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Ecological Diversity and Bioactive Compounds and their Potential for Aquaculture

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# Ecological Diversity and Proteomics of Cnidarians: Exploring Bioactive Compounds and their Potential for Aquaculture

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*Dedicated to the ones I love,*

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# Resumo

A biodiversidade marinha é uma componente fundamental dos serviços dos ecossistemas marinhos e desempenha um papel vital na sua conservação e gestão sustentável. Apesar da importância ecológica e económica das espécies marinhas, muitos grupos, como os cnidários, permanecem pouco estudados. Este diversificado filo ancestral, que compreende principalmente organismos marinhos pertencentes a Anthozoa (p.ex., anémonas-do-mar e corais) e Medusozoa (p.ex., medusas e hidrozoários), desempenha funções essenciais no ambiente marinho. No entanto, persistem lacunas no nosso conhecimento relativamente a estes organismos, devido às dificuldades associadas à sua amostragem, deteção e análise molecular. É essencial enfrentar estes desafios para alcançar uma conservação eficaz da biodiversidade e um avanço biotecnológico sustentável com base no potencial inexplorado destes organismos. Assim, esta tese investiga a diversidade dos cnidários, o potencial dos péptidos antimicrobianos (AMPs) de invertebrados aquáticos, onde se incluem os cnidários, e a proteómica de duas espécies de cnidários, com o objetivo de melhorar a compreensão dos seus papéis ecológicos e das suas aplicações biotecnológicas.

Foi compilado um conjunto de dados abrangente de espécies de cnidários da região ibérica, através da integração de dados ómicos globais, proporcionando novos conhecimentos sobre a sua biologia e ecologia (Capítulo 2). Este recurso não só identifica espécies de importância ecológica e socioeconómica, como também evidencia a necessidade urgente de mais estudos ómicos.

A investigação realizada nesta tese explora ainda as propriedades antimicrobianas dos invertebrados aquáticos, examinando a diversidade, estrutura e funções dos seus AMPs (Capítulo 3). Estas moléculas bioativas apresentam um potencial significativo para combater a resistência aos antimicrobianos (AMR), atuando como elementos-chave na luta contra os patógenos e oferecendo potenciais aplicações em várias indústrias, incluindo na aquacultura. Ao catalogar quase 350 AMPs provenientes de 10 filos de invertebrados, este estudo sublinha tanto o potencial destas moléculas como os desafios associados à sua validação e aplicação prática.

Contributos adicionais são fornecidos pela análise proteómica de duas espécies de cnidários, a anémona-do-mar *Actinia fragacea* (Anthozoa) (Capítulo 4) e a medusa-de-pintas *Phyllorhiza punctata* (Capítulo 5), que revela novos compostos bioativos—incluindo toxinas e AMPs—com promissoras aplicações biotecnológicas. A análise proteómica de *A. fragacea* elucida a composição do seu extrato de nematocistos, bem como dos seus tecidos e secreções do muco, enquanto o estudo integrado de

proteómica e transcriptómica de *P. punctata* recorre a abordagens computacionais e algoritmos preditivos baseados em inteligência artificial para identificar moléculas com relevância biotecnológica.

Globalmente, esta tese representa um avanço significativo na compreensão da ecologia dos cnidários, da diversidade ómica e do seu potencial biotecnológico, destacando os papéis cruciais destes organismos nos ecossistemas marinhos. Ao integrar a investigação da sua biodiversidade com a proteómica e a biologia computacional, esta tese oferece informações valiosas acerca do potencial inexplorado dos cnidários. Pesquisas futuras devem priorizar a ampliação dos dados moleculares, a validação dos AMPs em condições *in vitro* e *in vivo*, bem como a otimização da sua estabilidade, segurança e eficácia para aplicações sustentáveis, especialmente em áreas como a aquacultura.

**Palavras-chave:** invertebrados marinhos, Cnidaria, proteómica, péptidos antimicrobianos, toxinas, medusas, anémonas do mar, compostos bioativos, resistência antimicrobiana, peptidómica, medusozoários, patógenos da aquacultura.

# Abstract

Marine biodiversity is a fundamental component of marine ecosystem functions and vital in conservation and sustainable management. Despite marine species' ecological and economic importance, many groups, such as cnidarians, remain understudied. This diverse ancestral phylum, comprising mainly marine organisms from Anthozoa (e.g., sea anemones and corals) and Medusozoa (e.g., jellyfish and hydrozoans), plays essential roles in the marine environment. However, gaps in our knowledge regarding these organisms persist due to difficulties associated with their sampling, detection and molecular analysis. It is essential to face these challenges to achieve effective biodiversity conservation and sustainable biotechnological advancement based on the untapped potential of these organisms. Therefore, this thesis investigates the diversity of cnidarians, the potential of antimicrobial peptides (AMPs) from aquatic invertebrates, including cnidarians, and the proteomics of two species of cnidarians to improve our understanding of their ecological roles and biotechnological applications.

A comprehensive dataset of cnidarian species from the Iberian region has been compiled by integrating global omics data, providing new insights into their biology and ecology (Chapter 2). This resource identifies species of ecological and socioeconomic importance and highlights the urgent need for further omics studies.

The research further explores the antimicrobial properties of aquatic invertebrates by examining the diversity, structure, and functions of their AMPs (Chapter 3). These bioactive molecules hold significant promises for addressing antimicrobial resistance (AMR), as key players in combating pathogens and have potential applications in several industries including aquaculture. By cataloging nearly 350 AMPs across 10 invertebrate phyla, the study underscores the promise of these compounds and the challenges associated with their validation and practical use.

Additional insights are provided by the proteomic analysis of two cnidarian species, the sea anemone *Actinia fragacea* (Anthozoa) (Chapter 4) and the white-spotted jellyfish *Phyllorhiza punctata* (Chapter 5), which reveals novel bioactive compounds—including toxins and AMPs—with promising biotechnological applications. The proteomic analysis of *A. fragacea* elucidates the composition of its nematocyst extract, tissue, and mucus secretions, while the integrated proteomic and transcriptomic study of *P. punctata* employs computational approaches and AI-driven predictive algorithms to identify molecules of biotechnological relevance.

Overall, this work advances our understanding of cnidarian ecology, omics diversity, and biotechnological potential, emphasizing these organisms' critical roles in marine

ecosystems. By integrating research into their biodiversity with proteomics and computational biology, this thesis offers valuable information into the unexplored potential of cnidarians. Future research should focus on expanding molecular datasets, validating AMPs through both *in vitro* and *in vivo* studies, and optimizing their stability, safety, and efficacy for sustainable applications in fields such as aquaculture.

**Keywords:** marine invertebrates, Cnidaria, proteomics, antimicrobial peptides, toxins, jellyfish, sea anemone, bioactive compounds, antimicrobial resistance, peptidomics, medusozoans, aquaculture pathogens.

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# List of Abbreviations

| | |
|---|---|
| **AbHV** | Abalone Herpesvirus |
| **ALF** | Anti-Lipopolysaccharide Factor |
| **AMP** | Antimicrobial Peptide |
| **AMR** | Antimicrobial Resistance |
| **ANN** | Artificial Neural Networks |
| **APD3** | Antimicrobial Peptide Database |
| **BIC** | Bayesian Information Criterion |
| **BP** | Biological Process |
| **BPI** | Bactericidal Permeability Increasing Protein |
| **CAM** | Cell Adhesion Molecules |
| **CAMPR4** | Collection of Anti-Microbial Peptides |
| **Cat. No.** | Category Number |
| **CC** | Cellular Component |
| **cDNA** | Complementary Deoxyribonucleic Acid |
| **CNN** | Convolutional Neural Networks |
| **CIESM** | Mediterranean Science Commission |
| **CRISPR** | Clustered Regularly Interspaced Short Palindromic Repeats |
| **cryo-EM** | Cryogenic Electron Microscopy |
| **C-terminal** | Carboxyl-terminal |
| **DBAASP** | Database of Antimicrobial Activity and Structure of Peptides |
| **DNA** | Deoxyribonucleic Acid |
| **DRAMP** | Data repository of Antimicrobial Peptides |
| **EC** | Enzyme Commission |
| **EC** | Effective Concentration |
| **EEZ** | Exclusive Economic Zone |
| **EGF** | Epidermal Growth Factor |
| **EST** | Expressed Sequence Tag |
| **EUSC** | Exclusively Unique Spectrum Counts |
| **FASP** | Filter-Aided Sample Preparation |
| **FDA** | U.S. Food and Drug Administration |
| **FDR** | False Discovery Rate |
| **GBIF** | Global Biodiversity Information Facility |
| **GISD** | Global Invasive Species Database |

| | |
|---|---|
| **GO** | Gene Ontology |
| **GRAVY** | Grand Average of Hydropathy |
| **GRIIS** | Global Register of Introduced and Invasive Species |
| **HC** | Hemolytic Concentration |
| **HCl** | Hydrochloric Acid |
| **HPLC** | High-Performance Liquid Chromatography |
| **IgY** | Chicken Egg Yolk Immunoglobulin |
| **IHNV** | Infectious Hematopoietic Necrosis Virus |
| **InverPep** | Invertebrate Antimicrobial Peptide Database |
| **iProX** | Integrated Proteome Resources |
| **ISAV** | Infectious Salmon Anaemia Virus |
| **ISSG** | Invasive Species Specialist Group |
| **JFT** | Jellyfish Toxin |
| **KTx** | Potassium Channel Inhibitory Toxin |
| **LBD** | Lipopolysaccharide-Binding Domain |
| **LBP** | Lipopolysaccharide-Binding Protein |
| **LC** | Lethal Concentration |
| **LC-MS/MS** | Liquid Chromatography-Mass Spectrometry/Mass Spectrometry |
| **LGBP** | Lipopolysaccharide- and Beta-1,3-glucan Binding Protein |
| **LPS** | Lipopolysaccharides |
| **LPSN** | List of Prokaryotic Names with Standing in Nomenclature |
| **LTA** | Lipoteichoic Acid |
| **MassIVE** | Mass Spectrometry Interactive Virtual Environment |
| **MF** | Molecular Function |
| **MIC** | Minimum Inhibitory Concentration |
| **ML** | Maximum-likelihood |
| **MSA** | Multiple Sequence Alignment |
| **MW** | Molecular Weight |
| **NaCl** | Sodium Chloride |
| **NAMP** | Natural Antimicrobial Peptide |
| **NaTx** | Sodium Channel Inhibitory Toxin |
| **NCBI** | National Center for Biotechnology Information |
| **NMR** | Nuclear Magnetic Resonance |
| **N-terminal** | Amino-terminal |
| **OBIS** | Ocean Biodiversity Information System |
| **PAMP** | Pathogen-Associated Molecular Pattern |

| | |
|---|---|
| **Pd** | Proteome Discoverer |
| **PDB** | Protein Data Bank |
| **Pfam** | Protein Families Database |
| **PGRP** | Peptidoglycan Recognition Protein |
| **PI** | Isoelectric Point |
| **pI** | Protease Inhibitor |
| **PLA** | Phospholipase A |
| **pLDDT** | Predicted Local Distance Difference Test |
| **PRIDE** | Proteomics Identification Database |
| **PRP** | Pattern Recognition Proteins |
| **PRX** | Peroxiredoxin |
| **PTM** | Post-Translational Modification |
| **pTM** | Predicted Template Modeling |
| **QGIS** | Quantum Geographic Information System |
| **RF** | Random Forest |
| **RNA** | Ribonucleic Acid |
| **RNA-seq** | RNA Sequencing |
| **RNN** | Recurrent Neural Networks |
| **RT** | Room Temperature |
| **SAMP** | Synthetic Antimicrobial Peptide |
| **SCRIP** | Cnidaria Small Cysteine-Rich Protein |
| **SD** | Standard Deviation |
| **SDS** | Sodium Dodecyl Sulfate |
| **SHIV** | Shrimp Hemocyte Iridescent Virus |
| **ShK** | Stichodactyla Toxin |
| **sORF** | Small Open Reading Frames |
| **SR** | Scavenger Receptor |
| **SRA** | Sequence Read Archive |
| **SRA** | Sequence Read Archive |
| **SVM** | Support Vector Machine |
| **TIM** | Triose-Phosphate Isomerase |
| **TLR** | Toll-like Receptors |
| **ToxProt** | Animal Toxin Annotation Project |
| **TPM** | Transcripts Per Million |
| **Tris** | Tris(hydroxymethyl)aminomethane |
| **TRP** | Tachykinin-Related Peptide |

| | |
|---|---|
| **TSA** | Transcriptome Shotgun Assembly |
| **TSM** | Total Spectrum Matches |
| **UniProt** | Universal Protein Resource |
| **UniProt** | The Universal Protein Knowledgebase |
| **WAP** | Whey Acidic Protein |
| **WGA** | Whole Genome Amplification |
| **WGS** | Whole Genome Sequencing |
| **WoRMS** | World Register of Marine Species |
| **WSSV** | White Spot Syndrome Virus |
| **βGBP** | Beta-glucan Binding Protein |

# Chapter 1.

## General Introduction

## General Introduction

Oceans cover approximately 70% of the Earth's surface, yet marine biodiversity represents only about 13–16% of all currently described living species, totaling just under 250,000 species [1]. However, many evolutionary lineages are exclusively marine, and numerous marine taxa trace their ancestry to some of the oldest branches of the tree of life, highlighting the ocean's critical role in evolutionary history. While significant uncertainty surrounds estimates of undiscovered marine species [2], most known marine species have not yet been formally described [3]. However, marine biodiversity is essential for maintaining ecosystem stability, regulating biogeochemical cycles, and supporting crucial services, such as climate regulation and carbon sequestration, with the ocean absorbing approximately 25% of anthropogenic $CO_2$ emissions [4]. Therefore, given the escalating anthropogenic pressures on marine ecosystems, expanding our understanding of marine biodiversity is essential for its conservation and sustainable management.

A particularly rich, diverse, and underexplored group within marine ecosystems is the phylum Cnidaria, an ancient lineage of predominantly marine species that plays a crucial role in marine food webs and ecosystem services. This phylum is classified into three major clades: Anthozoa (corals, sea anemones, and sea pens), Medusozoa (true jellyfish, hydrozoans, box jellyfish, and stalked jellyfish), and Endocnidozoa (obligate endoparasites) [5]. A defining characteristic of cnidarians is the presence of cnidocytes, stinging cells containing nematocysts, specialized stinging organelles employed in both defense and prey capture [6]. These organisms exhibit a relatively simple body plan with radial symmetry, a gastrovascular cavity, and typically alternate between two main body forms: the sessile polyp and the free-swimming medusa [7].

Among cnidarians, Medusozoa includes four classes: Scyphozoa (true jellyfish), Hydrozoa (hydras, hydromedusae, and siphonophores), Cubozoa (box jellyfish), and Staurozoa (stalked jellyfish) [8]. Medusozoans display remarkable life cycle diversity, typically alternating between a benthic polyp stage and a pelagic medusa stage. Most species exhibit a metagenetic life cycle, in which sexual reproduction occurs in the medusa stage while polyps reproduce asexually. However, some species, have holoplanktonic life cycles with continuous reproduction in the water column [9,10]. Medusozoans have recently gained increased attention due to the global awareness of jellyfish blooms and their associated ecological and economic impacts, such as disrupting trophic webs, interfering with fishing, and damaging aquaculture [11,12]. Nevertheless, despite their central role in marine ecosystem dynamics, jellyfish remain

significantly understudied compared to other aquatic animals [13]. This knowledge gap stems from several factors, including their unpredictable distribution influenced by environmental and anthropogenic conditions, their transparent and gelatinous bodies that hinder detection and sampling, and the fragility of their tissues, which complicates the extraction of high-quality DNA, RNA, and proteins, limiting omics data availability [14–16]. As a result, the molecular and functional diversity of jellyfish remains poorly understood, restricting our ability to assess their evolutionary adaptations, ecological roles, and biotechnological potential.

In contrast to Medusozoa, the clade Anthozoa consists exclusively of sessile marine species permanently attached to substrates. They are distributed into two classes: Hexacorallia (stony corals, sea anemones, and tube anemones), and Octocorallia (soft corals, sea fans, sea pens, and gorgonians) [17]. Anthozoans exhibit diverse reproductive strategies with some species reproducing asexually through budding, fission, or fragmentation, while others engage in sexual reproduction, with some being hermaphroditic [18,19]. Despite their ecological significance, anthozoans remain understudied, particularly in molecular biology, limiting our understanding of their adaptive mechanisms, symbiotic relationships, and biotechnological applications.

Recently, cnidarians have gained increasing recognition as important sources of bioactive compounds, attracting significant interest for their biotechnological potential in pharmaceuticals, cosmeceuticals, and nutraceuticals [20]. Cnidarian toxins, known for their diverse neurotoxic, cytotoxic and immunomodulatory properties, though still understudied, have shown promising potential for therapeutic applications, including as analgesics and anti-cancer agents [21]. However, much remains to be explored in cnidarians, particularly regarding their antimicrobial peptides (AMPs). AMPs are small molecules produced by a wide variety of organisms, including cnidarians, that exhibit a wide range of bioactive properties, including immunomodulatory, anticancer, and antimicrobial activities against a wide range of pathogens, such as bacteria, fungi, viruses, and parasites [22]. Amid the growing global crisis of antimicrobial resistance (AMR), discovering new AMPs as alternatives to traditional antibiotics has become critical, particularly due to their significantly low propensity to induce resistance [23,24]. Despite their promising potential, the practical applications of AMPs, especially in aquaculture, remain primarily theoretical, with very few applied studies.

