XP_011413908	Cluster of PREDICTED RNA binding protein Nova 1 isoform X3	0.26	-0.34	0.13	0.60	-0.13
XP_011416301	Cluster of PREDICTED tektin 1	0.13	-0.32	0.28	0.45	0.15
XP_014788958	Cluster of PREDICTED transmembrane 9 superfamily member 3 like	0.39	-0.20	-0.28	0.59	-0.67
XP_021375922	Cluster of probable protein phosphatase 2C T23F11 1	0.31	-0.12	-0.29	0.43	-0.60
XP_022341530	Cluster of programmed cell death protein 4 like	-0.29	0.36	-0.11	-0.65	0.18
AKS48167	Cluster of protease inhibitor like protein D2	0.07	0.26	-0.49	-0.19	-0.56
XP_021379330	Cluster of proteasome subunit beta type 5 like	-0.22	-0.03	0.37	-0.19	0.59
XP_021377087	Cluster of protein disulfide isomerase A5 like	0.02	-0.30	0.43	0.32	0.41
F0V443	Cluster of Putative C1q domain containing protein MgC1q6	0.07	-0.49	0.64	0.56	0.57
XP_021341989	Cluster of ras related GTP binding protein C like	-0.33	0.39	-0.09	-0.72	0.23
A0A126Q9D7	Cluster of RNA helicase	0.33	-0.13	-0.30	0.46	-0.63
XP_021362448	Cluster of S adenosylhomocysteine hydrolase like protein 1 isoform X1	-0.31	0.04	0.41	-0.35	0.72
XP_021348096	Cluster of septin 2B like isoform X10	0.10	0.24	-0.51	-0.14	-0.61
XP_022319118	Cluster of serine hydroxymethyltransferase cytosolic like	-0.43	0.15	0.42	-0.58	0.84
AEP02968	Cluster of small heat shock protein 24 1	-0.62	0.89	-0.41	-1.51	0.21
G3GAE5	Cluster of Small heat shock protein 24 1	-0.43	0.48	-0.07	-0.91	0.36
XP_022304940	Cluster of splicing factor 3B subunit 1 like isoform X1	0.25	-0.37	0.18	0.62	-0.07
XP_022340925.1	Cluster of splicing factor 3B subunit 3 like	-0.03	0.41	-0.57	-0.43	-0.54
XP_021347443.1	Cluster of titin like	-0.12	0.46	-0.50	-0.58	-0.38
XP_021370764	Cluster of transportin 1 like isoform X4	-0.25	-0.03	0.41	-0.22	0.65
XP_022324431	Cluster of tryptophan tRNA ligase	0.23	0.07	-0.46	0.16	-0.70
A0A0B6ZPB7	Cluster of Ubiquitin like domain containing protein Fragment	-0.31	-0.04	0.52	-0.27	0.83
XP_020608397	Cluster of uncharacterized protein LOC110047016	-0.22	-0.04	0.38	-0.18	0.60
XP_021369729	Cluster of uncharacterized protein LOC110460860	0.18	0.20	-0.56	-0.02	-0.74
XP_022320073	Cluster of uncharacterized protein LOC111122577	0.44	-0.37	-0.10	0.81	-0.54
XP_022322557	Cluster of uncharacterized protein LOC111124008	-0.42	0.30	0.19	-0.72	0.61
A0A2T7NPT5	Cluster of Uncharacterized protein	-0.35	0.29	0.09	-0.64	0.44
XP_021340043	Cluster of V type proton ATPase subunit C 1 A like	0.00	-0.27	0.41	0.27	0.41
XP_022333787	Cluster of V type proton ATPase subunit D like	0.04	-0.31	0.40	0.35	0.36

XP_021342081	coiled coil domain containing protein 173 like	-0.34	0.13	0.32	-0.47	0.66
XP_022341081	collagen alpha 1 I chain like	0.25	-0.36	0.16	0.61	-0.09
XP_022323534	collagen alpha 1 I chain like	0.41	-0.47	0.09	0.88	-0.32
XP_021355139	collagen alpha 4 VI chain like	-0.58	0.57	0.01	-1.15	0.59
NA.22	comp62911_c0_seq1 aalen 341 99_partial clen 1025 BESTHIT	0.44	-0.23	-0.31	0.67	-0.75
NA.1	comp99906_c0_seq1 aalen 111 98_partial clen 337 BESTHIT	0.07	0.20	-0.40	-0.13	-0.47
AFQ32467	CuZn superoxide dismutase	0.05	-0.31	0.39	0.36	0.34
XP_021342314	cystathionine gamma lyase like	0.06	-0.44	0.57	0.49	0.51
XP_021371563	cysteine tRNA ligase cytoplasmic like	0.33	-0.16	-0.27	0.49	-0.60
AL158372	cytochrome c oxidase subunit Va	0.49	-0.61	0.18	1.10	-0.31
XP_021361172	cytosolic purine 5 nucleotidase like isoform X2	-0.32	0.29	0.05	-0.61	0.37
XP_022318978	dnaJ homolog subfamily B member 4 like	-0.01	0.26	-0.38	-0.27	-0.37
K1QHM2	Dolichyl diphosphooligosaccharide protein glycosyltransferase subunit 2	-0.45	0.29	0.24	-0.74	0.69
XP_022327361	endoplasmic reticulum resident protein 44 like	-0.34	0.29	0.07	-0.63	0.41
XP_021372964	ES1 protein homolog mitochondrial like	-0.30	0.36	-0.08	-0.66	0.22
XP_022345477	F box only protein 36 like	0.32	-0.42	0.15	0.75	-0.17
XP_021346087	FERM domain containing protein 5 like isoform X3	0.34	-0.05	-0.43	0.40	-0.78
XP_022341069	fibril forming collagen alpha chain like	0.24	0.11	-0.53	0.14	-0.77
XP_021372850	forkhead associated domain containing protein 1 like isoform X1	0.37	-0.21	-0.24	0.58	-0.61
XP_022307042	formimidoyltransferase cyclodeaminase like isoform X1	0.43	-0.10	-0.49	0.53	-0.92
XP_022289863	fumarylacetoacetate hydrolase domain containing protein 2 like	-0.26	-0.06	0.49	-0.20	0.75
AJW60777	galectin	-0.22	-0.14	0.54	-0.08	0.76
AFQ35985.1	glutathione S transferase sigma 3	-0.12	-0.16	0.43	0.04	0.55
XP_022328214	heat shock 70 kDa protein 12A like	0.39	-0.38	-0.02	0.77	-0.41
XP_021355259	heterogeneous nuclear ribonucleoprotein L like isoform X4	0.19	-0.41	0.32	0.60	0.13
OPL33454	hypothetical protein AM593_1137 partial	-0.10	0.32	-0.33	-0.42	-0.23
OPL33693	hypothetical protein AM593_3385 partial	-0.11	0.40	-0.44	-0.51	-0.33
OPL33405	hypothetical protein AM593_4064 partial	-0.23	0.51	-0.41	-0.74	-0.18
OPL20952	hypothetical protein AM593_10337 partial	0.30	-0.29	-0.03	0.59	-0.33
OWF49001	hypothetical protein KP79	-0.09	0.34	-0.37	-0.43	-0.28
XP_009051647	hypothetical protein LOTGIDRAFT_208961	-0.32	0.10	0.33	-0.42	0.64
XP_009052416	hypothetical protein LOTGIDRAFT_231723	-0.37	0.35	0.02	-0.72	0.39
XP_022332848	LOW QUALITY PROTEIN cilia and flagella associated protein 161 like	0.22	0.10	-0.48	0.11	-0.69
XP_022317804	LOW QUALITY PROTEIN digestive cysteine proteinase 2 like	-0.37	0.24	0.19	-0.62	0.57
XP_022340655	LOW QUALITY PROTEIN nodal modulator 1 like	-0.46	0.15	0.46	-0.61	0.92
XP_022286733	LOW QUALITY PROTEIN pyruvate dehydrogenase E1 component subunit alpha	0.03	0.27	-0.46	-0.24	-0.49
XP_021372081	LOW QUALITY PROTEIN tripeptidyl peptidase 2 like	0.17	0.19	-0.54	-0.02	-0.72