To bridge this gap, integrated high-throughput multi-omics approaches, combined with AI-driven predictive algorithms, such as machine learning and deep learning, offer a powerful framework for screening and optimizing bioactive compounds [25]. When paired with traditional biochemical and cellular approaches, these advancements can

accelerate the discovery of bioactive compounds, unlocking their therapeutic and biotechnological potential to address global challenges in healthcare and food production [26].

Considering all of this, this thesis aimed to address existing knowledge gaps in the ecological diversity and proteomic complexity of cnidarians, with a particular focus on identifying bioactive compounds with therapeutic and biotechnological potential, including applications in aquaculture. The scientific research has been organized into four main chapters as described below:

- **Chapter 2** compiles the occurrence records of cnidarian species from the Iberian region, integrating available global omics data. This comprehensive approach provides valuable insights into the biology and ecological significance of underexplored cnidarian groups, including Hydrozoa, Scyphozoa, Cubozoa, and Staurozoa. The chapter serves as an essential resource for identifying species of both ecological and socioeconomic importance, while emphasizing the need for further omics studies to fully explore their biotechnological potential.
- **Chapter 3** explores the diversity and richness of AMPs from aquatic invertebrates, focusing on their antimicrobial activities against pathogens relevant to human health and aquaculture. It connects the ecological importance of cnidarians with their potential as sources of bioactive compounds, highlighting AMP's promising applications in aquaculture and their role in combating AMR.
- **Chapter 4** presents the first proteomic study of the sea anemone *Actinia fragacea* (Anthozoa: Hexacorallia), a native cnidarian species from the Iberian region. This study contributes to bridging the gap in cnidarian omics data, integrating proteomics with predictive algorithms to trace and explore the proteomic profiles of the sea anemone's venom nematocyst extract, tissues, and mucus secretion, focusing on bioactive compounds such as toxins and AMPs that may have both ecological and biotechnological significance.
- **Chapter 5** expands the proteomic analysis, presenting the first proteomic study of the invasive jellyfish *Phyllorhiza punctata* (Medusozoa: Scyphozoa), a species recently detected in the Iberian region. By integrating transcriptomic data, proteomics, and advanced predictive algorithms, this pioneering study identifies and characterizes the jellyfish's bioactive compounds, with a particular focus on toxins and AMPs that hold significant therapeutic and biotechnological potential.

Overall, this Ph.D. thesis explores the regional ecology and diversity of some of the most understudied cnidarian groups, investigates AMPs derived from aquatic invertebrates,

and their potential applications in aquaculture. It characterizes the proteomes of two underexplored cnidarian species representing the two main cnidarian clades: the sea anemone *A. fragacea* and the jellyfish *P. punctata*. The focus is on identifying bioactive compounds, particularly toxins and AMPs, to evaluate their biotechnological potential.

# Chapter 2.

# A Comprehensive Compilation of Iberian Medusozoan Data: Diversity, Ecology, and Omics Insights

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Associated Dataset: Global Biodiversity Information Facility (GBIF)

DOI: https://doi.org/10.15468/j7349s

## Abstract

This study investigates the diversity and distribution of Medusozoa, a clade of the phylum Cnidaria, within the framework of the Iberian Peninsula and surrounding archipelagos. Medusozoa is notably recognized for encompassing jellyfish species with the capacity to exert detrimental ecological and socioeconomic impacts, this group of mostly marine organisms assumes a critical role in aquatic food webs and exhibits considerable biotechnological potential. A comprehensive dataset sourced from over 230 bibliographic references and public databases, comprising over 30,000 reports of Medusozoa in the Iberian region, reveals 593 species across the four medusozoan classes (549 hydrozoans, 37 scyphozoans, 5 staurozoans, and 2 cubozoans). Additionally, publicly available genomic, transcriptomic, and proteomic metadata from the phylum Cnidaria are incorporated. The analyses highlight Hydrozoa as the most frequently reported and diverse class in the Iberian region. Specifically, the order Leptothecata exhibits the highest diversity, while Siphonophorae stands out as the most documented with *Physalia physalis* emerging as the highest reported species. Spatial distribution patterns reveal that holoplanktonic species are more widespread and abundant than their benthic and meroplanktonic counterparts. Furthermore, medusozoans with a free-swimming life form display greater diversity and dispersal compared to sessile species. The Northeast Atlantic section of the region demonstrates greater medusozoan diversity compared to the Mediterranean. Key findings also include information into invasive and introduced species, bloom-forming organisms, and edible species. A sustained trend of discovering new species of Medusozoa over time was observed, underscoring untapped exploration potential in the Iberian region. The study reveals a clear limitation of omics data in cnidarians, particularly in the Medusozoa clade. This research provides an overall perspective on medusozoans in the Iberian region, underlining the importance of additional omics studies while highlighting growing interest in exploring the biotechnological potential of these organisms. This research serves as a valuable resource for future investigations, providing insights into underexplored species and those with greater ecological and socioeconomic importance.

**Keywords**: Iberian Peninsula; Cnidaria; Medusozoa; Reports; Jellyfish

## 2.1. Introduction

Medusozoans represent a predominantly marine clade of Cnidaria that comprises four classes: Hydrozoa (hydrozoans), Scyphozoa (true jellyfish), Cubozoa (box jellyfish), and Staurozoa (stalked jellyfish) (Fig. 1) [27,28]. These invertebrates inhabit various marine environments, ranging from shallow coastal waters to deep-sea habitats [29–32]. Nonetheless, some species have been observed in non-marine ecosystems, such as estuaries, lakes, and rivers [33–36]. These organisms play critical roles in aquatic ecosystems and food webs, preying on planktonic organisms, crustaceans, and small fishes, while also serving as prey for sea turtles, fish, sea slugs, and birds [11]. Moreover, they can act as hosts for a variety of symbiotic organisms, including algae [37], bacteria [38], bryozoans [39], crustaceans [40], and dinoflagellates [41].

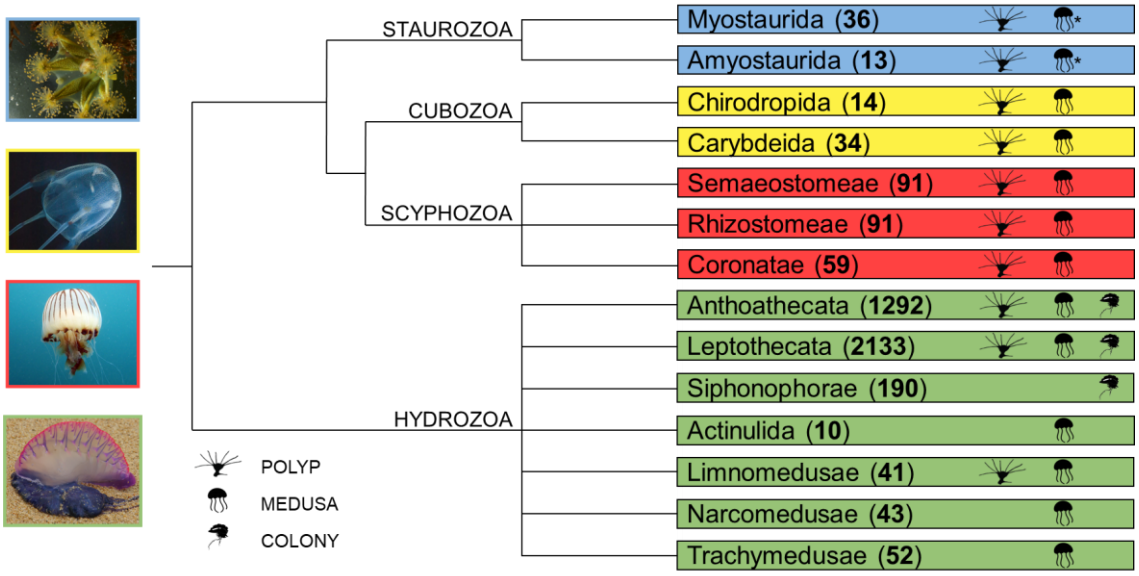


**Fig. 1** Phylogeny of the clade Medusozoa. The four classes of Medusozoa: Staurozoa (blue outline), Cubozoa (yellow), Scyphozoa (red), and Hydrozoa (green), with the life forms present in each order (suborder, in the case of Staurozoa). In parenthesis: the total number of species of each order (suborder, in the case of Staurozoa); asterisk: the sessile stauromedusae. Image credits (with colorful outlines): blue – licensed by HE Westlake under the CC BY-SA 4.0, source Wikimedia Commons (https://bit.ly/3jHfW9U); yellow – licensed by Seascapeza under the CC BY-SA 3.0, source Wikimedia Commons (https://bit.ly/3bhOYkF); red – licensed by Christophe Quintin under the CC BY-NC 2.0, source flickr (https://bit.ly/3BvLAO5); green –licensed by Biusch under the CC BY-SA 3.0, source Wikimedia Commons (https://bit.ly/3Emo2wU).

Hydrozoa, the most diverse class in Medusozoa, comprises over 3,700 species, consisting predominantly of small organisms, although certain hydroids within the Siphonophora order can remarkably reach over 40 m long [42]. Hydrozoans are primarily marine, yet some species are euryhaline, and inhabit brackish and freshwater environments [43]. In most cases, their life cycle begins with the formation of a planula

larva, that develops into a sessile polyp, which, through asexual reproduction, forms a colony [44]. The colony asexually produces medusae when a free-medusa stage is present. In the absence of this stage, the medusa transforms into a fixed sporosac. The cycle ends with the fusion of gametes and the formation of a zygote, which develops into a new planula larva [45]. Multiple variations exist and are well described in Piraino *et al.* (2004) [46]. Species from Anthoathecata, Leptothecata, and Limnomedusae orders often display meroplanktonic behavior, asexually producing jellyfish from the colonial hydroid stage. In contrast, the Actinulida, Narcomedusae, and Trachymedusae orders are holoplanktonic, lacking a sessile benthic stage. The order Siphonophorae comprises colonial, highly polymorphic, holoplanktonic jellyfish with polypoid and medusoid zooids [47].

According to the World Register of Marine Species (WoRMS, 2023), the class Scyphozoa includes about 240 marine species distributed in three orders (Coronatae, Rhizostomeae, Semaeostomeae). Often referred to as "true jellyfish", they are known to cause blooms with adverse effects on fisheries, aquaculture, and tourism [15,48–50]. Despite this, only 49 species have had their life cycles described, while the complete life cycle of most scyphozoans yet to be described [51]. Scyphozoans can exhibit either a meroplanktonic or a holoplanktonic life cycle [52]. Meroplanktonic species alternate between asexual polyps and sexual medusae stages, with medusae being more dominant and longer-lived [9]. The cycle begins with medusae releasing gametes for external fertilization. The fertilized eggs develop into planula larvae, that settle on the substrate to become scyphistomae polyps. Trough asexual reproduction, they produce ephyrae by strobilation, which then mature into adult medusae [9,53]. In contrast, holoplanktonic scyphozoans remain in the medusa planktonic state throughout their entire life cycle. They develop directly from fertilized eggs to planula larvae, which metamorphose into ephyrae or actinulae-like stages [53].

The class Cubozoa comprises 50 species of marine box jellyfish [54]. These marine, pelagic, metagenetic species, typically found in coastal warm waters, are remarkably known for their visual capacities (complex eyes) and extreme toxicity, posing a threat to human health [50,55–57]. The life cycles of cubozoans resemble those of scyphozoans, except they do not release ephyrae into the water. Instead, the entire scyphistoma metamorphoses into an ephyra, which then detaches from the substrate and develops into an adult medusa [56]. However, there are notable exceptions such as incomplete metamorphosis, swimming secondary polyps, or monodisc strobilation [58–61].

The class Staurozoa includes the stalked jellyfish and comprises about 49 species [54]. Staurozoans are benthic animals found in shallow temperate waters [62,63]. These

invertebrates also display a metagenetic life cycle with some remarkable changes as they present stauropolyps that metamorphose into non-free swimming stauromedusae that remain attached to the substrate by a basal peduncle [64,65].

Medusozoans have gained increasing attention mainly due to the growing global awareness of jellyfish blooms and their significant im- pacts in key areas. These blooms adversely affect fisheries by damaging or obstructing fishing equipment, resulting in reduced catches [12]. In aquaculture, the invasion of jellyfish into the cultures poses threats to fish and causes damage to facility infrastructures [66]. Additionally, in industries such as desalination plants and power stations, jellyfish can clog intake pipe systems, leading to increased operational and maintenance costs [67,68]. Furthermore, the proliferation of stinging jellyfish in bathing areas negatively impacts tourism, deterring tourists and impacting local businesses [69,70]. On the other hand, jellyfish aquaculture has gained interest aiming the prospect of bioactive compounds {71,72], used as food source, for human consumption [73] and aquafeed [74], and for aquaria and research [75,76].

The application of omics technologies has greatly enhanced the understanding of the biology and evolution of medusozoans [77,78]. Omics information can help identify the causes of jellyfish abundance and invasions [79], explore and identify new genes and compounds [80–82], search the evolution of develop- mental pathways and cell differentiation [83,84], and to investigate the potential of venom proteins [85,86].

The study of Medusozoans, although limited by the irregularity of their occurrence [87], has provided a wealth of information on their diversity in the Iberian Peninsula and surrounding archipelagos. However, data regarding medusozoans from this region is dispersed across numerous publications, including the studies by Medel and López-González (1996) [88], Cantero and Carrascosa (2002) [89], Altuna (2015) [90], and Gueroun *et al.* (2021) [91]. To address this issue, a comprehensive review conducted on the diversity of Medusozoa in the entire Iberian region, including Portugal, Spain, Azores, Madeira, and Canary Islands, is presented, extending the work previously initiated by Rodrigues *et al.* (2020) [8]. This research includes medusozoan reports in the Iberian region, integrated with genomic, transcriptomic, and proteomic metadata for the phylum Cnidaria, shedding light on the medusozoan biogeography and ecology, and emphasizing the need for further omics studies.

## 2.2. Methods

### 2.2.1. Diversity and distribution data

To assess Medusozoa diversity in the Iberian Peninsula and its surrounding archipelagos, an extensive literature review was conducted and complemented by data obtained from publicly accessible databases, resulting in the compilation of the publicly available medusozoan reports.

The study area is in the southwestern region of Europe, where the Northeast Atlantic Ocean and the Mediterranean Sea converge (24◦35'N - 46◦52'N, 35◦35'W - 06◦18'E), including the Exclusive Economic Zones (EEZs) of Portugal (with the EEZs of Azores and Madeira archipelagos) and Spain (with the EEZ of Canary Islands). Additionally, the study area also includes the minor Spanish territories in North Africa, such as the two autonomous cities, Ceuta and Melilla, and the "Plazas de Soberanía" (Alhucemas Islands, Chafarinas Islands, and Peñón de Vélez de la Gomera) (Fig. 2). Throughout this work, mainland Portugal and Spain will be referred as Portugal and Spain, respectively, and the entire study area as the "Iberian region".

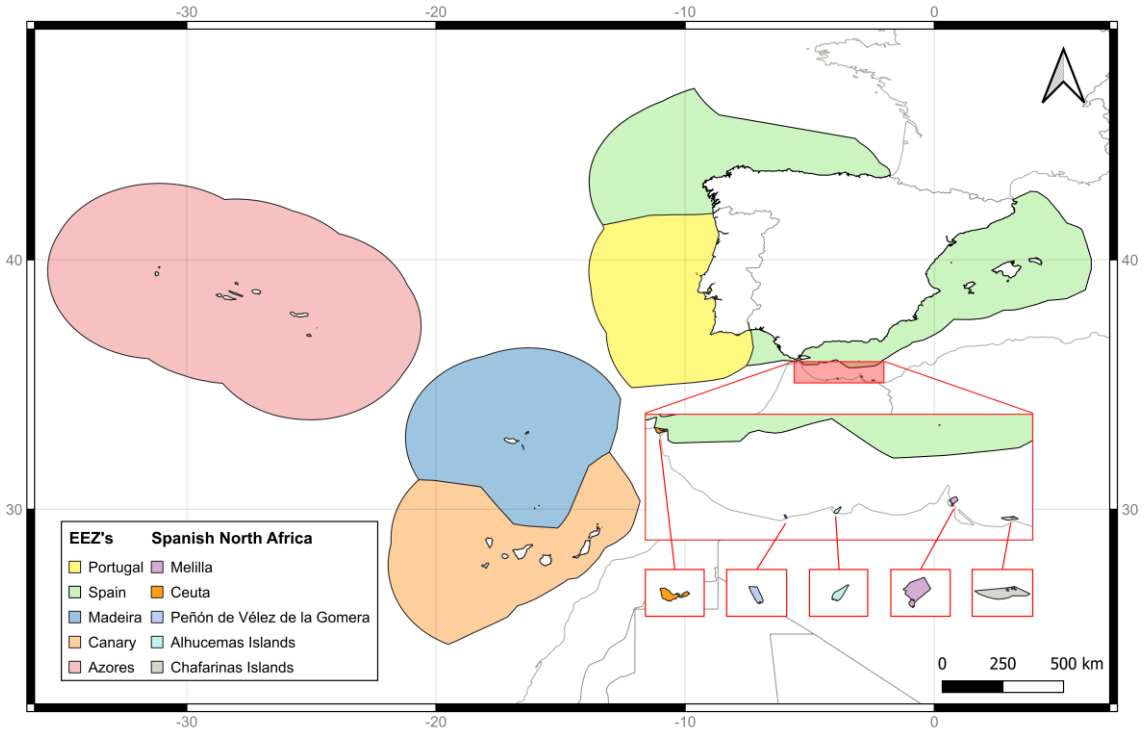


**Fig. 2** Study area. The Iberian region encompassing the Iberian Peninsula with the exclusive economic zones (EEZ's) of Continental Portugal and Spain, and the surrounding archipelagos of Macaronesia with the EEZ's of Azores, Madeira, and the Canary Islands.

When compiling the reports of Medusozoa, only records from the EEZs above mentioned were considered. Data were gathered from scientific literature, including articles, books, reports, and theses, published between 1829 and 2023 (see Supplementary material references). Data retrieval from Google Scholar involved systematically using the nomenclature of each of the 593 species found. Subsequently, the literature retrieved were examined to determine the presence of each species within the EEZs considered. Search results were not constrained by time or language, and only the first two pages results were considered, as subsequent pages did not provide relevant information. To expand the data compilation, three online databases, namely Global Biodiversity Information Facility [92], iNaturalist.org [93], and Ocean Biodiversity Information System [94], were accessed to retrieve data on medusozoan reports within the area of study (last retrieved in October 2023). To ensure reliability and relevance, data was manually curated. Redundant data was removed to prevent duplication and only records containing at least the genus-level identification and the year of the record were retained. For iNaturalist records, a "research grade" data quality level was required and observations with "private" coordinates were excluded. Additionally, reports of species that could not be confirmed in existing scientific literature were included and labelled as such in the datasheet for future considerations.

Taxonomic data were cross-checked in the WoRMS for accuracy and validation. Currently accepted nomenclature was used for invalid species names, and entries with "cf." qualifiers indicating uncertain identifications were recorded at the genus level. Species entries identified at the genus level were only included in the tally and analysis of species diversity if that genus had no other representatives in the collected data. This criterion applies consistently across all comparisons of species diversity counts, including those between different EEZs, the Mediterranean and Atlantic areas, and so on. Disputed species, non- marine species, and those with temporary nomenclature were included for future consideration. Data analysis (e.g., species counting and percentage calculations) were performed using an Excel spread- sheet. Life history information was obtained from bibliographical research, with particular attention paid to the works of Bouillon and Schuchert for Hydrozoa [95–101], Jarms and Morandini for Scyphozoa [102], Jarms and Straehler-Pohl for Cubozoa [58,103,104], and Miranda for Staurozoa [62,63,105,106]. For analytical purposes, scyphozoan species lacking disclosed details about their life cycle were tentatively categorized as meroplanktonic. Information about the natural environment of each species (marine, brackish, freshwater) was retrieved from WoRMS [54].

Distribution maps for medusozoans in the Iberian region were created using the QGIS 3.28.2 software [107]. EEZ shapefiles for the study area were retrieved from Marineregions.org [108]. Heatmap layers were generated with a radius of 1 map unit, layer rendering blending mode set to “Multiply”, and the remaining settings kept as default.

Non-marine species were excluded from the analysis comparing species distribution between the Atlantic and Mediterranean regions, as they are not present in either. Nevertheless, a separate distribution map for non-marine species was produced (Fig. S 1).

Species flagged as invasive or introduced were identified using the Global Invasive Species Database (GISD) [109], and the Global Register of Introduced and Invasive Species (GRIIS) catalogue [110].

### 2.2.2. Medusozoan reports datasheet description

The Medusozoan reports datasheet compiles public data on Medusozoa in the Iberian region, organized into columns. The "recordOrigin" column specifies the data source, with "referenceID" serving as the record identifier. For database records, it corresponds to the original occurrence id. The "refYear" column indicates the publication year for literature records. Date details are in “year”, “month”, and “day” columns, while location information includes “latitude”, “longitude”, "location", "location2" and "location3”. When available, reports coordinates are presented in decimal degrees format (WGS84) in the “latitude” and “longitude” columns. If not available, approximate coordinates from Google Maps were used, retaining descriptive site data in the “location3” column. The “location” column categorizes reports by EEZ, and “location2” labels them as from the Mediterranean Sea, Northeast Atlantic Ocean, or non-marine area. Taxonomic data is columns “class”, “subclass”, “order”, “suborder”, “family”, “genus”, and “species”. Species life history details are in "lifeCycle", "lifeForms," and "isFree-swim". The "lifeCycle" column categorizes species as holoplanktonic, meroplanktonic, or benthic. "lifeForms" column provides information on each species main life forms, and "isFree-swim" indicates if a species has a free-swimming life form. Data on the species’ natural environment is in columns labeled “isMarine”, “isBrackish”, and “isFresh”.

### 2.2.3. Omics data

Transcriptomic and genomic data were retrieved from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) [111], encompassing all cnidarian entries. The data were manually curated, categorized by class, and refined

by assay type, selectively including RNA-sequencing (RNA-seq) for transcriptomes, as well as whole-genome amplification (WGA) and sequencing (WGS) for genomes.

Simultaneously, comprehensive proteomic metadata were compiled from major repositories such as ProteomeXchange [112], PRIDE [113], MassIVE [114], iProX [115], and UniProt Proteome [116]. Additionally, a thorough literature review was conducted to identify and incorporate additional proteomic studies on Medusozoa not available in any repository.

## 2.3. Results

### 2.3.1. Diversity and distribution data

The investigation compiled a total of 233 studies and public data from GBIF, iNaturalist, and OBIS databases, resulting in a datasheet of public reports of medusozoans in the Iberian region (Table S 1). Additionally, a checklist of the reported medusozoan species is provided (Table S 2). Upon the acceptance of this manuscript, any data that is currently unavailable on GBIF will be uploaded and made accessible through the platform.

The compilation identified 30,121 reports, uncovering 593 medusozoan species from all four classes in the Iberian region. Hydrozoa dominates with 549 species and 26,390 reports, followed by Scyphozoa (37 species, 3680 records), Staurozoa (five species, 17 records), and Cubozoa (two species, 34 records) (Fig. 3). The total medusozoan diversity of the region encompasses 290 genera, 117 families, and 12 orders (Table 1). Leptothecata is the most specious order, and Siphonophorae is the most frequently observed order (Fig. 4), with *Physalia physalis* (Linnaeus, 1758) being the most reported species (Table S 3).

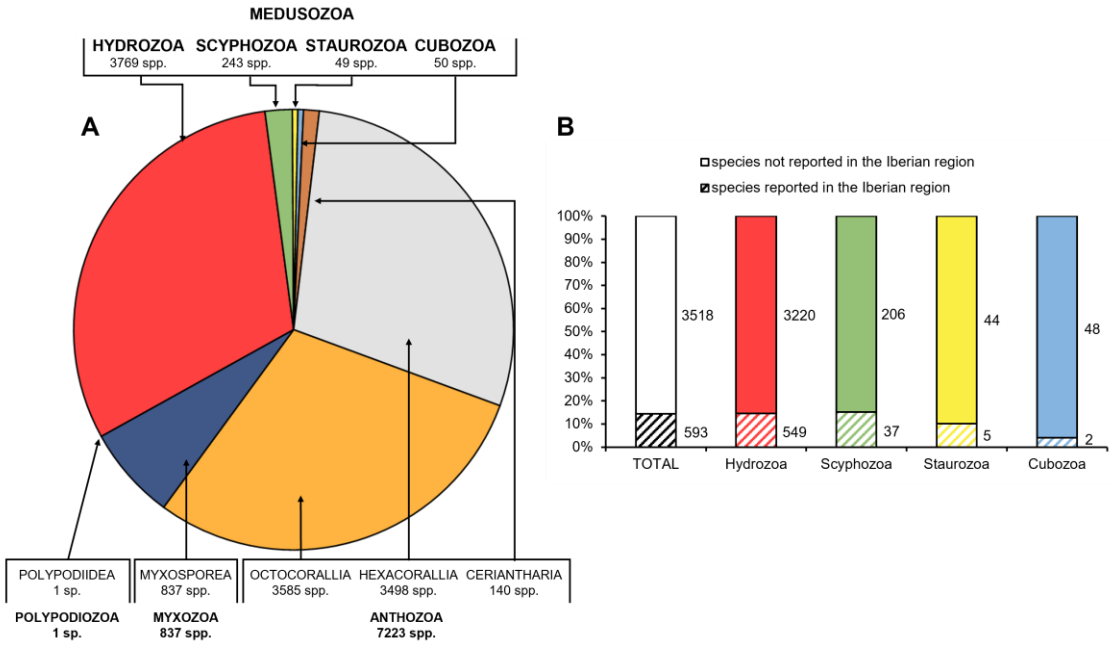


**Fig. 3** Schematic representation of the global and regional diversity of Medusozoa. A: General diversity of each Cnidaria class. (source: WoRMS Database accessed in October 2023) B: Global and regional diversity of Medusozoa in which the striped portions of each bar represent the species present in the Iberian region.

**Table 1** Medusozoan diversity in the Iberian Peninsula and Islands.

| | ***Iberian region*** | ***Portugal*** | ***Spain*** | ***Azores*** | ***Madeira*** | ***Canary*** |
|---|---|---|---|---|---|---|
| EEZ Area ($km^2$) | 2,736,391 | 315,501 | 561,763 | 960,421 | 452,796 | 445,910 |
| EEZ Species density (species/$km^2$) | $2.167\times10^{-4}$ | $7.258\times10^{-4}$ | $7.530\times10^{-4}$ | $2.749\times10^{-4}$ | $3.556\times10^{-4}$ | $5.203\times10^{-4}$ |
| **Number of species** | | | | | | |
| All | 593 | 229 | 423 | 264 | 161 | 232 |
| Hydrozoa | 549 | 205 | 394 | 246 | 152 | 213 |
| Scyphozoa | 37 | 20 | 26 | 18 | 9 | 17 |
| Staurozoa | 5 | 3 | 2 | 0 | 0 | 1 |
| Cubozoa | 2 | 1 | 1 | 0 | 0 | 1 |
| **Number of genera** | | | | | | |
| All | 290 | 143 | 234 | 154 | 106 | 145 |
| Hydrozoa | 261 | 127 | 213 | 145 | 100 | 133 |
| Scyphozoa | 22 | 12 | 18 | 9 | 6 | 10 |
| Staurozoa | 5 | 3 | 2 | 0 | 0 | 1 |
| Cubozoa | 2 | 1 | 1 | 0 | 0 | 1 |
| Range species per genus | 1–19 | 1–8 | 1–17 | 1–12 | 1–12 | 1–7 |
| Max. representative | *Lensia* | *Aglaophenia* / *Lensia* | *Lensia* | *Lensia* | *Lensia* | *Aglaophenia* |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Number of families** | | | | | | |
| All | 117 | 72 | 100 | 68 | 52 | 71 |
| Hydrozoa | 96 | 58 | 83 | 61 | 46 | 60 |
| Scyphozoa | 15 | 10 | 14 | 7 | 6 | 9 |
| Staurozoa | 4 | 3 | 2 | 0 | 0 | 1 |
| Cubozoa | 2 | 1 | 1 | 0 | 0 | 1 |
| Range genera per family | 1–12 | 1–8 | 1–10 | 1–10 | 1–7 | 1–11 |
| Max. representative | Rhopalonematidae | Diphyidae | Rhopalonematidae / Sertulariidae | Rhopalonematidae | Diphyidae | Rhopalonematidae |
| Range species per family | 1–35 | 1–17 | 1–30 | 1–23 | 1–22 | 1–15 |
| Max. representative | Diphyidae | Campanulariidae / Diphyidae | Diphyidae | Diphyidae | Diphyidae | Rhopalonematidae |
| **Number of orders** | | | | | | |
| All | 12 | 11 | 12 | 8 | 8 | 11 |
| Hydrozoa | 7 | 6 | 7 | 6 | 6 | 6 |
| Scyphozoa | 3 | 3 | 3 | 2 | 2 | 3 |
| Staurozoa | 1 | 1 | 1 | 0 | 0 | 1 |
| Cubozoa | 1 | 1 | 1 | 0 | 0 | 1 |
| Range family per order | 1–34 | 1–22 | 1–29 | 1–23 | 1–17 | 1–19 |
| Max. representative | Anthoathecata | Leptothecata | Leptothecata | Leptothecata | Anthoathecata | Anthoathecata, Leptothecata |
| Range genera per order | 1–95 | 1–54 | 1–79 | 1–55 | 1–40 | 1–46 |
| Max. representative | Leptothecata | Leptothecata | Leptothecata | Leptothecata | Leptothecata | Leptothecata |
| Range species per order | 1–240 | 1–106 | 1–174 | 1–116 | 1–76 | 1–86 |
| Max. representative | Leptothecata | Leptothecata | Leptothecata | Leptothecata | Leptothecata | Leptothecata |
| **Life cycle** | | | | | | |
| Holoplanktonic (%) | 192 (32%) | 57 (25%) | 131 (31%) | 111 (42%) | 61 (38%) | 98 (42%) |
| Meroplanktonic (%) | 164 (28%) | 67 (29%) | 119 (28%) | 54 (20%) | 29 (18%) | 57 (25%) |
| Benthic (%) | 237 (40%) | 105 (46%) | 173 (41%) | 99 (38%) | 71 (44%) | 77 (33%) |
| Free-swimming? | | | | | | |
| Yes (%) | 352 (59%) | 122 (53%) | 246 (58%) | 165 (63%) | 90 (56%) | 155 (67%) |
| No (%) | 241 (41%) | 107 (47%) | 177 (42%) | 99 (37%) | 71 (44%) | 77 (33%) |

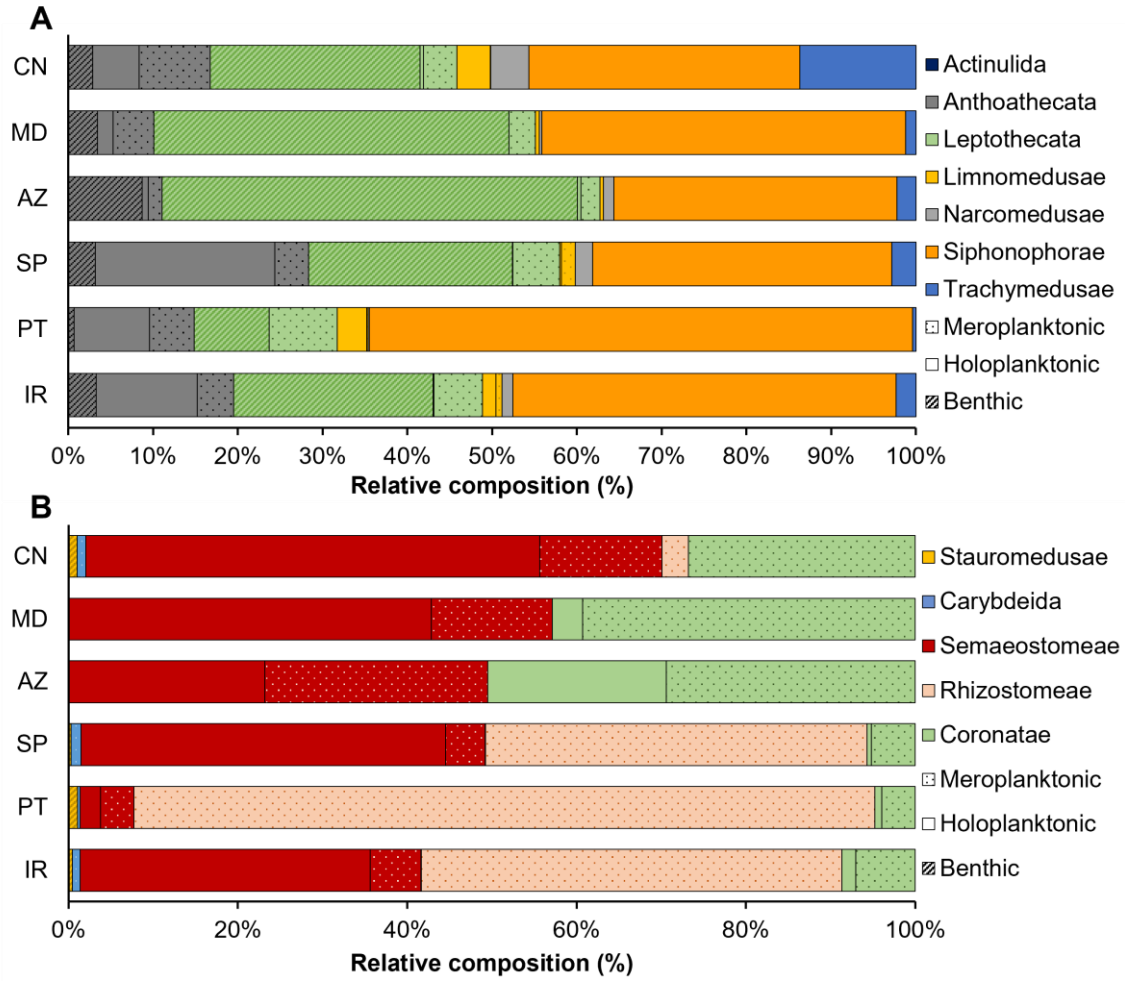


**Fig. 4** Iberian region relative percentage (%) composition of medusozoan reports by order. A: Hydrozoa; B: Scyphozoa, Cubozoa, and Staurozoa. IR: Iberian region; PT: Portugal; SP: Spain; AZ: Azores; MD: Madeira; CN: Canary Islands.