A0A0C5Q4G7	MACPF domain containing protein 4	-0.11	-0.21	0.48	0.10	0.59
AJQ21522	MACPF domain containing protein 7	-0.11	0.51	-0.59	-0.62	-0.48
OWF36413	MAGUK p55 subfamily member 2	0.08	0.19	-0.41	-0.11	-0.49
XP_021364577	major vault protein like isoform X2	0.17	-0.34	0.26	0.51	0.09
XP_022330328	mesenchyme specific cell surface glycoprotein like	0.04	-0.47	0.65	0.51	0.61
EKC41639	Myosin XV	-0.30	0.31	-0.02	-0.60	0.28
XP_022319861	neutral ceramidase like	-0.08	-0.19	0.41	0.11	0.49
XP_021352601	non POU domain containing octamer binding protein like isoform X2	-0.50	0.23	0.40	-0.74	0.90
XP_021371695	nucleolysin TIAR like isoform X2	-0.09	0.34	-0.38	-0.43	-0.29
XP_021341001	papilin like isoform X5	-0.31	0.02	0.44	-0.32	0.75
XP_022321158	phospholipid transfer protein C2CD2L like isoform X1	-0.04	0.33	-0.43	-0.37	-0.38
XP_022319633	polynucleotide 3 phosphatase ZDP like	0.35	-0.16	-0.29	0.51	-0.65
XP_011448859	PREDICTED 40S ribosomal protein S17	-0.02	0.27	-0.38	-0.30	-0.36
XP_011451537	PREDICTED actin interacting protein 1	-0.21	0.37	-0.24	-0.58	-0.03
XP_011441796	PREDICTED alanine glyoxylate aminotransferase 2 mitochondrial	0.08	0.25	-0.50	-0.17	-0.58
XP_011413000	PREDICTED annexin A7 like isoform X1	-0.46	0.04	0.63	-0.49	1.08
XP_019929295	PREDICTED arrestin domain containing protein 17 isoform X3	0.42	-0.33	-0.14	0.75	-0.56
XP_011415611	PREDICTED astacin like	0.07	-0.29	0.33	0.36	0.25
XP_013063871	PREDICTED calcium binding mitochondrial carrier protein SCA2C 2 like	-0.13	0.56	-0.65	-0.70	-0.51
I1VYX7	PREDICTED dehydrogenase reductase SDR family member 7	0.04	-0.26	0.33	0.30	0.28
XP_011440625	PREDICTED dolichyl diphosphooligosaccharide protein glycosyltransferase subunit 2	0.29	-0.04	-0.37	0.33	-0.66
XP_011426045	PREDICTED EF hand domain containing family member B	0.06	0.21	-0.40	-0.15	-0.46
XP_011437904	PREDICTED gelsolin like protein 2	-0.05	-0.26	0.46	0.20	0.52
XP_019924673	PREDICTED glutamine rich protein 2 isoform X3	0.08	0.22	-0.44	-0.14	-0.52
XP_011416455	PREDICTED importin 4	0.16	-0.47	0.47	0.63	0.32
XP_011416491	PREDICTED isoleucine tRNA ligase cytoplasmic like	0.33	-0.12	-0.31	0.45	-0.64
XP_011450015.1	PREDICTED mesenchyme specific cell surface glycoprotein like	0.37	-0.18	-0.28	0.55	-0.64
XP_019922173	PREDICTED mucin 16 isoform X6	-0.31	0.32	-0.02	-0.63	0.29
XP_011444273	PREDICTED neuroendocrine convertase 2	-0.01	0.34	-0.49	-0.36	-0.48
XP_011422378	PREDICTED neutral cholesterol ester hydrolase 1	0.18	-0.35	0.26	0.53	0.08
XP_011444269	PREDICTED peptidyl prolyl cis trans isomerase	-0.31	0.11	0.30	-0.42	0.61
XP_011421092	PREDICTED prenylcysteine oxidase isoform X2	0.31	-0.04	-0.40	0.34	-0.71
XP_011456198	PREDICTED sperm associated antigen 17 isoform X4	0.02	0.29	-0.47	-0.26	-0.49
XP_011444861	PREDICTED stromal cell derived factor 2 like	0.33	-0.07	-0.39	0.40	-0.72
XP_011430749	PREDICTED tetratricopeptide repeat protein 25 isoform X2	-0.26	0.04	0.34	-0.30	0.60
XP_013095195	PREDICTED troponin T like	-0.25	0.49	-0.35	-0.75	-0.10
XP_019922009	PREDICTED uncharacterized protein LOC105326232 isoform X35	-0.35	0.26	0.14	-0.60	0.48

XP_019921660	PREDICTED Williams Beuren syndrome chromosomal region 27 protein isoform X2	0.01	-0.30	0.43	0.31	0.42
XP_021354128	presequence protease mitochondrial like isoform X1	-0.26	-0.15	0.62	-0.11	0.89
XP_021373328	probable ubiquitin carboxyl terminal hydrolase FAF X isoform X3	-0.25	0.00	0.37	-0.25	0.62
XP_021354982	prostaglandin G H synthase 2 like	0.23	0.15	-0.57	0.09	-0.80
XP_022293421.1	proteasome subunit alpha type 1 like	0.35	-0.25	-0.15	0.60	-0.50
A0A210QN06	Proteasome subunit alpha type	0.43	-0.30	-0.19	0.73	-0.62
A0A3G1CJM1	Proteasome subunit beta Fragment	-0.37	0.42	-0.08	-0.79	0.29
XP_022342581	protein phosphatase 1 regulatory subunit 7 like isoform X2	0.27	0.15	-0.63	0.12	-0.90
OWF37582	Protein transport protein Sec23A	0.32	-0.29	-0.05	0.60	-0.36
F0V478	Putative C1q domain containing protein MgC1q41	0.34	-0.26	-0.12	0.60	-0.46
F0V4D4	Putative C1q domain containing protein MgC1q97	-0.23	-0.23	0.70	0.00	0.93
XP_022320087	radial spoke head 1 homolog	0.15	0.18	-0.49	-0.02	-0.64
XP_021358954	ras GTPase activating like protein IQGAP1 isoform X2	0.30	-0.06	-0.35	0.36	-0.65
XP_022335184	RNA binding protein lark like	0.42	-0.44	0.03	0.85	-0.39
XP_021360996	serine pyruvate aminotransferase like	0.27	-0.03	-0.35	0.30	-0.62
AKQ70853	shell myostracum nacrein like protein 1 partial	-0.32	0.31	0.02	-0.64	0.34
XP_022315645	sodium calcium exchanger 2 like isoform X6	-0.43	0.12	0.47	-0.55	0.90
XP_022293803	succinate dehydrogenase ubiquinone flavoprotein subunit mitochondrial like	0.38	-0.32	-0.08	0.71	-0.47
XP_022326686	sulfite oxidase like	-0.07	-0.25	0.48	0.18	0.55
XP_021377365	sulfotransferase 1A1 like	-0.23	-0.07	0.44	-0.17	0.68
XP_022313968	syntenin 1 like	0.02	0.33	-0.52	-0.32	-0.54
G9G141	TCTP	-0.22	-0.04	0.39	-0.19	0.61
XP_022301260	transforming growth factor beta induced protein ig h3 like	0.30	0.23	-0.79	0.06	-1.09
XP_022326764	tripartite motif containing protein 2 like	0.33	-0.15	-0.28	0.48	-0.62
XP_021374147	tripartite motif containing protein 45 like isoform X1	0.39	-0.35	-0.05	0.74	-0.44
XP_022328361	ubiquitin carboxyl terminal hydrolase like	-0.39	0.41	-0.03	-0.80	0.36
A0A3L5TT67	Uncharacterized protein Fragment	0.35	-0.34	-0.02	0.68	-0.36
A0A3L5TRS7	Uncharacterized protein Fragment	-0.54	0.03	0.76	-0.56	1.30
XP_021342083	uncharacterized protein LOC110442690	-0.36	0.06	0.44	-0.42	0.80
XP_021345049	uncharacterized protein LOC110445015 partial	-0.06	0.27	-0.32	-0.33	-0.26
XP_021358863	uncharacterized protein LOC110453939	-0.55	0.45	0.15	-1.00	0.71
XP_021358863.2	uncharacterized protein LOC110453939	0.29	-0.32	0.05	0.61	-0.24
XP_021364413.1	uncharacterized protein LOC110457451 isoform X2	0.09	-0.34	0.37	0.42	0.29
XP_021372384	uncharacterized protein LOC110462645	-0.25	-0.05	0.45	-0.20	0.70
XP_022315033	uncharacterized protein LOC111119297 isoform X3	0.36	-0.15	-0.32	0.51	-0.69
XP_022330117	uncharacterized protein LOC111128660 isoform X1	-0.32	0.02	0.46	-0.34	0.78
XP_022337955	uncharacterized protein LOC111133666 isoform X21	-0.39	0.33	0.09	-0.73	0.48

A0A0L8FU30	Uncharacterized protein	-0.36	0.34	0.03	-0.71	0.40
XP_021378408	vesicular integral membrane protein VIP36 like	-0.14	0.34	-0.30	-0.47	-0.16
XP_022328041	X14 kDa phosphohistidine phosphatase like	0.37	-0.13	-0.36	0.50	-0.72
XP_021365147	X26S proteasome non ATPase regulatory subunit 13 like	0.11	-0.37	0.39	0.49	0.27
XP_022344862	X40S ribosomal protein S10 like	-0.08	-0.24	0.49	0.16	0.57
XP_021370373	X40S ribosomal protein S25 like	-0.55	0.30	0.37	-0.84	0.92
XP_021379854	X60S ribosomal protein L10a 2 like	0.31	-0.44	0.19	0.75	-0.12
XP_021340663	X60S ribosomal protein L15 like	-0.15	0.40	-0.37	-0.55	-0.22
A0A077GZK3	X60S ribosomal protein L21 Fragment	0.46	-0.22	-0.36	0.68	-0.82
A0A3G1CJM6	X60S ribosomal protein L30 Fragment	0.38	-0.13	-0.37	0.50	-0.74

Supplementary Table S12. Blast results of the *Crassostrea gigas* Uniprot and Ensembl homologous sequences. obtained with blastp function on the Blast2GO basic version. Below are represented 3 tables with the differently expressed proteins from exposition to nocuolin A found for the 5 μ M concentration, the 10 μ M concentration, and common to both concentrations.

Reference Sequence	UniProt			Ensembl	
	Accession number	Annotation	e-Value	Accession number	e-Value
XP_022295017	A0A8W8N441	W2 domain-containing protein	0	G5223	0
XP_021375560	K1R1C6	Calpain-5	0	K1R1C6	0
XP_021374413	-	-	-	-	-
XP_021369794	K1QLZ1	Actin-related protein 3	0	K1QLZ1	0
XP_015927503	A0A8W8LFU1	ADP-ribosylation factor-like protein 3	2.70E-92	G27795	4.96E-92
XP_021353098	K1QAB1	AP-2 complex subunit alpha	0	K1QAB1	0
E1UJ66	A0A8W8JHD6	C1q domain-containing protein	3.72E-26	G18610	6.85E-26
XP_021362141	-	-	-	-	-
NA.19	-	-	-	-	-
XP_022329591	A0A8W8I9Z0	NAD(P)H oxidase (H2O2-forming)	0	G13129	0
AMK01816	A0A8W8KB31	Eukaryotic translation initiation factor 4 gamma 1	0	G22753	0
XP_021368420	A0A8W8INP3	Eukaryotic translation initiation factor 3 subunit A	0	G14873	0
XP_022314527	A0A8W8HZ31	Eukaryotic translation initiation factor 3 subunit B	0	G1175	0
XP_022296622	A0A8W8LWI1	glutathione transferase	6.66E-146	G30437	1.23E-145
XP_021375144	-	-	-	-	-
Q6Q5Y5	A0A8W8J8N3	glutathione transferase	6.78E-97	G17288	1.25E-96
XP_021348928	A0A8W8IE27	RRM domain-containing protein	0	G1373	0
XP_021379021	A0A8W8J2R1	SCP2 domain-containing protein	0	G1648	0
XP_009058775	A0A8W8MPX4	alpha-L-fucosidase	0	G34791	0
XP_009063021	A0A8W8M9C6	Arp2/3 complex 34 kDa subunit	6.55E-180	G31680	1.20E-179
XP_022300367	A0A8W8LTT6	Inter-alpha-trypsin inhibitor heavy chain H3	0	G29677	0
XP_021345331	K1PQD4	Phosphoglucomutase-1	0	K1PQD4	0
OPL33132	K1QMT9	Uridylate-specific endoribonuclease	1.63E-162	K1QMT9	3.00E-162
XP_011434053	K1QUE6	26S proteasome non-ATPase regulatory subunit 6	0	G30766	0
XP_011430094	K1RAL0	Aspartate aminotransferase, cytoplasmic	0	K1RAL0	0
XP_005089259	-	-	-	-	-
XP_011439173	-	-	-	-	-
XP_011427688	K1RB91	Neutral alpha-glucosidase AB	0	K1RB91	0
XP_011426298	A0A8W8LQQ9	PARP-type domain-containing protein	0	G11388	0
XP_014788958	A0A8W8MZX0	Transmembrane 9 superfamily member	0	G375	0

XP_021375922	A0A8W8L6L6	PPM-type phosphatase domain-containing protein	0.00	G26695	0
XP_021379330	A0A8W8KC84	Proteasome subunit beta	1.08E-165	G22577	1.99E-165
A0A126Q9D7	A0A8W8LI13	RNA helicase	0	G28120	0
XP_021362448	A0A8W8LM98	Adenosylhomocysteinase	0	G28681	0
XP_022319118	K1RD83	Serine hydroxymethyltransferase	0	K1RD83	0
XP_021370764	-	-	-	-	-
XP_022324431	A0A8W8KZC5	tryptophan-tRNA ligase	0	G25911	0
A0A0B6ZPB7	A0A8W8IAH1	Ubiquitin-like domain-containing protein	6.06E-58	G13130	1.12E-57
XP_020608397	-	-	-	-	-
XP_022322557	A0A8W8LAN9	Phosphonoacetaldehyde hydrolase	0	G27152	0
XP_021342081	A0A8W8M820	Trichohyalin-plectin-homology domain-containing protein	0	G31463	0
XP_021355139	-	-	-	-	-
NA.22	-	-	-	-	-
XP_021371563	-	-	-	-	-
K1QHM2	K1QHM2	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	0	K1QHM2	0
XP_021346087	-	-	-	-	-
XP_021372850	-	-	-	-	-
XP_022307042	A0A8W8MBV9	Formimidoyltransferase-cyclodeaminase	0	G32268	0
XP_022289863	A0A8W8IR96	Fumarylacetoacetase-like C-terminal domain-containing protein	0	G15309	0
XP_009051647	K1R7T2	Isocitrate dehydrogenase	0	K1R7T2	0
XP_022332848	K1RQ81	Uncharacterized protein C15orf26-like protein	0	G11651	0
XP_022340655	K1Q4H2	Nodal modulator 3	0	K1Q4H2	0
XP_021352601	-	-	-	-	-
XP_021341001	-	-	-	-	-
XP_022319633	A0A8W8LQU1	PARP-type domain-containing protein	2.20E-172	G2925	4.04E-172
XP_011440625	-	-	-	-	-
XP_011416491	-	-	-	-	-
XP_011450015.1	-	-	-	-	-
XP_011444269	K1Q5P7	Peptidyl-prolyl cis-trans isomerase	5.12E-161	K1Q5P7	9.42E-161
XP_011421092	A0A8W8NBG2	Prenylcysteine lyase domain-containing protein	0	G5104	0
XP_011444861	A0A8W8NHD7	MIR domain-containing protein	1.11E-172	G5820	2.04E-172
XP_011430749	-	-	-	-	-
XP_021373328	-	-	-	-	-
A0A210QN06	A0A8W8JYP9	Proteasome subunit alpha type	3.53E-162	G21626	6.49E-162
XP_021358954	A0A8W8HRH0	Ras GTPase-activating-like protein IQGAP1	0	G10733	0
XP_021360996	-	-	-	-	-
XP_022315645	K1RGC9	Sodium/calcium exchanger 3	0	K1RGC9	0

XP_021377365	A0A8W8NK63	Sulfotransferase domain-containing protein	7.91E-82	G6132	1.46E-81
G9G141	A0A8W8IH07	TCTP domain-containing protein	4.37E-47	G14250	8.04E-47
XP_022326764	A0A8W8KQJ8	Tripartite motif-containing protein 2	0	G24892	0
XP_021342083	A0A8W8L0E1	Profilin	4.87E-62	G26073	8.96E-62
XP_021358863	-	-	-	-	-
XP_021372384	A0A8W8KGG3	DUF1330 domain-containing protein	3.71E-42	G23787	6.83E-42
XP_022315033	A0A8W8J0P9	EF-hand domain-containing protein	0	G1675	0
XP_022330117	K1QR22	Ankyrin repeat domain-containing protein 50	0	G13762	0
XP_022328041	A0A8W8LAZ5	Sex-regulated protein janus-A	1.72E-93	G27443	3.16E-93
XP_021370373	A0A8W8LCY1	40S ribosomal protein S25	2.71E-42	G26985	4.99E-42
A0A077GZK3	A0A8W8L8N9	60S ribosomal protein L21	1.16E-91	G26901	2.13E-91
A0A3G1CJM6	A0A8W8NG46	Large ribosomal subunit protein eL30	1.14E-74	G5652	2.10E-74
XP_021361418	-	-	-	-	-
XP_021359006	K1Q019	Beta-galactosidase	0	K1Q019	0
XP_021375560	K1R1C6	Calpain-5	0	K1R1C6	0
XP_022293175	K1R973	Chloride intracellular channel exc-4	0	G17791	0
XP_022341970	A0A8W8JYD4	60S ribosomal protein L13	1.77E-146	G21588	3.3E-146
XP_021350043	A0A8W8ID93	calcium/calmodulin-dependent protein kinase	0	G1363	0
NP_001191582	A0A8W8MFI8	Cyclic nucleotide-binding domain-containing protein	0	G3254	0
XP_021364896	-	-	-	-	-
XP_021363917	-	-	-	-	-
XP_021375144	-	-	-	-	-
Q6Q5Y5	A0A8W8J8N3	glutathione transferase	6.78E-97	G17288	1.2E-96
XP_021348928	A0A8W8IE27	RRM domain-containing protein	0	G1373	0
XP_021358952	-	-	-	-	-
OPL33119.1	K1QRQ0	60 kDa SS-A/Ro ribonucleoprotein	0	K1QRQ0	0
XP_009063021	A0A8W8M9C6	Arp2/3 complex 34 kDa subunit	6.55E-180	G31680	1.2E-179
XP_022300367	A0A8W8LTT6	Inter-alpha-trypsin inhibitor heavy chain H3	0	G29677	0
OPL33132	K1QMT9	Uridylate-specific endoribonuclease	1.63E-162	K1QMT9	3.0E-162
XP_013402087	G21588. G15236. G34615. G26901	KOW domain-containing protein	2.78E-92	G15236	5.1E-92
XP_011444095	-	-	-	-	-
XP_019930280	-	-	-	-	-
XP_011436159	A0A8W8KPG9	Mitogen-activated protein kinase	0	G24024	0
XP_011413908	A0A8W8I486	K Homology domain-containing protein	0	G12312	0
XP_014788958	A0A8W8MZX0	Transmembrane 9 superfamily member	0	G375	0
XP_022341530	K1QQP1	Programmed cell death protein 4	0	G15222	0

XP_021341989	A0A8W8NGW2	Ras-related GTP-binding protein C	0	G5238	0
G3GAE5	A0A8W8JB64	SHSP domain-containing protein	4.47E-33	G17984	8.2E-33
XP_022304940	A0A8W8M508	Splicing factor 3B subunit 1 domain-containing protein	0	G31757	0
XP_022320073	A0A8W8KPG7	Mitochondria-eating protein C-terminal domain-containing protein	0	G24376	0
XP_022322557	A0A8W8LAN9	Phosphonoacetaldehyde hydrolase	0	G27152	0
A0A2T7NPT5	K1Q122	Myosin regulatory light chain sqh	8.22E-105	G153	1.5E-104
XP_022341081	A0A8W8NCS6	Collagen alpha-2(I) chain	0	G5252	0
XP_022323534	A0A8W8KPZ6	Fibrillar collagen NC1 domain-containing protein	0	G24458	0
XP_021355139	-	-	-	-	-
NA.22	-	-	-	-	-
XP_021361172	-	-	-	-	-
K1QHM2	K1QHM2	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	0	K1QHM2	0
XP_022327361	A0A8W8KVI2	Thioredoxin domain-containing protein	0	G25203	0
XP_021372964	A0A8W8IQJ1	DJ-1/Pfpl domain-containing protein	4.80E-116	G15139	8.80E-116
XP_022345477	A0A8W8IZD7	F-box domain-containing protein	4.57E-144	G16562	8.40E-144
XP_022328214	A0A8W8LI81	Heat shock 70 kDa protein 12B	0	G27617	0
OPL20952	A0A8W8IEQ9	NAD(P)-binding protein	1.18E-72	G13604	2.20E-72
XP_009052416	A0A8W8JUK3	Transglutaminase-like domain-containing protein	2.47E-167	G20569	4.50E-167
XP_022317804	A0A8W8NWQ5	Counting factor associated protein D	0	G78	0
XP_022340655	K1Q4H2	Nodal modulator 3	0	K1Q4H2	0
EKC41639	K1RD93	Myosin-XV	0	K1RD93	0
XP_021352601	-	-	-	-	-
XP_019929295	K1RXR0	Arrestin domain-containing protein 3	0	K1RXR0	0
XP_019922173	-	-	-	-	-
XP_019922009	-	-	-	-	-
XP_022293421.1	A0A8W8J5Q6	Proteasome subunit alpha type	0	G17351	0
A0A210QN06	A0A8W8JYP9	Proteasome subunit alpha type	3.53E-162	G21626	6.5E-162
A0A3G1CJM1	K1QBT2	Proteasome subunit beta	5.42E-120	K1QBT2	1.0E-119
OWF37582	A0A8W8IF30	Protein transport protein SEC23	0	G13615	0
F0V478	A0A8W8KP68	C1q domain-containing protein	1.32E-71	G24732	2.4E-71
XP_022335184	A0A8W8HY81	RNA-binding protein 4	2.54E-158	G11518	4.7E-158
AKQ70853	A0A8W8LGH4	Laminin G domain-containing protein	3.28E-27	G27866	6.0E-27
XP_022293803	A0A8W8N2K5	Succinate dehydrogenase	0	G5034	0
XP_021374147	A0A8W8MB56	Tripartite motif-containing protein 45	0	G32153	0
XP_022328361	K1PX47	Ubiquitin carboxyl-terminal hydrolase	1.40E-15	G27716	2.6E-15
A0A3L5TT67	A0A8W8L019	Heat shock 70 kDa protein 12B	0	G25778	0
XP_021358863	-	-	-	-	-