The only reported non-marine species in this study belong to class Hydrozoa, totaling six species and 1795 records, mainly from Hydra spp. in Spain. Marine species are distributed in both the Northeast Atlantic Sea area and the Mediterranean Sea area. The Northeast Atlantic has 21,756 records, including 537 species (314 exclusive), while the Mediterranean Sea has 6570 reports, with 273 species (50 exclusive, 223 shared). Of the reported medusozoans, 40% display a benthic life cycle strategy, 32% are holoplanktonic, and 28% are meroplanktonic. Additionally, 59% of all registered species have a free-swimming form in their life cycle, while the remaining are exclusively sessile. Medusozoans are documented along nearly the entire coastline of the study area, including all minor Spanish territories in North Africa, except for the Alhucemas Islands and Peñón de Vélez de la Gomera (Fig. 5). Notably, the public records of Medusozoa are concentrated in the coastal area of Portugal, the Spanish Valencian coast, and the central group of the Azores (Fig. S 2). A significant concentration of reports is observed in the Northwest of the Spain EEZ. The distribution pattern indicates that species with a

free-swimming life cycle stage are more widespread across the Iberian region, while sessile species are mostly concentrated in coastal areas (Fig. S 3). The number of publicly available sightings of medusozoans is increasing exponentially, and there is no decrease in the discovery of new species in recent times (Fig. 6).

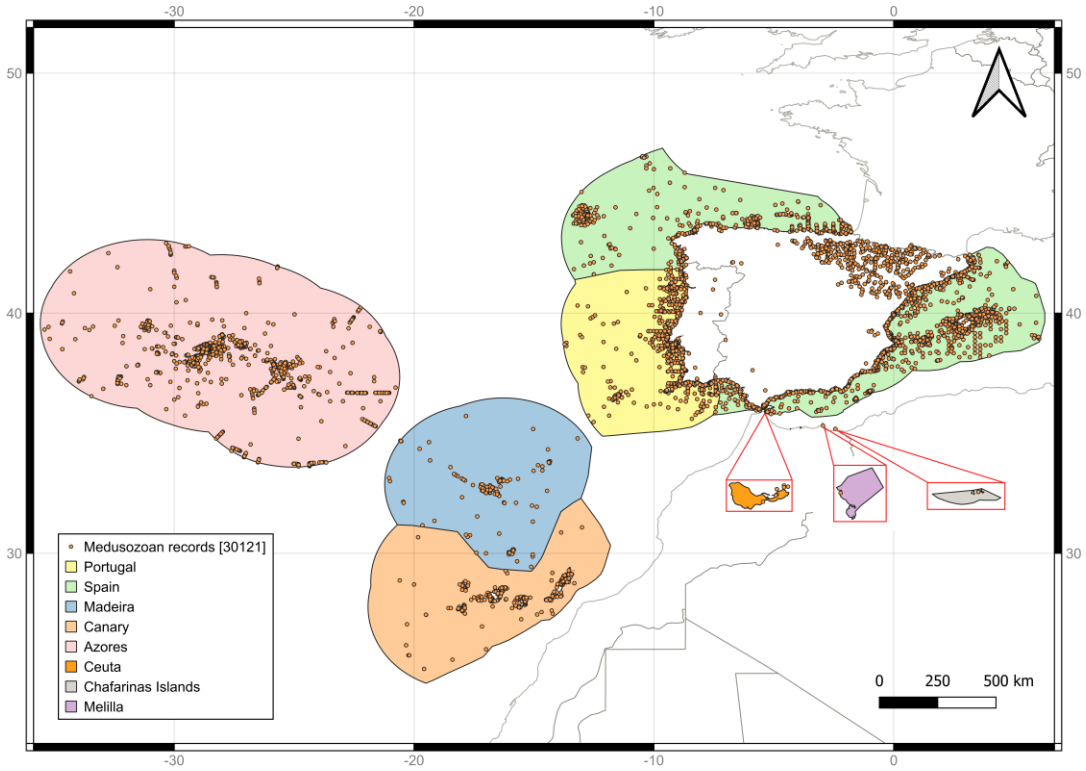


**Fig. 5** Distribution of medusozoans in the Iberian region. The points represent the reports available in Table S 1.

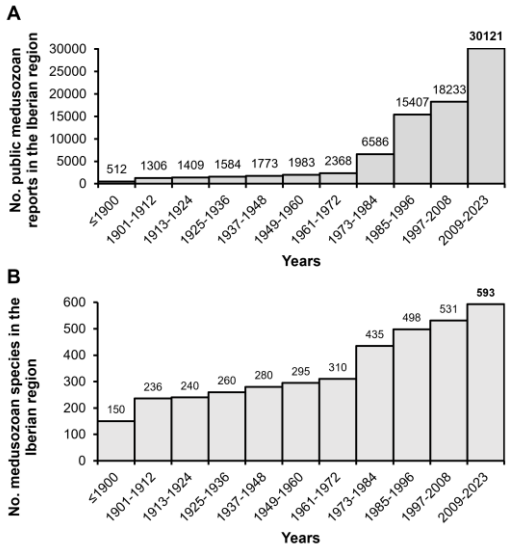


**Fig. 6** Medusozoans reported in the Iberian region over the years. A: Cumulative number of public reports of medusozoans in the Iberian region; B: Cumulative number of medusozoan species in the Iberian region.

The EEZ of Portugal has 10,104 records, documenting 229 species, 143 genera, 72 families, and 11 orders. The region has the largest number of staurozoan species, with three species recorded. Most records in Portugal's EEZ are of holoplanktonic species with a free-swimming life stage. Leptothecata is the most species-rich order, while Siphonophorae has the highest number of occurrence records. *Muggiaea atlantica* Cunningham, 1892 is the most frequently recorded hydrozoan in Portugal, and *Catostylus tagi* (Haeckel, 1869) is the most recorded scyphozoan.

Spain's EEZ has 13,088 records, hosting 423 species, 234 genera, 72 families, and representatives from all 12 orders, surpassing other regions in diversity, especially in hydrozoans and scyphozoans. It is the area of the Iberian region with the highest density of medusozoan species, with most records pertaining to holoplanktonic species with a free-swimming life stage. *Velella velella* (Linnaeus, 1758) is the most frequently recorded hydrozoan, and *Pelagia noctiluca* (Forsskål, 1775) is the most documented scyphozoan.

The Azores EEZ boasts 4892 records, cataloging 264 species, 154 genera, 68 families, and eight orders, with a prevalence of benthic species. Notably, it has the lowest medusozoan species density among the examined areas.

The EEZ of Madeira has the fewest records, with only 692 reports, and it is the least diverse area in the Iberian region, documenting 161 species across 106 genera, 52 families, and eight orders. Reports in this area are mainly from holoplanktonic species with a free-swimming life form and benthic species.

The Canary EEZ, with 1345 records, features 232 species, 145 genera, 71 families, and 11 orders, with most of the records documenting holoplanktonic species with a free-swimming life stage.

In the Azores, Madeira, and Canary EEZs, *P. physalis* emerges as the dominant hydrozoan, while *P. noctiluca* stands out as the most common scyphozoan.

Furthermore, a comprehensive compilation of the most frequently reported hydrozoan and scyphozoan species in both the overall region and individual EEZs has been provided (Table S 3). In addition, a list of reported invasive and introduced medusozoan species is presented (Table 2), along with compiled data on documented bloom-forming species (Table 3), and a gathered list of recorded edible species (Table 4). These tables serve as valuable references for a better understanding of the ecological dynamics of these organisms within the context of the Iberian region and shedding light on their ecological and socioeconomic potential.

**Table 2** Medusozoans with invasive and introduced status reported in the Iberian region.

| *Taxonomy* | *Portugal* | *Spain* | *Azores* |
|---|---|---|---|
| **Hydrozoa** | | | |
| Order Anthoathecata | | | |
| *Cordylophora caspia* (Pallas, 1771) | X | X | |
| *Ectopleura crocea* (Agassiz, 1862) | | | X |
| *Tubularia indivisa* Linnaeus, 1758 | X | | X |
| Order Leptothecata | | | |
| *Blackfordia virginica* Mayer, 1910 | X | | X |
| *Kirchenpaueria halecioides* (Alder, 1859) | X | | X |
| *Tridentata marginata* (Kirchenpauer, 1864) | | X | |
| Order Limnomedusae | | | |
| *Craspedacusta sowerbii* Lankester, 1880 | X | | X |
| *Gonionemus vertens* A. Agassiz, 1862 | X | | |
| *Maeotias marginata* (Modeer, 1791) | X | | |
| **Scyphozoa** | | | |
| Order Rhizostomeae | | | |
| *Phyllorhiza punctata** von Lendenfeld, 1884 | | X | |

*it is the only species with invasive status

**Table 3** Medusozoans with worldwide reports of blooms in the Iberian region.

| *Taxonomy* | *Location* | *Reference* |
|---|---|---|
| **Class Cubozoa** | | |
| Order Carybdeida | | |
| *Carybdea marsupialis* (Linnaeus, 1758) | Denia, Spain | [117] |
| *Copula sivickisi* (Stiasny, 1926) | Shirahama, Japan | [118] |
| **Class Hydrozoa** | | |
| Order Anthoathecata | | |
| *Velella velella* (Linnaeus, 1758) | Northern California Current, USA | [119] |
| Order Leptothecata | | |
| *Aequorea pensilis* (Haeckel, 1879) | North Arabian Sea, Pakistan | [120] |
| *Staurostoma mertensii* (Brandt, 1834) | Bay of Fundy, Grand Manan Island, Canada | [121] |
| Order Limnomedusae | | |
| *Gonionemus vertens** A. Agassiz, 1862 | Berre Lagoon, France | [122] |
| *Olindias muelleri* Haeckel, 1879 | Aegean Sea, Çeşme Peninsula, Turkey | [123] |
| Order Siphonophorae | | |
| *Apolemia uvaria* (Lesueur, 1815) | Norwegian waters, Norway | [124] |
| *Physalia physalis* (Linnaeus, 1758) | Mediterranean Sea | [125] |
| **Class Scyphozoa** | | |
| Order Coronatae | | |
| *Linuche unguiculata* (Swartz, 1788) | Karujeu Island, Solomon Islands | [126] |
| *Nausithoe punctata* Kölliker, 1853 | East China Sea, Northeast Taiwan | [127] |
| *Periphylla periphylla* (Péron & Lesueur, 1810) | Trondheimsfjord, Norway | [128] |

| | | |
|---|---|---|
| Order Rhizostomeae | | |
| *Cotylorhiza tuberculata* (Macri, 1778) | Northern Adriatic Sea | [129] |
| *Phyllorhiza punctata** von Lendenfeld, 1884 | Southern Brazil, Brazil | [130] |
| *Rhizostoma pulmo* (Macri, 1778) | Northern Adriatic Sea | [129] |
| Order Semaeostomeae | | |
| *Aurelia aurita* (Linnaeus, 1758) | Northern Yellow Sea, China | [131] |
| *Aurelia solida* Browne, 1905 | Gulf of Trieste, Croatia | [38] |
| *Chrysaora fuscescens* Brandt, 1835 | Northern California Current, USA | [132] |
| *Chrysaora hysoscella* (Linnaeus, 1767) | Gulf of Trieste, Italy | [133] |
| *Chrysaora quinquecirrha* (Desor, 1848) | Chesapeake Bay, USA | [134] |
| *Cyanea capillata* (Linnaeus, 1758) | Yangtze Estuary, China | [135] |
| *Discomedusa lobata* Claus, 1877 | Iran and Croatia | [136] |
| *Drymonema dalmatinum* Haeckel, 1880 | Puerto Rico | [137] |
| *Mawia benovici* (Piraino *et al.*, 2014) | Gulf of Trieste, Italy | [138] |
| *Pelagia noctiluca* (Forsskål, 1775) | Strait of Messina, Italy | [139] |

**Table 4** Edible jellyfish species recorded in the Iberian region.

| ***Scyphozoa*** | ***References*** |
|---|---|
| **Order Coronatae** | |
| *Periphylla periphylla* (Péron & Lesueur, 1810) | [140] |
| **Order Rhizostomeae** | |
| *Catostylus tagi* (Haeckel, 1869) | [141] |
| *Cotylorhiza tuberculata* (Macri, 1778) | [142] |
| *Phyllorhiza punctata** von Lendenfeld, 1884 | [143,144] |
| *Rhizostoma luteum** (Quoy & Gaimard, 1827) | [145] |
| *Rhizostoma pulmo* (Macri, 1778) | [146,147] |
| *Stomolophus meleagris* Agassiz, 1860 | [142,148,149] |
| **Order Semaeostomeae** | |
| *Aurelia aurita* (Linnaeus, 1758) | [150] |
| *Chrysaora hysoscella** (Linnaeus, 1767) | [145] |
| *Cyanea capillata** (Linnaeus, 1758) | [145] |
| *Pelagia noctiluca* (Forsskål, 1775) | [151] |

2.3.1.1. Class hydrozoa

The Iberian region accounts for 549 hydrozoan species across 261 genera, 96 families, and seven orders. Spain's EEZ boasts the highest hydrozoan diversity with 394 species, followed by Azores (246), Canary (213), Portugal (205), and Madeira (152). The average number of species per order is 78, with Leptothecata emerging as the most speciose order in all the EEZs, boasting 240 species belonging to 95 genera, and *Lensia* Totton, 1932 as the most speciose genus with 19 species. Notably, Leptothecata dominates in Azores and Madeira EEZ's, while Siphonophorae prevails in the other EEZs. Actinulida is exclusive to Spain, with the remaining orders represented in all areas of the Iberian

region. Approximately 57% of documented hydrozoans have a free-swimming life stage, and over 80% are colonial or present a colonial phase. Moreover, more than 62% are holoplanktonic. *Lensia* is the most frequently registered genus, and *P. physalis* is the most documented species in the public Iberian region records.

Introduced hydrozoan species in Portugal comprise *Blackfordia virginica* Mayer, 1910, *Cordylophora caspia* (Pallas, 1771), and *Craspedacusta sowerbii* Lankester, 1880 in brackish and freshwater environments, and *Gonionemus vertens* Agassiz, 1862, *Kirchenpaueria halecioides* (Alder, 1859), *Maeotias marginata* (Modeer, 1791), and *Tubularia indivisa* Linnaeus, 1758 in marine habitats [152]. In Spain, including the Canary Islands, the introduced hydrozoan species are *C. caspia* in freshwater environments, and *Tridentata marginata* (Kirchenpauer, 1864) in marine ecosystems [153]. None of the reported hydrozoans is considered invasive. The distribution heatmap of hydrozoan public records aligns closely with the total distribution of medusozoans, encompassing almost 88% of total medusozoan reports (Fig. 7). Reports of hydrozoans in the Iberian region are higher in the spring and summer months and lower in the autumn and winter months (Fig. S 4).

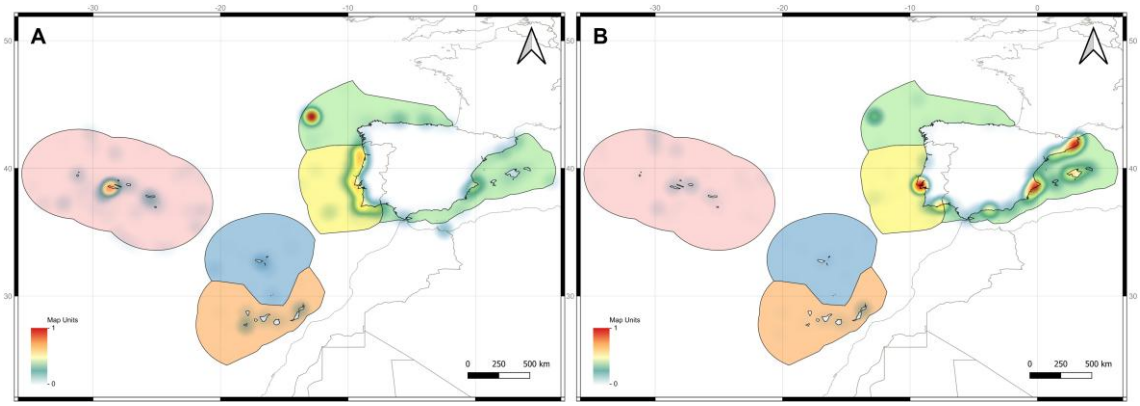


**Fig. 7** Heatmaps of Medusozoa distribution in the Iberian region. A: Hydrozoa. B: Scyphozoa. Heatmaps represent the marine reports available in Table S 1.

2.3.1.2. Class scyphozoa

In the study region, there are 37 species of Scyphozoa belonging to 22 genera, 15 families, and three orders. Spain's EEZ holds the highest scyphozoan diversity with 26 species, followed by Portugal (20), Azores (18), Canary (17), and Madeira (9). On average, each order contains 12 species, with Coronatae being the most speciose with 17 species belonging to six genera, and *Nausithoe* Kölliker, 1853 as the leading genus with eight species. Remarkably, the predominant scyphozoan order displays significant variation across different EEZs. Rhizostomeae overwhelmingly dominates in Portugal, shares dominance with Semaeostomeae in Spain, is absent in the Azores and Madeira

EEZs, and sees Semaeostomeae as the dominant order in the Canary EEZ. *P. noctiluca*, is the most frequently recorded scyphozoan species, while the Australian spotted jellyfish *Phyllorhiza punctata* von Lendenfeld, 1884 is the only invasive species recorded in the Iberian region. The distribution heatmap of scyphozoan public occurrences in the Iberian region shows a hotspot in the central area of the Portuguese coast and on the Spanish Valencian and Catalan coasts (Fig. 7). Scyphozoan reports show they start to increase in the beginning of spring until they reach a maximum in the warm August summer.