XP_021358863.2	-	-	-	-	-
XP_022337955	A0A8W8KGG9	DUF1330 domain-containing protein	0	G23788	0
A0A0L8FU30	K1QBM3	Ras-related protein Rab-2	1.03E-142	G21850	1.90E-142
XP_021370373	A0A8W8LCY1	40S ribosomal protein S25	2.71E-42	G26985	5.00E-42
A0A077GZK3	A0A8W8L8N9	60S ribosomal protein L21	1.16E-91	G26901	2.10E-91
XP_021375560	K1R1C6	Calpain-5	0	K1R1C6	0
XP_021375144	-	-	-	-	-
Q6Q5Y5	A0A8W8J8N3	glutathione transferase	6.78E-97	G17288	1.25E-96
XP_021348928	A0A8W8IE27	RRM domain-containing protein	0	G1373	0
XP_009063021	A0A8W8M9C6	Arp2/3 complex 34 kDa subunit	6.55E-180	G31680	1.20E-179
XP_022300367	A0A8W8LTT6	Inter-alpha-trypsin inhibitor heavy chain H3	0	G29677	0
OPL33132	K1QMT9	Uridylate-specific endoribonuclease	1.63E-162	K1QMT9	2.99E-162
XP_014788958	A0A8W8MZX0	Transmembrane 9 superfamily member	0	G375	0
XP_022322557	A0A8W8LAN9	Phosphonoacetaldehyde hydrolase	0	G27152	0
XP_021355139	-	-	-	-	-
NA.22	-	-	-	-	-
K1QHM2	K1QHM2	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	0	K1QHM2	0
XP_022340655	K1Q4H2	Nodal modulator 3	0	K1Q4H2	0
XP_021352601	-	-	-	-	-
A0A210QN06	A0A8W8JYP9	Proteasome subunit alpha type	3.52E-162	G21626	6.49E-162
XP_021358863	-	-	-	-	-
XP_021370373	A0A8W8LCY1	40S ribosomal protein S25	2.71E-42	G26985	4.99E-42
A0A077GZK3	A0A8W8L8N9	60S ribosomal protein L21	1.157E-91	G26901	2.13E-91

Supplementary Table S13. Functional analysis and enrichment of differentially expressed proteins identified in mussel plantigrades exposed to nocoulin A at 5 and 10 μM , employing the g:GOST function with multi-query in g:Profiler. GO categories represented include Molecular Functions (MF), Biological Processes (BP), and Cellular Components (CC). Statistically different GO terms ($p < 0.05$).

Category	GO Name	GO ID	Adjusted p -value	
			5 μM	10 μM
GO:MF	structural molecule activity	GO:0005198	0.17	0.01
GO:MF	RNA binding	GO:0003723	0.11	0.01
GO:MF	extracellular matrix structural constituent	GO:0005201	0.14	0.02
GO:MF	structural constituent of ribosome	GO:0003735	0.26	0.04
GO:BP	protein metabolic process	GO:0019538	0.08	0
GO:BP	organonitrogen compound metabolic process	GO:1901564	0.07	0.01
GO:CC	protein-containing complex	GO:0032991	0.00	0
GO:CC	ribonucleoprotein complex	GO:1990904	0.01	0
GO:CC	proteasome core complex	GO:0005839	0.01	0
GO:CC	eukaryotic 43S preinitiation complex	GO:0016282	0.01	1
GO:CC	translation preinitiation complex	GO:0070993	0.01	1
GO:CC	eukaryotic 48S preinitiation complex	GO:0033290	0.01	1
GO:CC	proteasome complex	GO:0000502	0.01	0.02
GO:CC	proteasome core complex, alpha-subunit complex	GO:0019773	0.06	0.01
GO:CC	ribosome	GO:0005840	0.05	0.01
GO:CC	endopeptidase complex	GO:1905369	0.01	0.02
GO:CC	vesicle coat	GO:0030120	0.01	0.14
GO:CC	peptidase complex	GO:1905368	0.01	0.02
GO:CC	eukaryotic translation initiation factor 3 complex	GO:0005852	0.01	1
GO:CC	actin cytoskeleton	GO:0015629	0.20	0.016
GO:CC	coated vesicle membrane	GO:0030662	0.02	0.16
GO:CC	cytoplasm	GO:0005737	0.02	0.02
GO:CC	coated vesicle	GO:0030135	0.02	0.17
GO:CC	intracellular anatomical structure	GO:0005622	0.05	0.02
GO:CC	organelle	GO:0043226	0.06	0.02
GO:CC	catalytic complex	GO:1902494	0.09	0.02
GO:CC	large ribosomal subunit	GO:0015934	0.10	0.02
GO:CC	membrane coat	GO:0030117	0.02	0.18
GO:CC	coated membrane	GO:0048475	0.02	0.18
GO:CC	endocytic vesicle membrane	GO:0030666	0.03	1
GO:CC	AP-2 adaptor complex	GO:0030122	0.03	1
GO:CC	clathrin coat of endocytic vesicle	GO:0030128	0.03	1
GO:CC	clathrin-coated endocytic vesicle	GO:0045334	0.03	1
GO:CC	clathrin-coated endocytic vesicle membrane	GO:0030669	0.03	1
GO:CC	mitochondrial inner membrane	GO:0005743	1	0.03
GO:CC	organelle inner membrane	GO:0019866	1	0.04
GO:CC	intracellular organelle	GO:0043229	0.06	0.04
GO:CC	myofilament	GO:0036379	1	0.04
GO:CC	acetyl-CoA carboxylase complex	GO:0009317	1	0.04
GO:CC	striated muscle thin filament	GO:0005865	1	0.04
GO:CC	troponin complex	GO:0005861	1	0.04
GO:CC	clathrin coat of coated pit	GO:0030132	0.05	1
GO:CC	COPI-coated vesicle	GO:0030137	0.05	1
GO:CC	endocytic vesicle	GO:0030139	0.05	1
GO:CC	Arp2/3 protein complex	GO:0005885	0.05	0.06
GO:CC	oligosaccharyltransferase complex	GO:0008250	0.05	0.06
GO:CC	COPI-coated vesicle membrane	GO:0030663	0.05	1
GO:CC	COPI vesicle coat	GO:0030126	0.05	1

Supplementary Material of Chapter 6

Supplementary Table S14. Environmental seawater parameters at the study site from September 2021 to January 2023.

Date	Temp (°C)	Sal (psu)	DO (mg L ⁻¹)	pH	Depth (m)
09/09/2021	20.4	35.6	5.10	7.41	1.4
16/09/2021	21.1	37.3	5.46	7.63	1.3
23/09/2021	14.3	37.9	4.64	7.46	1.1
01/10/2021	14.6	38.2	4.66	7.57	1.3
03/11/2021	14.6	36.0	6.11	8.08	1.2
17/12/2021	13.6	35.0	7.24	7.88	1.2
17/02/2022	14.4	36.0	8.58	8.18	1.2
21/04/2022	13.4	28.8	25.08	8.08	1.2
27/06/2022*	16.3	29.1	19.86	7.86	1.2
05/01/2023*	15.1	24.1	32.19	7.67	1.2

* time points correspondent to the collected biofilm samples analysed by amplicon sequencing (16S and 18S).

Supplementary Table S15. Summary of the collected samples and DNA quantification parameters.

Sample description	Sample ID	Sampling Date	Biomass of sequenced sample (g)	DNA concentration (ng/μl)	DNA of sequenced sample (ng)
Polyurethane-base coating (Control)	PU_C_1	Jun-22	0.094	22.9	229
	PU_C_2	Jun-22	0.107	29.1	291
	PU_C_3	Jun-22	0.228	56	560
	PU_C_4	Jun-22	0.162	57	570
Polyurethane-base coating with ECONEA®	PU_E_1	Jun-22	0.126	14.7	294
	PU_E_2	Jun-22	0.189	59	590
	PU_E_3	Jun-22	0.188	55	550
	PU_E_4	Jun-22	0.131	41.5	415
Polyurethane-base coating with Pam 0.7%	PU_Pam_1	Jun-22	0.203	25.2	504
	PU_Pam_2	Jun-22	0.143	42.6	426
	PU_Pam_3	Jun-22	0.172	55	550
	PU_Pam_4	Jun-22	0.152	43.8	438
PDMS-base coating ECONEA®	PDMS_E_1	Jan-23	0.093	38.3	383
	PDMS_E_2	Jan-23	0.097	53	530
	PDMS_E_3	Jan-23	0.13	52	520
PDMS-base coating with Pam 0.7%	PDMS_Pam_1	Jan-23	0.297	8.3	249
	PDMS_Pam_2	Jan-23	0.041	5.3	212
	PDMS_Pam_3	Jan-23	0.18	8.67	260.1

Supplementary Table S16. Summary of sequencing processing in collected samples.