#### 2.3.1.3. Class staurozoa

The Iberian region contains five staurozoans from the Stauromedusae order, distributed among four genera and three families. Notable sightings include *Calvadosia campanulata* (Lamouroux, 1815) in Portugal (Setúbal) and Spain (Southern Bay of Biscay) [90,154], *Depastrum cyathiforme* (M. Sars, 1846) in the Canary Islands (Lanzarote) [155], *Haliclystus auricula* James-Clark, 1863 in Portugal (Porto and Viana do Castelo) [154,156], *Lipkea ruspoliana* Vogt, 1886 in Portugal (Arrábida Natural Park) [106], and *Stylocoronella riedli* Salvini-Plawen, 1966 in Spain (Southern Bay of Biscay) [90,152].

#### 2.3.1.4. Class cubozoa

Two cubozoan species of the order Carybdeida were documented in the study area, rendering Chirodropida the sole unrepresented medusozoan order in the region. *Carybdea marsupialis* (Linnaeus, 1758) was reported in Portugal [153] and Spain [117,159–162], while *Copula sivickisi* (Stiasny, 1926) was reported in the Canary Archipelago [163].

### 2.3.2. Omics data

The comprehensive examination of Cnidaria data gathered from repositories such as NCBI SRA, ProteomeXchange, PRIDE, MassIVE, iProX, and UniProt Proteome, as well as a literature review focused specifically on medusozoan proteomic studies (until November 2023), unveiled interesting insights. Among the vast phylum Cnidaria, only about 650 out of the approximately 12,100 species have publicly available genomic (WGA, WGS), transcriptomic (RNA-Seq) (Table S 4), or proteomic data (Table S 5). Notably, only 20% of these belong to the Medusozoa clade, comprising 87 hydrozoans, 26 scyphozoans, nine cubozoans, and eight staurozoans, totaling 130 species. Remarkably, nearly half of these medusozoan species were reported in the Iberian region, encompassing 53 hydrozoans, 12 scyphozoans, two staurozoans and two cubozoans. Noteworthy representatives such as the scyphozoans *Chrysaora*

*fuscescens* Brandt, 1835, and *Chrysaora quinquecirrha* (Desor, 1848), and the hydrozoans *Clytia hemisphaerica* (Linnaeus, 1767), and *H. vulgaris*, are particularly distinguished for having all three types of omics data publicly accessible. Within the Anthozoa class, over 77% of genomic, transcriptomic, and proteomic data in the repositories originate from the last five years. This trend is even more pronounced for Medusozoa, (over 87%), indicating a growing interest in employing omics approaches to study these organisms. Many studies have employed various specimens or pooled tissues for the extraction of DNA, RNA or proteins from cnidarians. A broad array of tissues and structures, such as acrorhagi, bell margin, body wall, branch, gonad, head, nectosome, oral arm, siphosome, tentacle, umbrella, and zooid, have been used for genetic material extraction. Notably, the extensive use of nematocysts should be highlighted, as much research on Cnidaria is particularly focused on studying their venoms and toxins [164–166].

## 2.4. Discussion

### 2.4.1. Medusozoan diversity and distribution in the Iberian region: key findings and considerations

In biodiversity studies, the nature of compiled data plays a crucial role in result analysis. This study focuses on Medusozoa reports in the Iberian region, documented through sightings and samplings. Medusozoan sightings are influenced by organism size, morphology, and other visual characteristics, where smaller or more transparent organisms pose challenges in their detection [87,167]. Depth in the water column is also a factor to consider, as organisms from deeper waters may be less observable compared to those closer to the surface. Yet, diminished visibility in deeper waters does not necessarily signify lower organism abundance [168]. In some cases, the use of Remotely Operated Vehicles (ROVs) can help surpass this limitation [169]. Sampling methods including dredges, trawl nets, and plankton nets, present additional challenges. Indeed, various sampling methods demonstrate selectivity towards distinct animal groups [170]. Dredges and trawl nets are better suited for benthic medusozoans [171], while plankton nets, designed for planktonic medusozoans, may yield varied results influenced by mesh size [172,173].

#### 2.4.1.1. Diversity

Hydrozoans take the lead in the Iberian region, particularly with the prevalence of the dominant Siphonophora order, a trend consistent with their recognition as the most speciose class in the clade [54]. This aligns with findings from Hosia *et al.* (2017) [170],

who studied gelatinous organisms in the northern Mid-Atlantic Ridge from Iceland to the Azores, confirming siphonophores as the most abundant species in that region.

The highest reported medusozoan species is the Portuguese man-of-war, *P. physalis*. Known for its high toxicity and potent stinging capabilities, this species is easily recognized by local communities due to awareness campaigns, and its distinctive features such as vibrant colors, large tentacles, and a sizable pneumatophore, as well as for frequently washing up on beaches [174,175]. The reports are predominantly derived from human observations submitted on the accessed databases, which may help explain why this is the most reported species in the region. Within Scyphozoa, the order Coronatae, which is the least speciose order, exhibited the highest species richness in the region. The pre- dominant scyphozoan order varied a lot between EEZs. Interestingly, the EEZs of Azores and Madeira archipelagos did not present any Rhizostomeae, contradicting the supposed distribution of *C. tagi* (Scyphozoa, Rhizostomeae) from the Tagus estuary to the African coast [176]. Currently, the predominantly meroplanktonic nature of scyphozoans is challenged solely by the holoplanktonic *Pelagia* and *Periphylla*, lacking a polyp stage in their life cycle [51]. However, comprehensive knowledge of scyphozoan life cycles remains limited, with only about 50 out of nearly 250 species having fully described life cycles [51,102]. In this study, scyphozoan species with undisclosed life cycle details were pro- visionally classified as meroplanktonic for analytical purposes. Yet, a more thorough understanding requires additional data, especially from deep-sea species that can have different life cycle features (Morandini, personal communication). Among the scyphozoan genus documented in the region, *Atolla* Haeckel, 1880, *Discomedusa* Claus, 1877, Mawia Avian *et al.*, 2016, *Paraphyllina* Maas, 1903, Periphyllopsis Vanhöffen, 1902, and *Poralia* Vanhöffen, 1902 have only their free-swimming stage described, with their complete life cycles yet to be documented.

The study region observes five staurozoan species, which may appear limited in number but represent 10% of the class's diversity. The absence of recorded staurozoans in the Azores and Madeira is likely due to limited investigations on this group. Global distribution patterns, concentrated in mid-latitudes, suggest the unlikelihood of staurozoan absence in both archipelagos [63]. Scarcity of scientific research locally and globally, contributes to the limited available information on this taxonomic group. Notable exceptions are the publications by Miranda *et al.* [62–65,105,106,177–179].

Limited data on Cubozoa in the Iberian region comprises two species: *C. marsupialis*, reported throughout the Mediterranean and considered native [180,181], and *C. sivickisi*, recently reported in the Canary [163]. The latter report raises questions given the

absence of other Iberian reports and misidentifications observed in previous studies mistaking other cubozoan species for *C. marsupialis* [181]. On the other hand, Schlaefer *et al.* (2020) [182] propose *C. sivickisi* as a cosmopolitan species, supporting its presence in the Mediterranean Sea. The absence of cubozoans and staurozoans in the Azores and Madeira archipelagos aligns with Gueroun *et al.* (2021) [91], who also observed no cubozoan records in the Macaronesia region, of which the Azores and Madeira archipelagos are a part.

This research extends the data compilation initiated by Rodrigues *et al.* (2020) [8], originally focused solely on Portugal. The current analysis reveals a substantial increase of 135 species reported in Portugal, encompassing mainland Portugal, Azores, and Madeira, rising from 272 in Rodrigues *et al.* (2020) [8] to 407 in the present study. This expansion is attributed to the use of GBIF, iNaturalist and OBIS data, along with an expansion of the literature volume revisited, especially older publications predating the 1960 s. It is important to acknowledge that certain species likely no longer inhabit the region, possibly facing local extinctions. This assumption is supported by the absence of recent reports for several species documented in the past, such as *Atolla bairdii* Fewkes, 1886 [183,184], *Atolla verrillii* Fewkes, 1886 [184,185], *D. cyathiforme* [155], *Nausithoe rubra* Vanhöffen, 1902 [186], and *Zygophylax pinnata* (G. O. Sars, 1874) [187]. Conversely, it is equally or more plausible that other species may inhabit the area but have not yet been recorded. In fact, comparing the gathered results to other diversity studies provides an intriguing perspective. Gravili *et al.* (2015) [188] reported 115 non-siphonophore hydrozoan species in the Mediterranean Salento Peninsula, while the present study identified around 200 from Mediterranean records. Additionally, Bouillon *et al.* (2004) [189] documented 457 hydrozoan species in the entire Mediterranean, whereas the current investigation found 549, with 256 belonging to Mediterranean reports. When accounting temporal evolution, following Gravili *et al.* (2015) [190] consideration, and focusing on species reported in the last decade, the total number of medusozoan species in the Iberian region decreases to 226, with less than 7000 records.

2.4.1.2. Distribution

Medusozoans in the Iberian region concentrate mainly along the coasts of Portugal and Spain, and in the Azores central group, with a notable concentration of records in the Northwest of Spain's EEZ. This "hotspot" is explained by a substantial number of Siphonophora records from the OBIS database, specifically sourced from the National Oceanography Centre Southampton dataset "Discovery Collections Midwater Database" [191], reflecting extensive sample campaigns during Discovery sailings. This illustrates the importance of analytical caution. While compiled reports may not precisely reflect the

actual prevalence of Medusozoa in the study area, the commendable effort acknowledges data limitations. To enhance diversity and distribution analysis, obtaining additional data through monitoring programs, such as the Portuguese GelAvista program, a citizen science project monitoring gelatinous organisms [175], and the CIESM JellyWatch Program, dedicated to recording jellyfish outbreaks across the Mediterranean Sea [192] is crucial.

Results show that holoplanktonic medusozoans are more widely distributed and abundant than benthic and meroplankton species in the Iberian region (Fig. S 5). This pattern aligns with the distribution of pelagic medusozoans and ctenophores in Macaronesia [91] and global patterns of medusozoan distribution and abundance [10]. These results confirm global trends indicating benthic and meroplanktonic medusozoans primarily found in coastal waters, while holoplanktonic species exhibit no specific preference for coastal or open waters [10]. The observed medusozoans diversity by life cycle showed higher diversity of benthic species, followed by holoplanktonic, and meroplanktonic. These results partially contradict the global trend that suggests that benthic species are indeed the highest diverse, but holoplanktonic species are typically less diverse than meroplankton [193]. The importance of addressing the gaps in the current understanding of medusozoan life cycles becomes evident, as new knowledge can potentially alter observed trends. The higher diversity of medusozoans in the Northeast Atlantic Ocean compared to the Mediterranean Sea can be attributed to several factors. For example, the Strait of Gibraltar, a narrow passage connecting the two seas, plays a crucial role in shaping water flow dynamics. The denser and saltier Atlantic waters form a surface layer that flows over the Mediterranean waters, while a subsurface current flows out of the Strait and into the Atlantic, with the latter having a lesser impact on the volume of water exchanged. [194]. The Northeast Atlantic Ocean, with its larger size, complex oceanographic regime, and enhanced connectivity to other waters, may provide a more diverse ecosystem with a wider range of habitats for medusozoans to thrive. In contrast, the Mediterranean Sea is a more stable and predictable environment, characterized by less niche differentiation and fewer nutrients that may limit the diversity of medusozoans [195]. Additionally, human activities such as fishing, pollution, and coastal development may have greater impacts on the semi-enclosed Mediterranean Sea than in the wide-open Northeast Atlantic Ocean, leading to reduced global diversity of medusozoans.

There is a significant scarcity of information worldwide concerning the nature - whether native, introduced, or invasive - of medusozoan species, and the Iberian region is no exception. The GISD (2023) [196] recognizes only one medusozoan species as invasive,

the white spotted jellyfish *P. punctata*. The GRIIS catalogue (2022) [110] provides further insights into invasive and introduced medusozoans in the Iberian region. However, recent sightings of *P. punctata* along the southwestern Atlantic coast of Spain [197], have yet to be included. Additionally, the GRIIS acknowledges the rhizostomeae *Rhopilema nomadica* Galil *et al.*, 1990 in its checklist for Spain as an introduced species, but despite extensive data compilation efforts, the occurrence of this species in the Iberian region could not be confirmed.

2.4.1.3. Omics disparity in Cnidaria: unraveling Anthozoa's wealth and Medusozoa's potential for bioprospection of natural compounds

The analysis of cnidarian omics metadata collected various repositories and literature, highlights a significant disparity between Anthozoa and Medusozoa, the two primary clades of the phylum. Approximately 80% of available omics data pertains to Anthozoa (Fig. S 6), resulting in four times the amount of information available for Medusozoa. This aligns with the prevalent focus on bioprospecting for natural products within the Cnidaria phylum. Cnidaria stands as the second most extensively explored group of marine invertebrates in the search for novel natural products [198,199], with most of these natural products being sourced from benthic Anthozoan species [200]. This imbalance may be due the easier access to samples from anthozoans compared to medusozoans. Moreover, the available information on Cnidaria is concentrated on a few species, with the top seven cnidarian species holding over half of the total omics data of the phylum. These include anthozoans *Nematostella vectensis* Stephenson, 1935 and four species of *Acropora* Oken, 1815, along with the hydrozoan *H. vulgaris*. Notably, *H. vulgaris* constitutes approximately 60% of the total Medusozoa data, primarily due to its status as a model organism.

Genomes annotation is crucial for functional gene identification and protein characterization, aiding in metagenomics, transcriptomic, and proteomics studies. However, the annotated genomes from Cnidaria are limited to 33 from Anthozoa, six from Hydrozoa, and three from Myxozoa. Remarkably, there is an absence of annotated genomes from Scyphozoa, emphasizing the insufficient attention that jellyfish has received in omics studies despite the recent increasing interest. Most of the medusozoan omics data has been generated in the past five years, with a predominant focus on venom studies. The main objective of such studies is to identify and characterize the molecular structure, function, and mode of action of toxins. This information serves valuable purposes, including comparative analyses, exploration of bioactive compounds, and the development of more effective treatments for jellyfish stings in humans [201–204]. Furthermore, omics studies can aid unravel the evolutionary and phylogenetic

relationships of Medusozoans, shed light on their developmental processes and mechanisms of gene regulation, and provide insights into host-microbiota interactions [84,205–208]. Omics projects focusing on underexplored species holds promise for significant advancements in bioprospecting natural compounds with numerous relevant biotechnological applications [209,210].

Exploring the omics of Cnidaria, with its rich diversity, involves extracting genetic material from various tissue types. The choice depends on the inherent characteristics of the organisms and the study objectives. Extract genetic material from small or low-cell content organisms, such as rich in water gelatinous medusozoans, can be challenging [14]. Often, a collective pool of specimens or tissues is used to concentrate samples, addressing biological heterogeneity and enhancing quality control [211]. Moreover, studies may encompass organisms at different life stages, providing a holistic understanding of the genetic landscape across developmental phases [212]. Some investigations focus on organisms or tissues under distinct laboratory conditions, offering insights into the impact of environmental variables on genetic expression [213]. Other research may analyze specific tissues or structures to achieve their objectives [205,214]. This versatility of omics studies ensures the alignment of research objectives and the characteristics of the organism under study.

## 2.5. Conclusion

Information on Medusozoa in the Iberian region is spread across more than 200 publications and thousands of reports available in public databases. This investigation brings together all this vast information in a collective dataset, comprising over 30,000 records of 593 Medusozoa species across four classes along with omics metadata covering the phylum Cnidaria. Addresses the challenge of dispersed and varied data formats, presenting a valuable preliminary resource for researchers and stakeholders.

Key findings from our comprehensive analysis shed light on critical aspects of medusozoan species, including the most prevalent with each class, those reaching highest abundances in specific EEZs, invasive and introduced species, bloom-forming organisms, and edible species. A sustained trend of discovering new Medusozoa species over time was observed in the examination, underscoring the untapped potential for further exploration in the region.

The surge in available omics data in recent years reflects a growing interest in unlocking the biotechnological potential of Cnidaria, and Medusozoa in particular. Although limited, studies have explored a range of topics, including analysis of novel toxins and other non-toxin compounds, evolutionary and phylogenetic relationships, developmental

processes, gene regulation, and microbiome investigations. Additional data would be needed to obtain a more accurate view of Cnidaria in the Iberian context. As future work, it would be interesting to add Anthozoa data compiled from the literature, and integrate data from monitoring programs, such as citizen science initiatives.

This comprehensive compilation of data on medusozoan diversity and omics research establishes a sturdy groundwork for future research. It aids in pinpointing less-explored target organisms and underscores the pivotal role of certain species in the environment, society, and economy.

# Chapter 3.

# Aquatic Invertebrate Antimicrobial Peptides in the Fight Against Aquaculture Pathogens

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## Abstract

The intensification of aquaculture has escalated disease outbreaks and overuse of antibiotics, driving the global antimicrobial resistance (AMR) crisis. Antimicrobial peptides (AMPs) provide a promising alternative due to their rapid, broad-spectrum activity, low AMR risk, and additional bioactivities, including immunomodulatory, anticancer, and antifouling properties. AMPs derived from aquatic invertebrates, particularly marine-derived, are well-suited for aquaculture, offering enhanced stability in high-salinity environments. This study compiles and analyzes data from AMP databases and over 200 scientific sources, identifying approximately 350 AMPs derived from aquatic invertebrates, mostly cationic and α-helical, across 65 protein families. While *in vitro* assays highlight their potential, limited *in vivo* studies hinder practical application. These AMPs could serve as feed additives, therapeutic agents, or in genetic engineering approaches like CRISPR/Cas9-mediated transgenesis to enhance resilience of farmed species. Despite challenges such as stability, ecological impacts, and regulatory hurdles, advancements in peptidomimetics and genetic engineering hold significant promise. Future research should emphasize refining AMP enhancement techniques, expanding their diversity and bioactivity profiles, and prioritizing comprehensive *in vivo* evaluations. Harnessing the potential of AMPs represents a significant step forward on the path to aquaculture sustainability, reducing antibiotic dependency, and combating AMR, ultimately safeguarding public health and ecosystem resilience.

**Keywords**: antimicrobial peptides; aquatic invertebrates; aquaculture; bioactive compounds; cryptides; pathogens; antimicrobial resistance; antibacterial; antifungal; antiviral

## 3.1. Introduction

The transition to intensive aquaculture has increased population densities, resulting in stress and weakened immune systems in farmed species, which increases their susceptibility to infectious diseases. These diseases contribute to high mortality rates, reduced productivity, and significant economic losses [215]. To address these issues, antibiotics were introduced, initially reducing disease-related mortality and supporting industrial growth [216]. However, antibiotic overuse promoted the resurgence of bacterial diseases, with the emergence of new pathogens and the reoccurrence of older infections, driven by the rapid spread of AMR [217,218]. Bacteria develop resistance through various mechanisms, such as the use of efflux pumps to actively expel antibiotics, the production of enzymes that degrade or modify antibiotics, and horizontal gene transfer, which allows the exchange of resistance genes between bacterial populations, including from nonpathogenic to pathogenic strains [219–221]. Furthermore, bacterial mutations change antibiotic targets, resulting in the development of treatment-resistant strains that cause more severe and prolonged infections, leading to increased healthcare costs [222]. Nevertheless, there is currently no comprehensive global system to regulate and monitor the use of antimicrobial agents in aquaculture. Despite the approval of only a limited number of antibiotics for use, they are still widely and indiscriminately applied, especially in major aquaculture-producing countries in Asia [223,224]. The unrestricted use of antibiotics in aquaculture poses environmental risks, including accumulation in culture systems, disruption of microbial communities, and harm to non-target organisms. It promotes the development of antibiotic-resistant microbial strains, reducing antibiotic efficacy, threatening aquaculture sustainability, and posing human health risks through the consumption of seafood contaminated with resistant bacteria [225]. Therefore, it is crucial to explore alternative strategies to address pathogens and AMR in aquaculture, such as the use of AMPs.

AMPs are evolutionarily conserved, gene-encoded natural molecules with diverse functional and structural properties. They have a wide range of biological activities, including antibacterial, antifungal, antiviral, antiparasitic, anticancer, antibiofilm, immunomodulatory, wound healing, and anti-inflammatory [226–233]. These molecules provide distinct advantages, including rapid and direct antimicrobial action, broad-spectrum activity against bacteria, viruses, fungi, and parasites, and a reduced risk of contributing to AMR, making them highly attractive for clinical applications [24,234]. In metazoans, AMPs evolved through recurrent gene duplications, leading to the emergence of paralogs, and balancing/positive selection in response to bacterial

pathogens [23,235,236]. The first AMP, gramicidin, was isolated in 1939 from a soil Bacillus strain [237]. Since then, numerous AMPs have been isolated, many of which are specific to certain taxa or species [238]. Their common features include being composed of 10–100 amino acids, having a molecular weight below 25–30 kDa, and a positive net charge [239–241]. Despite their common features, AMPs vary widely in structural motifs and secondary structures—such as α-helical, β-sheet, αβ motifs, and random coils—with similar sequences often adopting different conformations, leading to diverse classifications [242,243]. AMPs can be natural (NAMPs), directly isolated from natural sources, or produced synthetically (SAMPs) [244]. While NAMPs may have issues like instability, low solubility, toxicity, and salt sensitivity [245], SAMPs are designed based on NAMPs with modifications to enhance bioactivity. These modifications include the addition of specific amino acid residues, sequence truncation to retain only active regions, cyclization, and peptidomimetics for improved stability [238,246–248].

AMPs exhibit diverse modes of action to target and disrupt microbial cells. Most AMPs primarily target cell membranes through several proposed mechanisms, including the toroidal pore, barrel-stave, carpet, and agglutination models. In the toroidal pore model, peptides integrate into the membrane, forming pores that remain associated with the membrane's polar groups [249]. In the barrel-stave model, peptides create transmembrane pores that form a central cavity and detach after formation [250]. The carpet model involves peptides interacting with the membrane surface, disrupting its structure and causing the formation of multiple pores [251]. In the agglutination model, peptides aggregate bacterial cells into a micellar complex, preventing membrane penetration [252]. Alternatively, some AMPs interfere with intracellular processes, such as protein synthesis, nucleic acid functions, and enzyme activity, impairing cell wall formation. Other AMPs disrupt cellular energy metabolism by inhibiting ATP synthase or interfering with the electron transport chain, ultimately leading to cell death [253]. New AMPs are discovered through several approaches, including direct extraction from cells or tissues followed by identification via mass spectrometry, cDNA cloning of AMP genes using primers targeting conserved regions, and omics-based methods that integrate genomic, proteomic, or transcriptomic data with bioinformatics analysis [254]. Additionally, the development of advanced AMP prediction tools like amPEPpy [255] and AMPlify [256], which use artificial intelligence and machine learning tailored to predicting and characterizing AMPs, alongside AlphaFold2 [257], which utilizes deep learning to predict protein structures from amino acid sequences, has greatly improved the discovery and characterization of new AMPs. While amPEPpy and AMPlify focus specifically on AMP prediction, AlphaFold2 provides a more generalized solution for

predicting the structures of proteins, offering faster and more accurate predictions than traditional methods like X-ray crystallography, Nuclear Magnetic Resonance (NMR), and cryogenic electron microscopy (cryo-EM), which are accurate but time-consuming and labor-intensive. While advances in DNA sequencing have vastly increased the number of known protein sequences, the structures of many remain undetermined. AlphaFold has revolutionized the field by providing a faster and more accurate way to predict protein structures, helping bridge the gap between the substantial number of known sequences and the limited number of experimentally determined structures. Several AMP databases, including APD3 [258], CAMPR4 [259], DBAASP [260], dbAMP 2.0 [261], DRAMP 3.0 [262], and Inverpep [263], serve as useful repositories for cataloging known AMPs. However, these resources face limitations such as data redundancy, lack of standardization, and inadequate curation. The StarPep graph database, which integrates over 45,000 peptide sequences and metadata from 42 databases, including more than 22,600 AMPs, represents a notable effort to address these issues [264]. Nonetheless, a significant data gap for AMPs derived from aquatic invertebrates still persists, given the sparsity of relevant information, which is dispersed across numerous studies, and often lacks structural information.

Our study provides a comprehensive review of AMPs derived from aquatic invertebrates. We focus on the AMPs' activities against pathogens relevant to both aquaculture and human health, including antibacterial, antifungal, antiviral, antiparasitic, antibiofilm, and anticancer properties. Furthermore, we address gaps in knowledge about the structures of these AMPs and explore their potential applications for the aquaculture industry.

## 3.2. Data Collection and Analysis

A comprehensive data review was conducted to compile AMPs derived from aquatic invertebrates, classified within the Marine, Brackish, and Freshwater categories as defined by the World Register of Marine Species (WoRMS) database [265].

Data on the AMPs were sourced from over 200 scientific articles and several databases, including the National Center for Biotechnology Information (NCBI) GenBank [266], UniProt [267], RCSB Protein Data Bank (RCSB PDB) [268], and StarPep [264]. The selection of articles was based on specific inclusion criteria, prioritizing peer-reviewed studies with clear methodologies, and peptides with documented experimental evidence of antimicrobial activity, either through antimicrobial assays or gene expression analyses following antimicrobial challenges. Articles lacking sufficient data or relevance to the review's focus were excluded. Duplicate records and inconsistencies were systematically checked and removed.

Given the focus on AMP activity against pathogens relevant to aquaculture and human health, the pathogenic status of the reported microbial agents was verified by consulting over 180 articles and books, e.g., [269–271] (Table S 6). The study adhered to the accepted nomenclature from the List of Prokaryotic names with Standing in Nomenclature (LPSN) [272] for bacteria, MycoBank [273] for fungi and fungi-like organisms, and the NCBI Taxonomy browser [274] for viruses and parasites.

The physical and chemical properties of AMPs, including net charge, molecular weight (MW), isoelectric point (pI), and the grand average of hydropathy (GRAVY), were calculated using a custom Biopython script based on the ProtParam tool from the Expasy Proteomic Server [275]. Signal peptides were predicted using SignalP 6.0 [276], and propeptides were predicted using ProP 1.0 with minimum probability confidence of 70% [277]. Mature AMP sequences were analyzed against the Pfam database [278] using InterProScan [279] to identify protein domains and functional annotations. Structural predictions were made using ColabFold [280] with the AlphaFold2 model [257], generating for each peptide, five independent models with 10 recycles to enhance prediction accuracy. Model relaxation ensured physical plausibility and energetic stability, and the highest-ranked model was selected for structure visualization using ChimeraX [281]. ColabFold and AlphaFold2 provide significant benefits in terms of speed, accuracy, and cost-effectiveness for predicting AMP structures. However, they may face challenges with highly flexible or disordered peptides, which are common in AMPs and may not fully capture the dynamic nature of AMP-pathogen interactions essential to their antimicrobial activity. Despite these limitations, these tools accelerate the understanding of AMP structures in the absence of experimental data, thereby supporting further experimental validation.

## 3.3. Aquaculture Pathogens

Pathogen control is a major challenge in aquaculture, where densely populated, intensive farming systems facilitate the rapid spread of infections, emphasizing the need for effective disease management strategies [282]. A comprehensive approach includes proper husbandry practices, the development of disease-resistant strains, and the implementation of robust biosecurity protocols, such as quarantine procedures, water treatment methods, and regular health monitoring. Additionally, preventive strategies, including vaccination, the use of immunostimulants, and the application of probiotics, as well as advances in pathogen biology and epidemiology, are essential for improving treatment strategies and reducing disease outbreaks [283–285].

Pathogens, including bacteria, fungi, viruses, and parasites, cause significant diseases in farmed aquatic organisms in both marine and freshwater systems. These diseases result in high mortality rates, reduced productivity, and substantial economic losses [286]. Among these, bacterial pathogens are the most prevalent, causing diseases such as columnaris disease, edwardsiellosis, furunculosis, septicemia, streptococcosis, ulcerative diseases, and vibriosis. Genera such as *Aeromonas*, *Edwardsiella*, *Enterobacter*, *Flavobacterium*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, and *Vibrio* have developed multidrug-resistant strains, which complicate treatment efforts [287,288]. These bacteria use mechanisms like biofilm formation, iron acquisition, extracellular polysaccharide production, and lytic enzyme production to enhance their virulence and persistence in aquaculture systems [289].

Fungal and fungi-like pathogens also pose significant threats. Water molds, like *Saprolegnia* and *Aphanomyces*, were initially classified as fungi due to their morphology and feeding behavior but are now recognized as oomycetes [290]. Differentiating between true fungi (e.g., members of *Ascomycota*, *Basidiomycota*, and *Mucoromycota*) and oomycetes is crucial for developing effective treatments [291]. While true fungi typically infect external fish tissues, oomycetes can invade internal organs and are commonly found in aquaculture systems [285,292].

Viral diseases represent an increasing concern in aquaculture, with significant pathogens including Infectious Salmon Anaemia Virus (ISAV), viral hemorrhagic septicemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV), Shrimp hemocyte iridescent virus (SHIV), Abalone Herpesvirus (AbHV), and White Spot Syndrome Virus (WSSV) [293]. Several factors contribute to the growing concern, including the high mortality rates associated with viral infections and the lack of effective therapeutic options compared to bacterial infections, which can often be treated with antibiotics. In contrast, viral diseases primarily rely on preventive measures, such as vaccination, which is not available for all pathogens [294]. Moreover, viral infections can spread rapidly in densely populated aquaculture systems and may remain asymptomatic in the early stages, complicating early detection and containment. The rapid mutation rates and ability of viruses to evolve quickly further challenge control efforts, as new strains may emerge that evade existing measures [294]. Additionally, parasitic infestations caused by sea lice and protozoans further reduce production yields and increase treatment costs [295,296].

In this study, the antimicrobial activities of aquatic invertebrate-derived AMPs were compiled, with a focus on pathogens relevant to aquaculture (Fig. 8). A detailed list of

microorganisms, along with their pathogenic significance for aquaculture and human health, is provided in Table S 6.

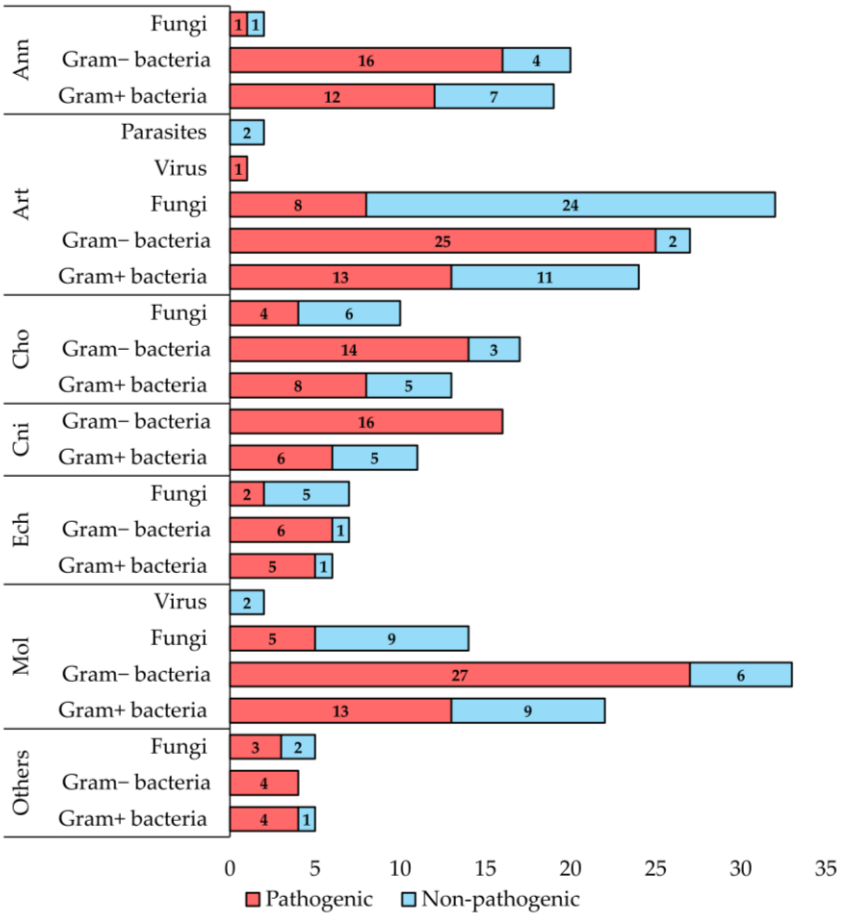


**Fig. 8** Distribution of antimicrobial activities of aquatic invertebrate AMPs against various pathogens. The chart categorizes microorganisms into Gram-positive bacteria, Gram-negative bacteria, fungi, viruses, and parasites, showing their pathogenic (red) and non-pathogenic (blue) statuses. Each taxonomic group (*Ann: Annelida, Art: Arthropoda, Cho: Chordata, Cni: Cnidaria, Ech: Echinodermata, Mol: Mollusca, Others: Nematoda, Placozoa, Platyhelminthes, Porifera*) highlights the diversity and potential antimicrobial targets of AMPs, emphasizing their relevance to aquaculture pathogens.

Notably, aquatic invertebrate-derived AMPs demonstrated activity against a wide range of pathogens, including 47 Gram-negative bacteria, 25 Gram-positive bacteria, 11 fungi and fungi-like organisms, and the virus WSSV. Among these, the Gram-negative bacterium *E. coli*, the Gram-positive bacterium *S. aureus*, and the fungus *C. albicans* were identified as the primary targets of these AMPs.

## 3.4. AMPs and Other Antimicrobial Agents Isolated from Aquatic Invertebrates

Aquatic invertebrates are globally distributed and remarkably diverse, representing ancient animal lineages with diversified adaptations to overcome microbial threats in

environments increasingly impacted by anthropogenic factors [297]. Traditionally, invertebrates, unlike vertebrates, were thought to lack an adaptive immune system, relying solely on an innate immune system that includes both cellular and humoral responses mediated by pattern recognition receptors [298]. However, recent evidence suggests that some invertebrates exhibit immunological priming leading to enhanced long-term immune responses, although the underlying mechanisms remain largely unexplored [299]. The cellular immune response in invertebrates involves defense mechanisms such as encapsulation, nodule formation, and phagocytosis, mediated by motile cells known as hemocytes found in the hemolymph. The humoral immune response, on the other hand, is characterized by the presence of antimicrobial substances in the blood cells and plasma, together with responses such as hemolymph coagulation [300]. AMPs are an integral part of the humoral defense system of invertebrates, protecting against infection primarily by disrupting microbial plasma membranes and compromising microbial integrity [295]. AMPs from aquatic invertebrates typically possess cationic and hydrophobic properties, which are critical for effectively targeting key components of microbial cell walls and membranes [301]. Specifically, marine-derived AMPs have evolved to thrive in the unique conditions of the seawater, characterized by its high salinity. This adaptation has led to enhanced electrostatic interactions, increased stability, and a broader antimicrobial spectrum, ultimately making them more potent antimicrobial agents [302]. Invertebrates are the major source of AMPs among aquatic organisms, with approximately 40 families of AMPs characterized to date [234]. This study focuses on invertebrates and the content is organized into sub-chapters based on phyla, providing a comprehensive overview of the diverse range of AMPs derived from aquatic invertebrates (Fig. 9, Table S 7).