Target Gene	SampleID	Input	Filtered	Denoised	Merged	Non chimera
16S rRNA	PU_C_1	66608	59653	53259	31730	23096
	PU_C_2	62587	56691	50497	27988	21003
	PU_C_3	63013	57223	50290	26645	19620
	PU_C_4	70452	63759	56813	33057	24640
	PU_E_1	63150	56949	50619	29852	21188
	PU_E_2	62156	56504	49884	26804	20196
	PU_E_3	65792	59733	54555	39053	30520
	PU_E_4	73668	66392	62071	48202	37604
	PU_Pam_1	62580	56281	51300	29529	21578
	PU_Pam_2	65984	59028	53287	29796	22512
	PU_Pam_3	64636	57909	51854	25395	19592
	PU_Pam_4	65877	58817	54158	24466	18408
	PDMS_E_1	62782	57190	50002	26209	20988
	PDMS_E_2	66130	60369	52497	28364	22793
	PDMS_E_3	64153	57826	49762	23482	18265
	PDMS_Pam_1	62662	57380	50383	25142	19076
	PDMS_Pam_2	65429	59205	52247	28054	20738
	PDMS_Pam_3	67788	61451	54251	27976	19667
18S rRNA	PU_C_1	64884	58637	57823	53971	46464
	PU_C_2	62143	34162	33664	31783	28699
	PU_C_3	66591	58956	58046	53053	42846
	PU_C_4	74388	66598	65723	61329	50883
	PU_E_1	65908	54649	54032	50889	46676
	PU_E_2	66348	52909	52090	48139	42235
	PU_E_3	71990	64075	63380	60434	53264
	PU_E_4	74044	64615	63814	60397	51715

PU_Pam_1	63538	52645	52045	48539	43147
PU_Pam_2	74144	63353	62642	59767	48540
PU_Pam_3	65060	58645	58179	56511	51368
PU_Pam_4	64781	57990	57721	56750	54013
PDMS_E_1	79273	69269	68215	61100	53989
PDMS_E_2	64031	57934	56771	51827	44263
PDMS_E_3	64380	57299	55962	48114	42853
PDMS_Pam_1	63168	57453	56284	28326	25193
PDMS_Pam_2	64762	52798	51804	48997	45962
PDMS_Pam_3	62990	56018	54752	34411	31176

Supplementary Table S17. Predicted environmental concentrations (maximum, median and minimum) obtained through the Marine Antifoulant Model to Predict Environmental Concentrations (MAMPEC; Version 3.0; Deltares, Netherlands), and putative Risk Assessment based on the maximum and minimum MAMPEC-predicted environmental concentrations (in mg.L⁻¹) in an OECD marina.

Environment / Emission scenario	PEC mg L ⁻¹ (Harbour)			PEC mg L ⁻¹ (Surroundings)		
	Max	Median	Min	Max	Median	Min
OECD-EU Commercial harbour	8.56E ⁻⁰⁵	4.72E ⁻⁰⁵	5.55E ⁻⁰⁶	6.61E ⁻⁰⁶	1.00E ⁻⁰⁶	1.11E ⁻¹⁵
OECD-EU Marina	6.44E ⁻⁰⁴	4.88E ⁻⁰⁴	7.37E ⁻⁰⁵	2.38E ⁻⁰⁵	7.33E ⁻⁰⁷	8.94E ⁻¹⁴
OECD-EU Shipping Lane	2.23E ⁻⁰⁷	0.00E ⁺⁰⁰	0.00E ⁺⁰⁰	-	-	-

Endpoint used for PNEC estimation (mg.L ⁻¹)	PEC OECD marina (mg L ⁻¹)		Risk Characterization*	
	Max	Min	PEC _{MAX} /PNEC	PEC _{MIN} /PNEC
6.254	6.44E-04	7.37E-05	1.03	1.17E-02

*worst-case scenario (PEC_{MAX} and PEC_{MIN}), divided by the PNEC, to which an assessment factor of 10000 (overprotective) was applied.

Supplementary Table S18. Summary of persistence, bioaccumulation, and toxicity (PBT) criteria, regulatory status, key biological effects, and environmental risk characterization of conventional and natural AF agents.

Compound	Regulatory status (BPR)	Key effects	Toxicity			Persistence	Bioaccumulation	Risk Characterization	Key references
			Crustaceans	Mussels	Zebrafish				
Tributyltin (TBT)	Banned for AF usage (IMO AFS convention)	Endocrine disruption, imposex, reproductive toxicity, gastropod local populations extinction	effects on the $\text{ng}\cdot\text{L}^{-1}$ range	effects on the $\text{ng}\cdot\text{L}^{-1}$ range	effects on the $\text{ng}\cdot\text{L}^{-1}$ range	Months-years in sediments	Yes, described for several species	Very High (historical) PEC/PNEC > 300	(Antizar-Ladislao, 2008; Oliveira <i>et al.</i> , 2014; A. C. A. Sousa <i>et al.</i> , 2014)
Copper (dissolved Cu^{2+})	Allowed with mitigation	Oxidative stress, immunotoxicity, lysosomal damage in bivalves	$\text{LC}_{50} \text{ A. salina}_{48h} = 0.14\text{-}0.33 \text{ mg}\cdot\text{L}^{-1}$	D-veliger larvae development $\text{EC}_{50} \text{ M. galloprovincialis}_{48h} = 16 \text{ }\mu\text{g}\cdot\text{L}^{-1}$	ZFET $\text{LC}_{50} = 0.174 \text{ mg}\cdot\text{L}^{-1}$	Binds to sediments; persistent near sources	Yes in bivalves	Moderate-High in hotspots	(Dafforn <i>et al.</i> , 2011; Hallare <i>et al.</i> , 2005; His <i>et al.</i> , 1999; Nunes <i>et al.</i> , 2006; K. V. Thomas & Brooks, 2010)
Tralopyril (ECONEA®)	Approved active substance	uncoupler of ATP production in mitochondria, oxidative stress, inhibit energy metabolism, alter gene expression, endocrine	$\text{LC}_{50} \text{ T. battagliai}_{48h} = 0.9 \text{ }\mu\text{g}\cdot\text{L}^{-1}$	D-veliger larvae development $\text{EC}_{50} \text{ M. galloprovincialis}_{48h} = 3.1 \text{ }\mu\text{g}\cdot\text{L}^{-1}$	ZFET $\text{LC}_{50} = 0.005 \text{ mg}\cdot\text{L}^{-1}$	Low persistence Half-life of ~6h in seawater	Potentially high ECOSAR log Kow > 4.7	High PEC/PNEC > 4	(X. Chen <i>et al.</i> , 2021; B. Liu <i>et al.</i> , 2024; B. Liu, Li, He, Xing, Cao, <i>et al.</i> , 2022; B. Liu, Li, He, Xing, Chen, <i>et al.</i> , 2022;

		disruption, interfere with biomineralization in oysters							Oliveira <i>et al.</i> , 2014, 2017; Oliveira, Groh, <i>et al.</i> , 2016; Oliveira, Schönenberger, <i>et al.</i> , 2016; Xu Wang <i>et al.</i> , 2022)
Medetomidine	Approved (until 12/2026) with measures to minimize environmental impact during application and disposal	acts as a potent $\alpha 2$ -adrenoceptor (neuroreceptor) agonist, interferes with swimming behavior, disrupts settlement cues, endocrine disruption	LC ₅₀ <i>A. tonsa</i> _{24h} = 48 $\mu\text{g}\cdot\text{L}^{-1}$ prevent barnacle cyprid settlement from 0.02 to 2 $\mu\text{g}\cdot\text{L}^{-1}$ whilst larvae mortality is 10 ⁵ lower	EC ₅₀ <i>A. nitida</i> _{24h} = 0.9 $\mu\text{g}\cdot\text{L}^{-1}$ for sediments reworking EC ₅₀ <i>A. nitida</i> _{24h} = 86 $\mu\text{g}\cdot\text{L}^{-1}$ for burrowing response	considered toxic to fish in SDS** but no data available	Half-life < 1.5 days under sunlight; low persistence in illuminated surface waters	Rapid uptake & elimination (1-24h) for different marine organisms except mussel <i>A. nitida</i> that showed slower depuration rate (~120h)	Low PEC _{marine sediment} /PNEC < 1	(Bellas <i>et al.</i> , 2006; Cai, Apell, <i>et al.</i> , 2021; Dahlström <i>et al.</i> , 2000; Fox <i>et al.</i> , 2014; Hilvarsson <i>et al.</i> , 2009; Wendt <i>et al.</i> , 2016)
DCOIT (4,5-dichloro-2-n-octyl-4-isothiazolin-3-one) SeaNine211	Approved/widely used under regulatory scrutiny	endocrine disruption potential, hormonal imbalances; cardiotoxicity and transgenerational effects in marine organisms	LC ₅₀ <i>Artemia sp.</i> _{48h} ≈ 163 $\mu\text{g}\cdot\text{L}^{-1}$ <i>Balanus improvisus</i> settlement EC ₅₀ = 250 $\mu\text{g}\cdot\text{L}^{-1}$	D-veliger larvae development EC ₅₀ <i>P. perna</i> ≈ 8.3 - 12.4 $\mu\text{g}\cdot\text{L}^{-1}$ Larvae settlement (byssus thread production) EC ₅₀ <i>P. perna</i> = 96.1 $\mu\text{g}\cdot\text{L}^{-1}$	Data unavailable	Moderate persistence (days-weeks), influenced by light and microbial activity	Potentially high ECOSAR log Kow > 6.4	PEC/PNEC > 1 for areas with constant input such as marinas, ports, maritime traffic zones	(Lianguo Chen & Lam, 2017; B. G. de Campos <i>et al.</i> , 2022; Santos <i>et al.</i> , 2020)