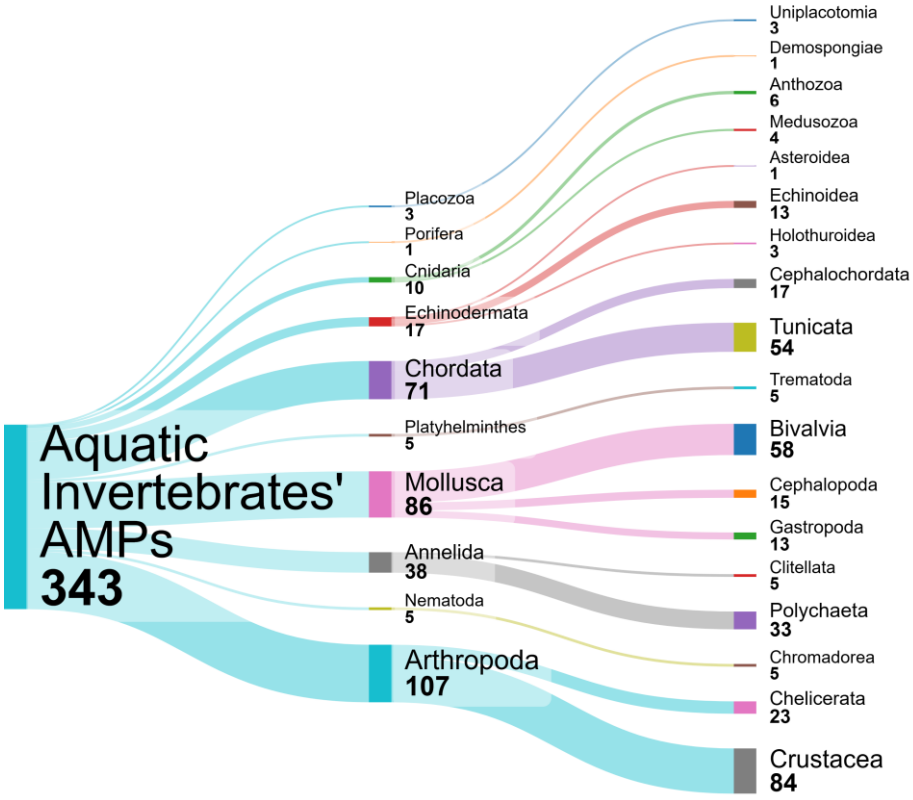


**Fig. 9** Phylogenetic distribution of currently known AMPs from aquatic invertebrates by phylum and subphylum/class.

### 3.4.1. Non-Phylum-Specific AMPs

#### 3.4.1.1. Defensins

Defensins are small, cysteine-rich cationic AMPs involved in innate immunity across a wide range of organisms, including invertebrates. These proteins are classified into α-, β-, and θ-defensins, with invertebrates producing β-defensins and big defensins. β-defensins feature a conserved β-sheet structure stabilized by three disulfide bridges and show antimicrobial activity against bacteria, fungi, and viruses [303]. Big defensins, considered ancestral to vertebrate β-defensins, feature a bipartite structure with an N-terminal hydrophobic domain and a C-terminal β-defensin-like domain, making them particularly effective against Gram-negative bacteria by disrupting their outer membranes. This disruption occurs as the hydrophobic N-terminal domain inserts into the membrane, forming pores that cause leakage of the cellular content, leading to bacterial death [304]. Defensins have been identified in various invertebrate phyla, including Arthropoda [305–309], Chordata [310], Cnidaria [311–313], Mollusca [314–329], and Porifera [330].

#### 3.4.1.2. Macins

Macins are a family of cationic, cysteine-rich AMPs with a disulfide-stabilized αβ structural motif. The first macin, theromacin, was isolated from the shallow-water leech *Theromyzon tessulatum* [331]. Since then, macins have been identified in various aquatic invertebrates, including other annelids [332–334], cnidarians [335], and mollusks [336–344]. Despite their high sequence identity, macins exhibit distinct biological activities, such as membrane-aggregating and permeabilizing effects against Gram-positive bacteria and Gram-negative bacteria [331–334,342,344–346]. Some macins also promote central nervous system regeneration [332,346] and display antibiofilm properties [342,344].

### 3.4.2. Annelida

The phylum Annelida, comprising approximately 20,200 species, is categorized into three groups: Clitellata, Polychaeta, and Sipuncula [347]. These aquatic and semi-aquatic organisms include clam worms, sand worms, tube worms, and leeches [348]. Extremophile worms are adapted to inhabit harsh environments like abyssal depths, hydrothermal vents, polar regions, and polluted areas, making them valuable for studying novel bioactive compounds [349]. AMPs derived from these organisms often exhibit exceptional properties, such as acid resistance, thermostability, salt tolerance, and broad-spectrum antibacterial activity [350]. In this study, 38 AMPs distributed into six AMP families were identified from 14 aquatic annelids (three Clitellata, 11 Polychaeta). These AMPs have antimicrobial activity against 41 microbial species, including 29 pathogens relevant to aquaculture: 12 Gram-positive bacteria, 16 Gram-negative bacteria, and the fungus *Candida albicans* (Table S 8).

#### 3.4.2.1. Clitellata AMPs

Lumbricins are proline-rich AMPs first identified in the earthworm *Lumbricus rubellus* [351] and later found in other earthworms [352–354]. Hm-lumbricin was discovered in *H. medicinalis* where its expression is upregulated upon exposure to *D. nishinomiyaensis*, indicating a role in innate immunity. Additionally, Hm-lumbricin has been shown to promote central nervous system regeneration, linking immune defense with tissue repair [332]. Theromyzin is a linear anionic α-helical peptide extracted from the coelomic fluid of *T. tessulatum* (Fig. 10). It is the first anionic AMP identified in invertebrates [331].

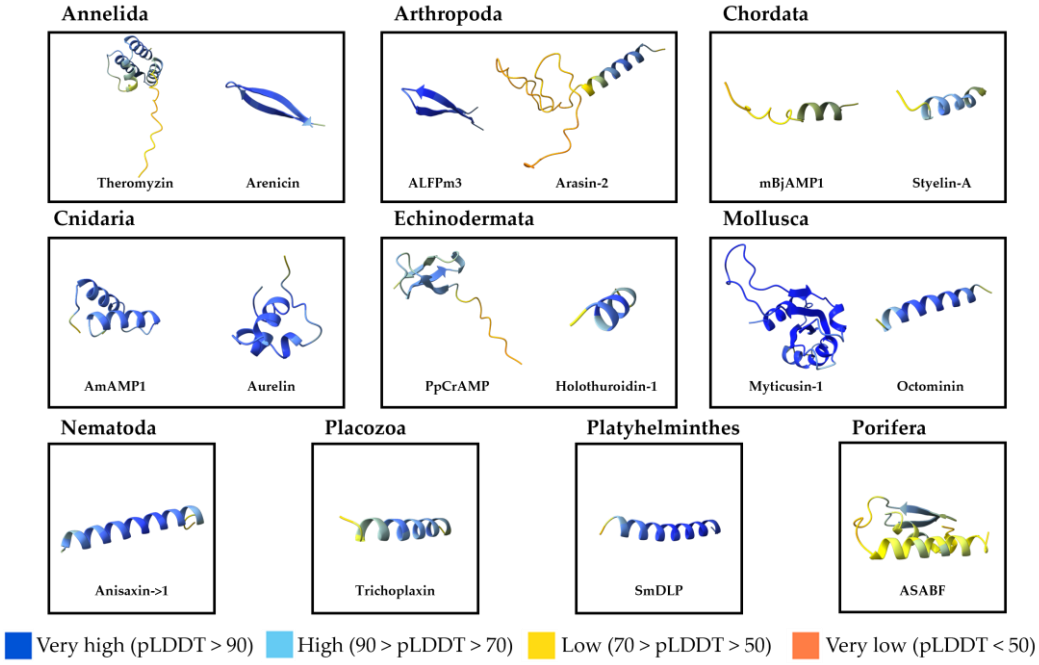


**Fig. 10** Examples of AMPs from aquatic invertebrate phyla.

### 3.4.2.2. Polychaeta AMPs

Polychaete AMPs are often found in species from fluctuating thermal habitats, where factors like temperature, salinity, and pressure may influence their evolution and function. BRICHOS-related AMPs are characterized by a conserved structure comprising a hydrophobic region (signal peptide or transmembrane domain), a proregion containing the BRICHOS domain, and a carboxy-terminal (C-terminal) double-stranded β-sheet [355]. The BRICHOS domain acts as an intramolecular chaperone, facilitating protein targeting to the secretory pathway and protease-mediated processing [356]. Highly conserved across species, this domain is associated with diseases such as Alzheimer's, Parkinson's, cancer, diabetes, dementia, and respiratory distress [357,358]. In annelids, BRICHOS-related peptides demonstrate diverse precursor peptides, reflecting adaptation to fluctuating thermal habitats [359,360]. The first BRICHOS-related AMP, arenicin, was found in the extremophile lugworm *A. marina*. This β-hairpin peptide, stabilized by a disulfide bond, inspired synthetic derivatives with enhanced biological properties [361–368]. Subsequent discoveries include abarenicin from AbA. pacifica and UuBRI-21 from *Urechis unicinctus* [369]. Other BRICHOS-related AMPs include α-helical nicomicin from the Arctic polychaeta *Nicomache minor*, which displays anticancer properties [254], and alvinellacin, the first AMP from a deep-sea organism, found in the thermotolerant Pompeii worm *Alvinella pompejana*. Alvinellacin and its homolog capitellacin, from *Capitella teleta*, form a double-stranded β-sheet stabilized by two disulfide bonds [370–372]. Recent discoveries of polaricin from the Antarctic worm

*Amphitritides* sp., the α-helical HfBRI-28 and β-hairpin HfBRI-25 from *Heteromastus filiformis*, and AmBRI-44a, a defensin-like peptide stabilized by four disulfide bonds from *A. marina*, expand the structural repertoire of BRICHOS-related AMPs [360,373,374].
Hedistin is a cationic α-helical peptide extracted from the coelomic fluid of the sandworm *Hediste diversicolor*. It contains two brominated tryptophan residues and a C-terminal amide [375]. Bromination, a rare post-translational modification, occurs at the 6-position of the tryptophan indole ring, typically found in marine organisms due to the high bromide content and (halo)peroxidases in seawater [376]. Perinerin, a cationic α-helical peptide, was isolated from the Asian clam worm *P. aibuhitensis*. Its full structure remains partially unknown, but it likely contains two intramolecular disulfide bridges [377].

### 3.4.3. Arthropoda

Arthropods represent the most abundant and diverse group of animals, comprising four subphyla: Chelicerata, Crustacea, Hexapoda, and Myriapoda. Despite the continuous discovery of new species, only a small fraction is aquatic. It is estimated that there are between 100,000 and 110,000 arthropod species, with nearly 100,000 species of aquatic insects from 12 orders, representing 60% of all aquatic animal species [5,378]. However, the assessment of aquatic arthropod diversity is biased due to several factors. There are more taxonomic studies on terrestrial arthropods than on aquatic ones, geographic data is limited, identification efforts primarily target insects and large crustaceans, and there is a global decline in taxonomists and scientific expeditions [379]. Arthropods represent one of the most prominent taxa for the discovery of AMPs with several already identified. In this study, 107 AMPs distributed into 19 AMP families were identified from 22 aquatic arthropods (four Chelicerata, 18 Crustacea). These AMPs were tested and showed antimicrobial activity against 86 microbial species, including 47 pathogens relevant to aquaculture: 13 Gram-positive bacteria, 25 Gram-negative bacteria, eight fungi, and the WSSV (Table S 9).

#### 3.4.3.1. Anti-Lipopolysaccharide Factor

Anti-lipopolysaccharide factors (ALFs) are essential immune peptides in arthropods (both Chelicerata and Crustacea) that bind and neutralize bacterial lipopolysaccharides (LPS), the primary component of Gram-negative bacterial outer membranes, thereby regulating immune responses [380]. They primarily target the lipid A component of LPS, disrupting membrane integrity and neutralizing LPS-induced toxicity through electrostatic and hydrophobic interactions, with some ALFs sequestering LPS to prevent host receptor binding [302,381]. Their main structural feature is the lipopolysaccharide-binding domain (LBD), a conserved region with a disulfide loop formed by two cysteines,

crucial for antimicrobial activity [381]. First identified in horseshoe crabs *Limulus polyphemus* and *Tachypleus tridentatus* [382], ALFs have since been found in crabs [383–387] as well as shrimp and prawns [388–391].

3.4.3.2. Chelicerata AMPs

Horseshoe crabs, the closest living relatives of trilobites, have thrived for up to 500 million years, originating in Mesozoic European waters before migrating as seas receded. Today, *Carcinoscorpius rotundicauda*, *T. tridentatus*, and *Tachypleus gigas* inhabit Asia's coasts, while *Limulus polyphemus* is found along North America's Atlantic coast. These ancient arthropods produce unique AMPs with diverse bioactivities [392–394]. Tachyplesin, first discovered in *T. tridentatus* and later in *C. rotundicauda* and *T. gigas*, is a short cationic peptide with two disulfide bonds that exhibits antibacterial, antifungal, anticancer, and antibiofilm activities. Cyclized forms show similar bioactivities with enhanced stability and therapeutic potential [395–399]. Polyphemusin, isolated from *L. polyphemus*, is structurally similar to Tachyplesin, and includes variants with optimized amphipathic properties and comparable bioactivities [396,398,400]. Tachycitin, discovered from *T. tridentatus*, features a stabilized αβ-motif structure with a chitin-binding domain and a C-terminal region with 10 cysteine residues. Its antimicrobial activity is synergistically enhanced with big defensin [401,402]. Tachystatin is a β-sheet chitin-binding peptide from *T. tridentatus* stabilized by three disulfide bonds. Isoforms A and B are homologous, while isoform C has amphiphilic and hemolytic properties [403–405]. A thermally stable, non-cytotoxic variant, tachystatin-A2, was identified in *T. gigas* [403,406]. Tatritin is a chitin-binding peptide from *T. tridentatus* with an N-terminal α-helix and a C-terminal β-sheet stabilized by six disulfide bonds, which showed interesting antimicrobial effects [407,408].

3.4.3.3. Crustacea AMPs

Arasins are proline-rich, cationic AMPs with two distinct domains: a proline/arginine-rich N-terminal region and a cysteine-rich C-terminal region with four cysteines [409]. The first arasin identified, callinectin, was found in the blue crab *Callinectes sapidus* [410,411]. Arasins were then found in the hemocytes of various crustaceans, including crabs [409,412,413], crayfish [414], and prawns [415]. Notably, the proline/arginine-rich N-terminal region of Ha-arasin1 exhibits *in vitro* chitin-binding activity, indicating its potential role in targeting pathogens with chitin-containing structures [416]. Crustins are cationic AMPs primarily found in crustaceans, first identified in the hemocytes of the shore crab *Carcinus maenas* as a cysteine-rich 11.5-kDa peptide [417]. The term "crustins" was introduced when similar peptides were discovered in penaeid shrimp

[418]. They typically feature one or two whey acidic protein (WAP) domains at the C-terminal, each with eight cysteines linked by four disulfide bonds, and a signal sequence at the N-terminal with a variable region between the signal sequence and WAP domain [419]. Crustins are classified into several types based on structural variations. Type I crustins have a cysteine-rich region between the signal peptide and WAP domain, further divided into Ia, Ib, and Ic, with Type Ib having a longer C-terminal region and Type Ic featuring two linked cysteine-rich domains [420]. Type II crustins have both glycine-rich and cysteine-rich regions, with subgroups IIa and IIb differing in the glycine-rich domain length [421]. Type III crustins have a short proline/arginine-rich N-terminal region [419]. Additional types include Type IV, which has two WAP domains [422,423], Type V, which is exclusive to insects and has a cysteine-rich domain and an aromatic amino acid-rich region preceding the WAP domain [424], Type VI, consisting of a glycine-rich region and a WAP domain, and Type VII, with a serine/leucine-rich region followed by a cysteine-rich region and a WAP domain [420]. Crustins are known for their antibacterial and antifungal activities, which vary by type. Type I crustins are particularly effective against Gram-positive bacteria, while Types II and III target both Gram-positive and Gram-negative bacteria [425,426]. Type IV crustins are antiproteolytic, with some exhibiting antibacterial and antifungal properties [427]. Certain crustins have demonstrated antiviral activity [428], antibiofilm properties [429], and wound-healing potential [430], highlighting their multifunctional roles in immune defense and tissue repair. Many AMPs from crabs are rich in specific amino acids that contribute to their antimicrobial properties. Proline-rich AMPs, for instance, exhibit membrane permeability and non-lytic mechanisms that inhibit protein synthesis and induce bacterial death [431,432]. Notable examples include the 6.5-kDa proline-rich peptide from *C. maenas*, the first AMP discovered in crustaceans [433], and SpPR-AMP1 from *S. paramamosain* [434]. Glycine-rich AMPs include GRPSp featuring glycine-rich motifs flanked by two C-terminal cysteine residues, and the thermally stable Spgly-AMP26-62, both from *S. paramamosain* [435,436]. Hyastatins are multi-domain chitin-binding cationic peptides with a conserved six-cysteine C-terminal region. The first hyastatin, discovered in the spider crab *Hyas araneus*, contains a glycine-rich N-terminal region followed by a proline/arginine-rich short region. Their antibacterial and chitin-binding functions are mainly attributed to the cysteine-rich domain, but both the cysteine-rich and proline/arginine-rich regions contribute to their antimicrobial activity [437,438]. Recent transcriptomic studies in *P. trituberculatus* have identified 14 distinct hyastatin variants, indicating significant peptide diversity [439]. Paralithocins, identified in the red king crab *Paralithodes camtschaticus*, are cysteine-rich cationic peptides with eight cysteine residues forming four disulfide bridges [440].