Nocuolin A	Investigation	strong AF activity but reported ecotoxicity in recent study	$LC_{50} \text{ A. salina}_{48h} = 0.74 \text{ mg} \cdot \text{L}^{-1}$	Larvae settlement (byssus thread production) $EC_{50} \text{ M.galloprovincialis} = 1.165 \text{ mg} \cdot \text{L}^{-1}$	ZFET $LC_{50} = 0.0174 \text{ mg} \cdot \text{L}^{-1}$	In ECOSAR ultimate biodegradation: days-weeks and in aerobic conditions: days	Potentially high ECOSAR log Kow > 4.5	High PECMAX/PNEC 17.72	(S. Pereira <i>et al.</i> , 2024)
Capsaicin	Investigation	prevent biofilme formation and inhibits byssal attachment (reversible clean water) in zebra mussel	$LC_{50} \text{ T. batagla}_{48h} = 1.25 \text{ mg} \cdot \text{L}^{-1}$	D-veliger larvae development $EC_{50} \text{ M.galloprovincialis}_{48h} = 3.868 \text{ mg} \cdot \text{L}^{-1}$	ZFET $LC_{50} = 5.98 \text{ mg} \cdot \text{L}^{-1}$	Degradable; low persistence; unlikely to persist in the environment	Moderate potential (log Kow~3)	Low PEC/PNEC = 0.06	(Angarano <i>et al.</i> , 2007; Oliveira <i>et al.</i> , 2014, 2017; J. Wang <i>et al.</i> , 2014)
Synoxazolidinone A e C	Investigation	Derived from coral-associated bacterium <i>Acinetobacter</i> sp.; potential non-toxic AF candidates. Anti-adhesion, antibacterial, deterrent settlement	<i>Amphibalanus improvisus</i> settlement $EC_{50} \text{ Synoxazolidinone A} = 15 \text{ mg} \cdot \text{L}^{-1}$ $EC_{50} \text{ Synoxazolidinone C} = 2 \text{ mg} \cdot \text{L}^{-1}$	Data unavailable	Data unavailable	Data unavailable	Data unavailable	Data unavailable	(Trepas <i>et al.</i> , 2014)
Barettin	Investigation	Sponge metabolite with dual antioxidant and AF activity;	<i>Amphibalanus improvisus</i> settlement $EC_{50} = 0.44 \text{ mg} \cdot \text{L}^{-1}$	Data unavailable	Data unavailable	Data unavailable	Data unavailable	Data unavailable	(Lind <i>et al.</i> , 2013; Sjögren <i>et al.</i> , 2006)

		Anti-settlement, anti-biofilm							
Phidianidine A	Investigation	Isolated from marine gastropod <i>Phidiana militaris</i> ; promising low-toxicity AF candidate; potent, nontoxic inhibitor of barnacle cyprid metamorphosis; microfouler inhibitor; anti-inflammatory potential	<i>Amphibalanus improvisus</i> settlement $EC_{50} = 2.88 \text{ mg} \cdot \text{L}^{-1}$	Data unavailable	Data unavailable	due to its amphiphilic character, it is not expected to be highly persistent	Moderate potential (predicted log Kow < 3)	Data unavailable	(Labriere <i>et al.</i> , 2020; Q. Xu <i>et al.</i> , 2022)
Madeirone Neomarinone	Investigation	Secondary metabolite from <i>Streptomyces aculeolatus</i> ; AF, antibacterial, and quorum-sensing inhibitory activity; potentially acts through	Data unavailable	<i>M. galloprovincialis</i> larvae settlement (byssus thread production) $EC_{50} \text{ Madeirone} = 1.76 \text{ } \mu\text{g} \cdot \text{L}^{-1}$ $EC_{50} \text{ Neomarinone} = 0.12 \text{ } \mu\text{g} \cdot \text{L}^{-1}$ no mortality up to $10 \text{ } \mu\text{g} \cdot \text{L}^{-1}$	Data unavailable	Data unavailable	Predicted low bioaccumulation	Data unavailable	(Wissner <i>et al.</i> , 2024)

		inhibition of larval adhesion							
Napyradiomycins	Investigation	Metabolites isolated from <i>Streptomyces aculeolatus</i> ; strong AF and antibacterial activity; bioactive against marine micro and macrofoulers	LC ₅₀ <i>A. salina</i> 48h = 1.2–2.5 mg·L ⁻¹ <i>Amphibalanus improvisus</i> settlement EC ₅₀ ≈ 0.8–3.5 mg·L ⁻¹	Larvae settlement (byssus thread production) EC ₅₀ <i>M. galloprovincialis</i> < 12 mg·L ⁻¹	Data unavailable	Moderately stable under light; likely biodegradable under natural conditions	Moderate potential (predicted log Kow 3.5–4.5)	Low to moderate depending on local exposure	(F. Pereira <i>et al.</i> , 2020)
Adenosine 2'-deoxyadenosine	Investigation	Mussel settlement inhibition; Growth inhibition of <i>Navicula</i> sp.	No mortality against <i>A. salina</i> up to 12.56 mg·L ⁻¹ nor to <i>Amphibalanus amphitrite</i> cyprids up to 8 mg·L ⁻¹	Larvae settlement (byssus thread production) EC ₅₀ Adenosine = 1.77 mg·L ⁻¹ EC ₅₀ 2'-deoxyadenosine = 2.20 mg·L ⁻¹	Data unavailable	In ECOSAR ultimate biodegradation: weeks and in aerobic conditions: days-weeks	Low ECOSAR log Kow log Kow adenosine = -1.38 log Kow 2'-deoxyadenosine = -0.71	Data unavailable	(S. Pereira <i>et al.</i> , 2025)
Portoamides A and B (<i>Pam</i>)	Investigation	Mussel settlement inhibition (reversible in clean water);	No mortality against <i>A. salina</i> up to 24.4 mg·L ⁻¹	Larvae settlement (byssus thread production) EC ₅₀	ZFET LC ₅₀ = 6.25 mg·L ⁻¹ (this study)	under study	Low ECOSAR log Kow -5.488	Low/Moderate PEC/PNEC = 1.03 (this study)	(Antunes <i>et al.</i> , 2019; Leão <i>et al.</i> ,

		low acute toxicity in many assays		<i>M. galloprovincialis</i> 4.84 mg·L ⁻¹	≈					2010; Ribeiro <i>et al.</i> , 2017)

*Data were collected from peer-reviewed literature and regulatory reports. BPR: Biocidal Product Regulation (EU) 528/2012);

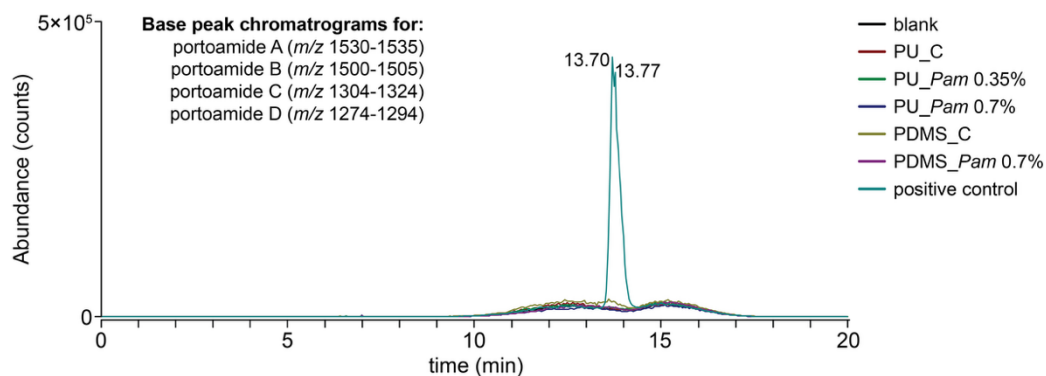
** Safety data sheet; NA: no data available

Supplementary Table S19. Morpho-taxonomical analyses of prototypes.

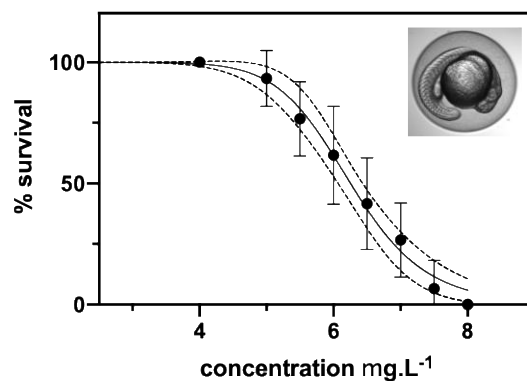
Sampling month	November	December	February	June	January
Condition	2 months immersion	3 months immersion	5 months immersion	9 months (Jun 2022)*	16 meses (Jan 23)*
PU_C	Presence of thin biofilm (diatoms, cyanobacteria); Low abundance of calcareous tubes of polychaetes (Annelida); erect hydroyzoans (Cnidaria)	Abundant Biofilm; Low abundance of calcareous tubes of polychaetes (Annelida); presence of few barnacles (Balanidae, Arthropoda); erect hydroyzoans (Cnidaria)	Abundant Biofilm; Low abundance of calcareous tubes of polychaetes (Annelida); presence of few barnacles; erect hydroyzoans (Cnidaria)	Abundant Biofilm; Low abundance of calcareous tubes of polychaetes (Annelida); presence of few barnacles (Balanidae, Arthropoda); erect hydroyzoans (Cnidaria)	Abundance of calcareous tubes of polychaetes (Annelida); presence of adult barnacles (Balanidae, Arthropoda); erect hydroyzoans (Cnidaria); mussels (Mytilidae, Mollusca); encrusting sponge (<i>Halichondria</i> sp., Demospongiae, Porifera)
PU_E	Presence of abundant biofilm (diatoms, cyanobacteria); Low abundance of calcareous tubes of polychaetes (Annelida); erect hydroyzoans (Cnidaria)	Abundant Biofilm; Low abundance of calcareous tubes of polychaetes (Annelida); presence of few barnacles (Balanidae, Arthropoda) and mussels (Mytilidae, Mollusca)	Abundant Biofilm; calcareous tubes of polychaetes (Annelida); presence of few barnacles (Balanidae, Arthropoda) and mussels (Mytilidae, Mollusca); erect hydroyzoans (Cnidaria); solitary ascidians (<i>Molgula</i> sp. and <i>Styela</i> sp., Tunicata, Urochordata)	Abundant Biofilm; green algae (Ulvoephyceae; Trebouxiophyceae); calcareous tubes of polychaetes (Annelida); presence of few barnacles (Balanidae, Arthropoda) and mussels (Mytilidae, Mollusca); erect hydroyzoans (Cnidaria); encrusting sponge (<i>Halichondria</i> sp., Demospongiae, Porifera); solitary (<i>Molgula</i> sp.; <i>Styela</i> sp.) and colonial ascidian (<i>Botrylloides</i> sp., Tunicata, Urochordata)	Abundance of mussels (Mytilidae, Mollusca); Presence of calcareous tubes of Cirratulidae polychaetes (Annelida); presence of adult barnacles (Balanidae, Arthropoda); erect hydroyzoans (Cnidaria); encrusting sponge (<i>Halichondria</i> sp., Demospongiae, Porifera); solitary ascidians (<i>Molgula</i> sp. and <i>Styela</i> sp., Tunicata, Urochordata)
PU_Pam	Low abundance of calcareous tubes of polychaetes (Annelida)	Low abundance of calcareous tubes of polychaetes (Annelida); presence of few barnacles (Balanidae, Arthropoda)	Thin biofilm; Low abundance of calcareous tubes of polychaetes (Annelida); presence of few barnacles (Balanidae, Arthropoda); colony of encrusting bryozoan <i>Watersipora subtorquata</i> ; colonial ascidian (<i>Botrylloides</i> sp., Tunicata, Urochordata)	Thin biofilm; Abundance of calcareous tubes of polychaetes (Annelida); presence of few barnacles (Balanidae, Arthropoda); colony of encrusting bryozoan <i>Watersipora subtorquata</i>	Abundance of calcareous tubes of polychaetes (Annelida); presence of adult barnacles (Balanidae, Arthropoda); mussels (Mytilidae, Mollusca); encrusting sponge (<i>Halichondria</i> sp., Demospongiae, Porifera)
PDMS_C	-	-	Presence of thin biofilm	Presence of thin biofilm	Thin Biofilm; Low abundance of calcareous tubes of polychaetes (Annelida); prevalence of encrusting sponge (<i>Halichondria</i> sp., Demospongiae, Porifera)
PDMS_E	-	-	Presence of thin biofilm	Presence of thin biofilm	Low abundance of thin calcareous tubes of polychaetes (Annelida)
PDMS_Pam	-	-	Presence of thin biofilm	Presence of thin biofilm	Thin Biofilm; Low abundance of thin calcareous tubes of polychaetes (Annelida)

Supplementary Table S20. β -diversity of the eukaryotic communities (18S) from the tested prototypes. Sample dissimilarity and distance analysis were calculated with the Bray-Curtis dissimilarity index. The table represents the summary of the PERMANOVA results using "coating" and "additive" as fixed factors, as well as the β -dispersion tests for each factor.

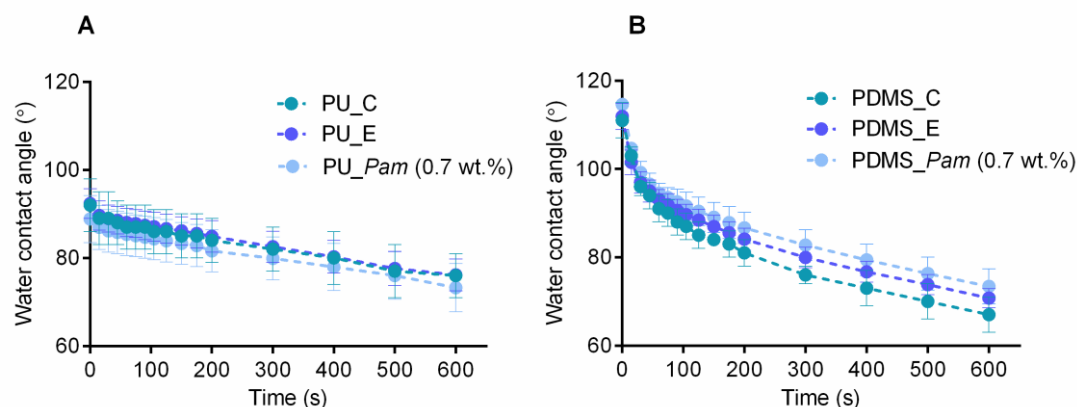
Factor	Df	R2	p-value
Coating (PU vs PDMS)	1	0.25	0.001
Additive	2	0.16	0.006
Coating:Additive	1	0.07	0.045
Residuals	13	0.51	-
Coating beta dispersion	1	-	0.454
Additive beta dispersion	2	-	0.020



Supplementary Figure S2. Extracted chromatogram for the m/z compatible with portoamides (*Pam*) obtained from the LC-MS analysis. Mass spectra obtained in the interval between 0 and 20 min for the 4 m/z areas where it is expected to find m/z values compatible with the 4 different *Pam*.

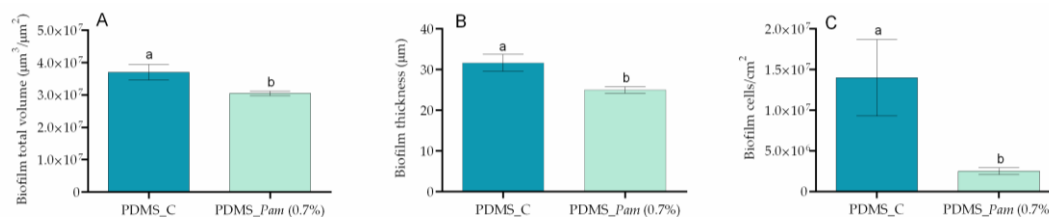


Supplementary Figure S3. Dose-response curve of *Danio rerio* embryos survival at 96-hour post fertilization (full line) and respective confidence intervals (intermittent line) following exposure to *Pam*.

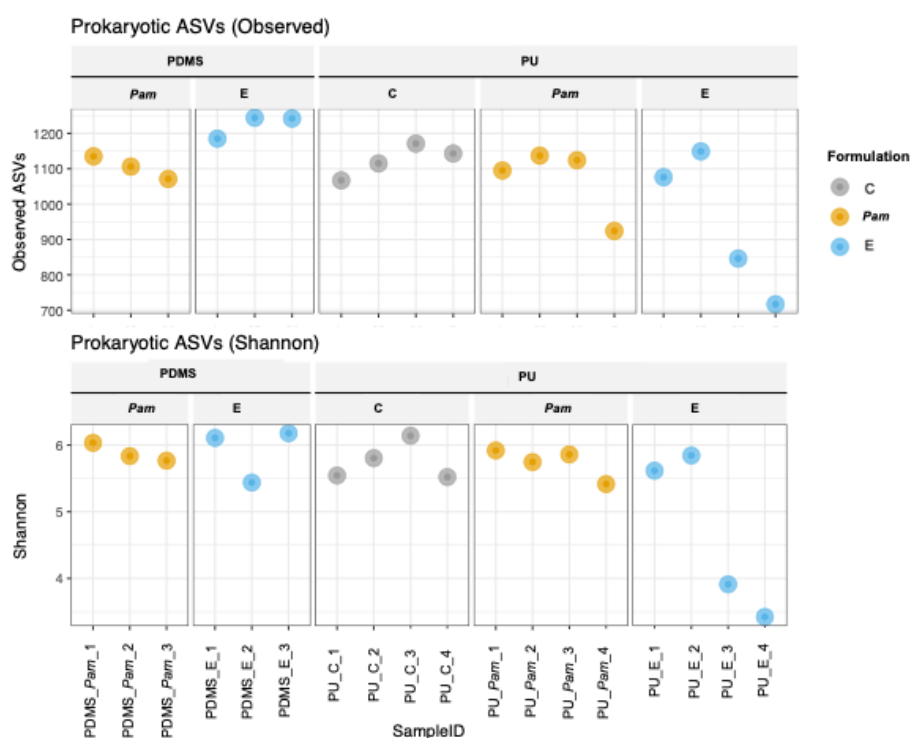


Supplementary Figure S4. Static contact angles of water on (A) PU- (PU_C, PU_E, PU_Pam (0.7 wt.%)) and (B) PDMS- (PDMS_C, PDMS_E, PDMS_Pam (0.7 wt.%)) based marine coatings as a function of time.

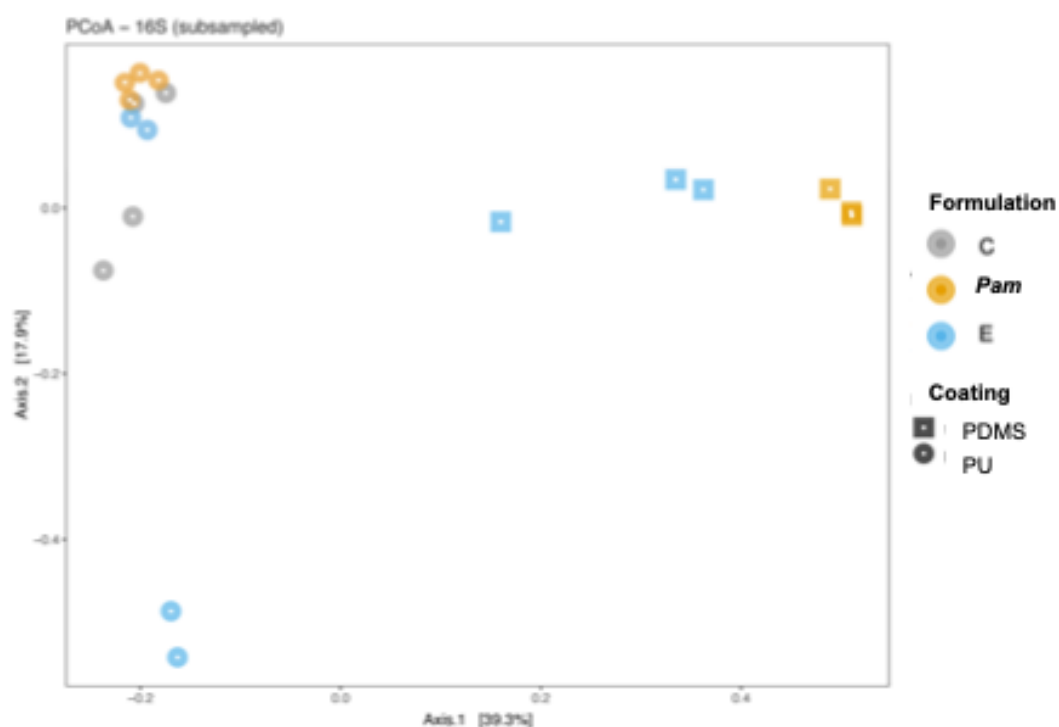
Supplementary Figure S4 shows that PU formulations exhibited relatively stable wettability over time compared to PDMS-based coatings. On PU surfaces (Supplementary Figure S2.A), contact angles decreased moderately from 92° at $t = 0$ s to 73° at $t = 600$ s, whereas PDMS surfaces declined more sharply from around 115° to 67°, reflecting their inherently higher hydrophobicity. The incorporation of Econe[®] (PU_E) and Pam (PU_Pam) did not significantly affect PU wettability, as all PU formulations showed similar contact angle values and temporal behavior ($p > 0.05$, Figure S1A). These findings are consistent with a previous study⁷, showing minimal influence of biocide addition (Irgarol or Econe[®]) on the wettability of PU-based coatings. In contrast, PDMS-based coatings exhibited more dynamic wettability behavior (Supplementary Figure S2.B). Over time, PDMS_Pam maintained higher contact angles than both PDMS_E and the control formulation (PDMS_C), with statistically significant differences at $t = 600$ s ($p < 0.05$), suggesting that Pam incorporation may enhance the hydrophobic character of PDMS coatings.



Supplementary Figure S5. Antifouling performance of *Pam*-functionalized coating formulation (PDMS_*Pam* 0.7 wt.%) during 14-days against *C. marina* biofilms. Biofilm quantitative parameters refer to biofilm total volume (A), biofilm thickness (B), and biofilm cells (C). Means and standard deviations (SD) are shown (letters show statistical differences at $p < 0.05$, *t*-test).

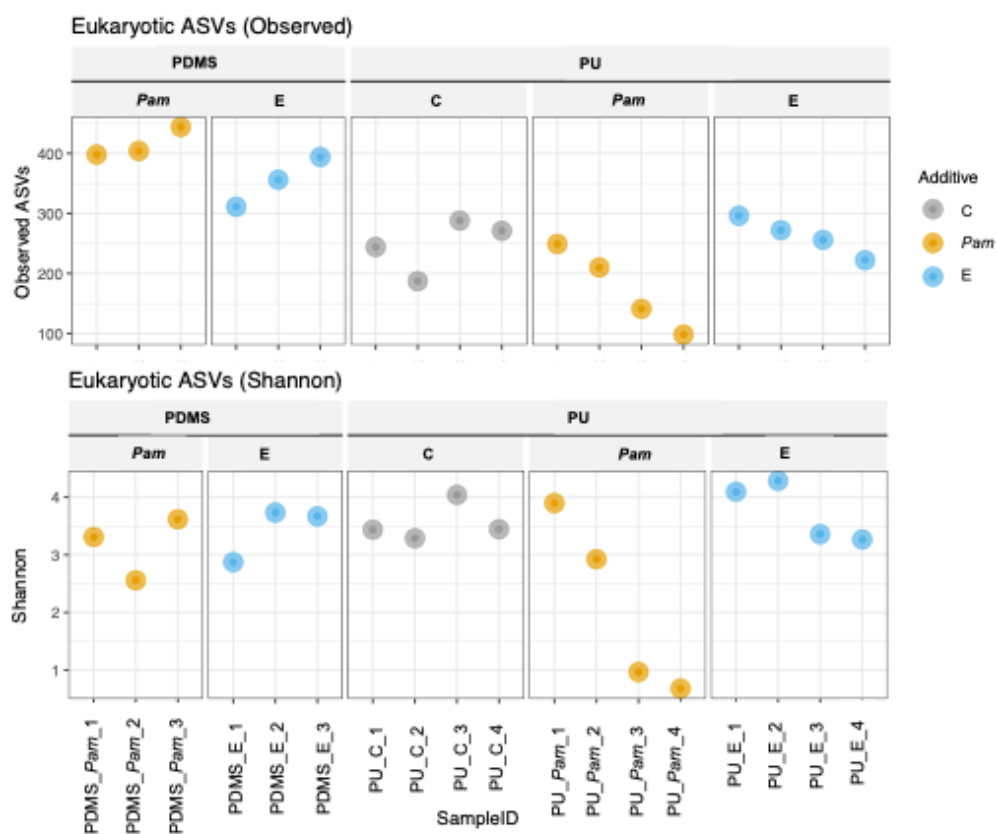


Supplementary Figure S6. Prokaryotic species richness (observed ASVs) and α -diversity (Shannon) from the tested prototypes estimated by 16S rRNA gene sequencing. Points represent individual biofilm samples collected by surface scratching.

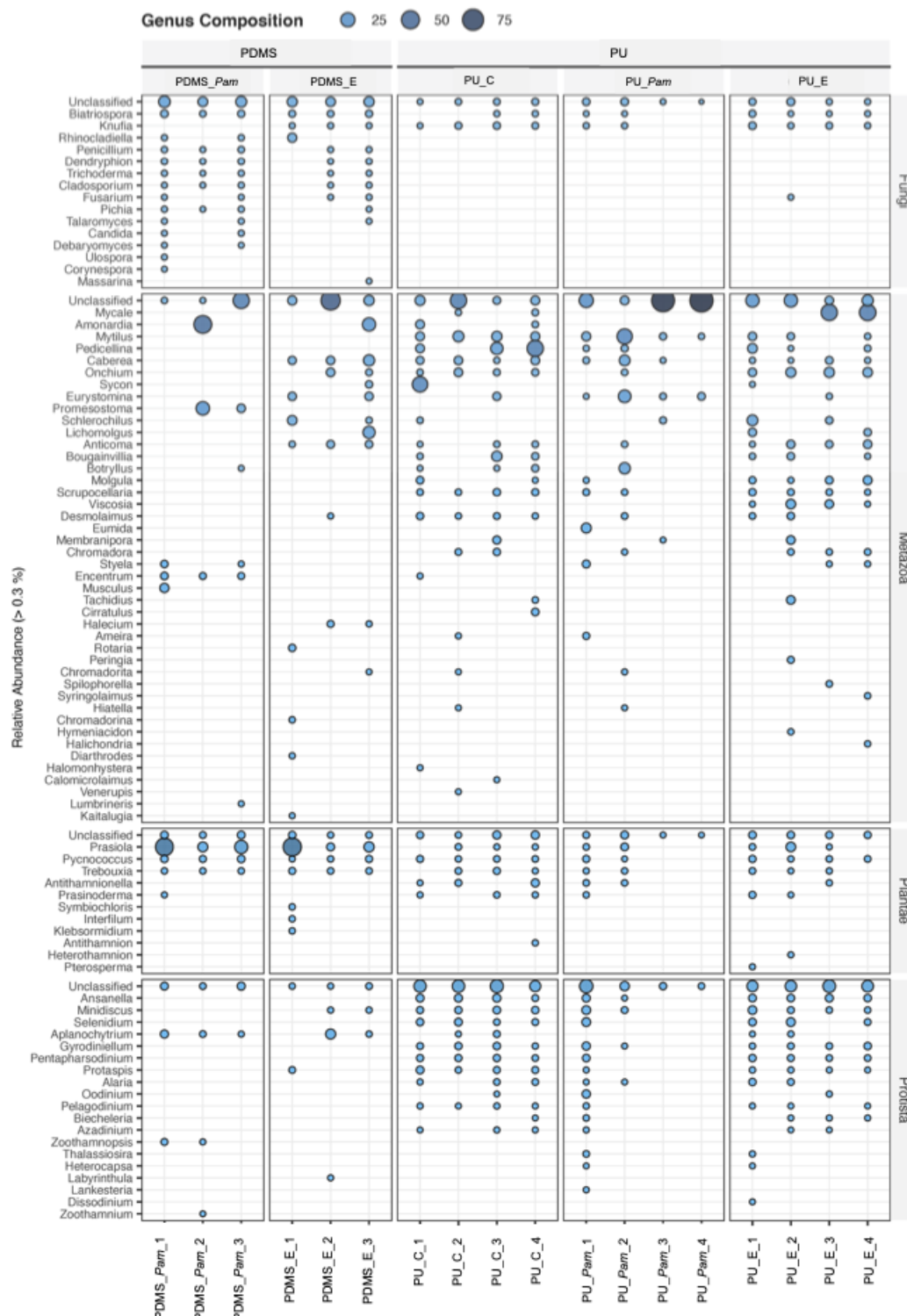


Factor	Df	R2	p-value
Coating (PU vs PDMS)	1	0.37	0.001
Additive	2	0.13	0.026
Coating:Additive	1	0.09	0.019
Residuals	13	0.40	-
Coating beta dispersion	1	-	0.778
Additive beta dispersion	2	-	0.008

Supplementary Figure S7. Principal coordinate analysis (PCoA) plot representing the β -diversity of the prokaryotic communities (16S) from the tested prototypes. Sample dissimilarity and distance analysis were calculated with the Bray-Curtis dissimilarity index. The table below represents the summary of the PERMANOVA results using “coating” and “additive” as fixed factors, as well as the β -dispersion tests for each factor.



Supplementary Figure S8. Eukaryotic species richness (observed ASVs) and α -diversity (Shannon) from the tested prototypes estimated by 18S rRNA gene sequencing. Points represent individual biofilm samples collected by surface scratching.



Supplementary Figure S9. Bubble plot representation of the eukaryotic community composition at the genus level, estimated by 18S rRNA gene sequencing from biofilm samples collected on the tested prototypes.