Other AMPs from crabs include scygonadin, an anionic peptide from the seminal plasma of *S. serrata*. It is expressed in the ejaculatory duct and plays a role in maintaining a sterile environment essential for successful fertilization [441–444]. Sparanegtin, an anionic peptide from *S. paramamosain* containing three α-helices, binds to pathogen-associated molecular patterns (PAMPs) like LPS and peptidoglycan. It plays a crucial role in immune defense during spermatogenesis and modulates immune gene expression in response to bacterial infections. By reducing bacterial loads in critical tissues like the gills and hepatopancreas, sparanegtin enhances the survival of infected crabs [445]. Astacidins are small, cationic, proline/arginine-rich AMPs found exclusively in crayfish, where they bind to PAMPs and play a crucial role in host defense. Initially identified in the invasive *Pacifastacus leniusculus* and later in *P. clarkii*, astacidins are classified into two groups: astacidin 1, derived from the C-terminal region of hemocyanin, and astacidin 2, which consists of proline/arginine-rich peptides with an amidated C-terminus [446–448]. Recent research also identified four proline-rich, non-cytotoxic, astacidin 2-related peptides in the transcriptome of *P. clarkii* [449]. Prawns and shrimp are aquatic crustaceans that differ in taxonomy and morphology. Prawns belong to Dendrobranchiata, and have larger, straighter bodies, longer legs, and branching gills, inhabiting both freshwater and saltwater environments. Shrimp, under Pleocyemata, are smaller, with curled bodies, shorter legs, and lamellar gills, primarily found in marine habitats. Misclassifications exist, such as “penaeid shrimp” from the Penaeidae family, which are actually prawns. Penaeidins, cationic AMPs unique to *Penaeus*, have a proline-rich N-terminal and cysteine-rich C-terminal [450]. First identified in *P. vannamei*, they are classified into four subgroups: PEN2, PEN3, and PEN4, which primarily target Gram-positive bacteria and fungi, and PEN5, which also targets Gram-negative bacteria and viruses [451–456]. Some penaeidins exhibit chitin-binding properties [251], while structural variants like MjPen- II in *P. japonicus* with a serine-rich N-terminal and BigPEN in *P. vannamei* with an extra repeat region at its N-terminus further highlight their diversity [456–459]. Stylicins are anionic AMPs exclusive to *Penaeus*, featuring a proline-rich N-terminal and a cysteine-rich C-terminal with 13 conserved cysteines [460–464]. Stylicins target filamentous fungi, exhibit strong LPS, prevent biofilm formation, and aid immunity against WSSV [461–465].

### 3.4.4. Chordata

The phylum Chordata includes a diverse array of animals primarily defined by the presence of a notochord and a dorsal hollow nerve chord. It is divided into three subphyla: Cephalochordata (amphioxus), Tunicata (ascidians, larvaceans, and

thaliaceans), and Vertebrata (amphibians, birds, fishes, reptiles, and mammals) [377]. The limited number of known AMPs derived from invertebrate chordates is restricted to one amphioxus species and five ascidians. In this study, 71 AMPs distributed into 11 AMP families were identified from seven invertebrate chordates (one Cephalochordata, six Tunicata). These AMPs were tested and showed antimicrobial activity against 40 microbial species, including 26 pathogens relevant to aquaculture: eight Gram-positive bacteria, 14 Gram-negative bacteria, and four fungi (Table S 10).

3.4.4.1. Cephalochordata AMPs

A single AMP, BjAMP1, has been identified in the amphioxus *Branchiostoma japonicum*, exhibiting a unique mode of action. Its structure comprises two α-helices connected by a reverse turn, forming an amphipathic arrangement of polar and hydrophobic residues. This configuration enables BjAMP1 to penetrate bacterial cell membranes without causing structural disruption. Instead, it employs a membranolytic mechanism that induces membrane depolarization and permeabilization. BjAMP1 also binds to LPS and LTA within bacterial membranes and may further inhibit biological functions by interacting with DNA or RNA after membrane penetration, ultimately inducing cell death. It shows broad-spectrum antibacterial activity while remaining non-toxic to mammalian cells [466,467]. Synthetic analogs of mBjAMP1, developed through amino acid modifications, have shown enhanced antimicrobial and antibiofilm properties [468].

3.4.4.2. Tunicata AMPs

The majority of invertebrate AMPs derived from the phylum Chordata have been identified in ascidians [469]. In *Styela clava*, a variety of AMPs have been identified, including phenylalanine-rich styelins, α-helical clavanins, and histidine-rich clavaspirin. These AMPs have been shown to exhibit antibacterial activity against both Gram-positive and Gram-negative bacteria, as well as fungi [470–473]. A unique group of amphipathic α -helical AMPs isolated from the hemocytes of *Halocynthia aurantium* have been described which includes halocidin and dicynthaurin. Both peptides have an unusual structural motif consisting of two amphipathic helices that are covalently linked through a single disulfide bond formed between adjacent single cysteine residues. Dicynthaurin is composed of two 30 amino acid monomers bounded together by a single cysteine disulfide bond. Both the peptide monomers and the dimer have an α-helical conformation. Dicynthaurin has a broad-spectrum antibacterial activity [474,475]. Halocidin is composed of an 18 amino acid residues monomer and a 15 residues monomer covalently linked by a single cysteine disulfide bond. Antimicrobial assays verified that the 18-residue monomer is more active than the 15-residue monomer.

Moreover, several synthetic Halocidin analogs have been developed with enhanced antimicrobial activities including di-K19Hc [476]. In *Halocynthia papillosa*, the AMPs halocyntin and papillosin demonstrated broad-spectrum antibacterial activity [477]. The identification of AMPs in *Ciona intestinalis* commenced with a search of its expressed sequence tag (EST) database, which yielded the identification of Ci-MAM-A24 and Ci-PAP-A22 [478,479]. Another study predicted over 180 potential AMPs from the small open reading frames (sORF) of *C. instestinalis*. The researchers synthesized the ten most promising candidates, of which five (P-02, P-03, P-04, P-05, and P-10) demonstrated antibacterial activity [480]. Recently, two novel cysteine-rich AMPs, designated as turgencins, along with their oxidized derivatives, were isolated from the Arctic ascidian *Synoicum turgens*. The peptides are post-translationally modified, containing six cysteines with unusual disulfide connectivity of C1-C6, C2-C5, and C3-C4, as well as an amidated C-terminus. Furthermore, the peptides contain methionine residues, resulting in the isolation of peptides with different degrees of oxidation. The most potent peptide, turgencin AMox1, which contains one oxidized methionine, displayed antimicrobial activity against both Gram-negative and Gram-positive bacteria. Additionally, the peptide inhibited the growth of the melanoma cancer cell line A2058 and the human fibroblast cell line MRC-5 [481]. Moreover, 11 synthetic peptides based on turgencin were designed and designated StAMP-1 to StAMP-11. The most potent overall was StAMP-9, which demonstrated potent antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi, as well as non-hemolytic activity against sheep red blood cells. It also exhibited non-cytotoxic effects against the human melanoma cell line A2058 and the non-malignant human lung fibroblast cell line MRC-5, indicating high selectivity [482].

### 3.4.5. Cnidaria

Cnidaria is a diverse and ancient phylum of predominantly marine organisms, comprising approximately 12,000 extant species [77,483]. These organisms are characterized by the presence of cnidae—organelle-like capsules containing venomous eversible tubules used for defense and prey capture [6,28]. Cnidarians are categorized into three main clades: Anthozoa (anemones, corals, sea fans, sea pens, zoanthids), Medusozoa (hydroids, jellyfish, siphonophores), and Myxozoa (obligate endoparasites) [8,484]. Their defense mechanisms primarily rely on innate immunity, although there is evidence suggesting the presence of immune memory in some species [485]. The cnidarian immune system encompasses immune recognition, intracellular signaling cascades, effector responses, and tissue repair mechanisms [486]. In this study, 10 AMPs

distributed into 10 AMP families were identified from eight cnidarians (six Anthozoa, two Medusozoa). These AMPs were tested and showed antimicrobial activity against 27 microbial species, including 22 pathogens relevant to aquaculture: six Gram-positive bacteria and 16 Gram-negative bacteria (Table S 11).

#### 3.4.5.1. Anthozoa AMPs

A few AMPs have been extracted from coral species, including damicornin from the stony coral *Pocillopora damicornis*. This peptide, containing six cysteine residues, exhibits activity against Gram-positive bacteria and the fungus *Fusarium oxysporum*. Notably, the coral pathogen *Vibrio coralliilyticus* represses the expression of the damicornin gene, providing the first evidence of AMP gene repression mediated by *Vibrio* [487]. Another six-cysteine peptide, AmAMP1, was identified in the stony coral *Acropora millepora*. It is expressed in ectodermal cells during late coral development and shows antibacterial activity against a broad range of Gram-positive and Gram-negative bacteria [488]. Additionally, Pd-AMP1, a peptide with a β-hairpin structure isolated from the soft coral *Phyllogorgia dilatata*, showed activity against Gram-positive bacteria [489]. Many AMPs have been identified in sea anemones, such as crassicorin isolated from *Urticina crassicornis*. This peptide adopts a double β-hairpin structure, contains six cysteines, and exhibits antibacterial activity [490]. Recently, equinins were discovered in *Actinia equina*, demonstrating low antibacterial activity and no hemolytic effects on human cells [491].

#### 3.4.5.2. Medusozoa AMPs

The first AMP identified in a cnidarian, aurelin, was isolated from the mesoglea of the moon jellyfish *Aurelia aurita*. This six-cysteine peptide, composed of two helices linked by a random coil, shows antibacterial activity [492,493]. In *Hydra*, the arminin family of α-helical peptides was discovered, with arminin 1a-C (the C-terminal region of arminin 1a) showing strong antibacterial activity and selective anticancer effects, suppressing leukemia cell viability without causing hemolysis in human erythrocytes [494,495]. Another *Hydra*-derived AMP, periculin-1, features an anionic N-terminal region and a cationic C-terminal region with eight cysteine residues. Expressed in the germline of female *Hydra*, periculin-1 plays a crucial role in selectively targeting bacterial colonization during embryogenesis, demonstrating potent antimicrobial activity [334,496].

### 3.4.6. Echinodermata

The phylum Echinodermata (from the Greek echinos, “spiny”, and derma, “skin”) includes over 7500 species [265]. These marine organisms, with few species found in brackish

waters [497,498], are divided into five classes: Asteroidea (sea stars), Crinoidea (feather stars and sea lilies), Echinoidea (sea urchins, sand dollars, and sea biscuits), Holothuroidea (sea cucumbers), and Ophiuroidea (brittle stars) [347]. Echinoderms possess an innate immune system mediated by coelomic fluid, containing coelomocytes that defend against pathogens and injuries, along with antimicrobial components such as lysozymes and AMPs [499]. Although genetic sequencing has revealed a complex repertoire of immune genes, the echinoderm immune system remains poorly understood, though recent studies have led to the discovery of new AMPs [500]. In this study, 17 AMPs distributed into five AMP families were identified from six echinoderms (one Asteroidea, four Echinoidea, and one Holothuroidea). These AMPs were tested and showed antimicrobial activity against 20 microbial species, including 13 pathogens relevant to aquaculture: five Gram-positive bacteria, six Gram-negative bacteria, and two fungi (Table S 12).

3.4.6.1. Asteroidea AMPs

The only known AMP derived from a sea star, *Patiria pectinifera*, was named P. pectinifera cysteine-rich antimicrobial peptide (PpCrAMP). It is predicted to adopt a β-hairpin structure with two extended β-strands linked by a random coil region and exhibit antibacterial activity [501].

3.4.6.2. Echinoidea AMPs

Sea urchins produce interesting AMPs with notable properties. Strongylocins, first identified in *Strongylocentrotus droebachiensis*, are cationic peptides featuring a distinct cysteine pattern of six residues and a brominated tryptophan. These peptides exhibit strong antibacterial activity and have been also identified in *Strongylocentrotus purpuratus* and *Echinus esculentus* [502–504]. Centrocins, also from *S. droebachiensis*, are heterodimeric peptides composed of a 30-residue antimicrobial heavy chain linked by a disulfide bond to a 12-residue inactive light chain. Similar to strongylocins, native centrocins contain a post-translational brominated tryptophan. These peptides display strong antibacterial activities, some antifungal activity, and have also been found in *E. esculentus* [504,505].

3.4.6.3. Holothuroidea AMPs

Holothuroidins isolated from *Holothuria tubulosa* are the only characterized AMPs derived from a sea cucumber. While these small cationic α-helical peptides exhibit weak antibacterial potency, they also display interesting antibiofilm activity [506,507].

### 3.4.7. Mollusca

Mollusca, the second largest phylum of invertebrates, includes over 50,000 species [265]. Of its eight classes, Bivalvia (clams, cockles, mussels, oysters, and scallops), Cephalopoda (octopuses, squids, cuttlefishes, and nautilus), and Gastropoda (snails and slugs) account for more than 95% of its diversity [377]. Mollusks are extensively studied for AMPs, with several AMPs reported, mostly from bivalves [469]. In this study, 86 AMPs distributed into 17 AMP families were identified from 28 mollusks (17 Bivalvia, three Cephalopoda, and eight Gastropoda). These AMPs were tested and showed antimicrobial activity against 71 microbial species, including 45 pathogens relevant to aquaculture: 13 Gram-positive bacteria, 27 Gram-negative bacteria, and five fungi (Table S 13).

#### 3.4.7.1. Molluscidin

Molluscidins are AMPs with repeated dibasic residues, identified in the gills of mollusks such as the bivalves *Crassostrea gigas* [508] and *Atrina pectinata* [509], and the gastropod *Haliotis discus* [510]. Their predicted non-amphipathic structures alternate between α-helical and random coil conformations. Molluscidins exhibit antimicrobial activity against Gram-positive and Gram-negative bacteria while maintaining low cytotoxicity.

#### 3.4.7.2. Bivalvia AMPs

Bivalves, particularly mussels, produce diverse cysteine-rich AMPs essential for their immune defense. These include mytilins [313,316,511], mytimycins [313,512,513], myticins [514–518], and myticusins [342,519,520], all derived from precursor peptides comprising a signal peptide, a mature cysteine-rich region, and a C-terminal extension. Mytilins, mytimycins, and myticins show activity against bacteria, fungi, and viruses, while myticusins also target parasites. Mussels also produce other AMPs, such as myticalins, cationic peptides with α-helical and random coil structures active against Gram-positive and Gram-negative bacteria [521–523], and mytichitins, which contain chitin-binding domains that enhance antimicrobial activity against bacteria, fungi, and parasites [524,525]. In non-mussel bivalves, AMPs include Ap from the shellfish *Argopecten purpuratus*, a cationic proline-rich peptide with strong antifungal properties [526], and Cg-Prp from *C. gigas*, a proline-rich peptide with limited direct antimicrobial effects but significant synergy with defensin Cg-Def. This synergistic activity may compensate for the low concentrations of antimicrobials in the oyster tissues [527,528]. Additionally, in *C. hongkongensis*, the cationic α-helical peptide URP20 exhibits potent

antimicrobial activity against bacteria and fungi without cytotoxicity to mammalian cells [529].

#### 3.4.7.3. Cephalopoda AMPs

Despite extensive research on cephalopod extracts, the isolation and characterization of peptides with antimicrobial properties remain limited [530–532]. A notable discovery is octopartenopin, a random-coiled pentapeptide from the suckers of *Octopus vulgaris* [533]. Cationic α-helical AMPs have been identified in *Octopus minor*, including octominins [534,535], octopromycin [536], and octoprohibitin [537]. Additionally, screening of the cuttlefish *Sepia officinalis* revealed peptides with antimicrobial potential: three (KT19, VA20, and GR21) identified by Houyvet *et al.* [538] and three others (NF19, AV19, and GK28) by Benoist *et al.* [539].

#### 3.4.7.4. Gastropoda AMPs

Several AMPs have been identified in aquatic snails. In marine snails, the α-helical peptide Cm-p1 and its derivatives obtained from *Cenchritis muricatus* exhibited potent antifungal activity without toxicity to mammalian cells [540,541]. Two proline-rich peptides from the invasive *Rapana venosa*, the random-coiled peptide 4 and the α-helical peptide 7, showed antibacterial activity against Gram-negative bacteria [542]. In freshwater snails, Bb-AMP4 from *Filopaludina bengalensis* displayed antibacterial effects against Gram-positive bacteria [543]. Additionally, two fragments of α-helical peptides, Pom-1 (Closticin 574) and Pom-2 (Cecropin D-like), identified in *Pomacea poeyana*, exhibited antibacterial activity, with Pom-1 also showing antiviral effects against ZIKV [544]. Moreover, the α-helical peptide Dolabellanin B2, isolated from the sea hare *Dolabella auricularia* and the sea slug *Peronia peronii*, displayed antifungal and antibacterial properties [545,546].

### 3.4.8. Other phyla

Despite the extensive research on AMPs across many aquatic invertebrate phyla, certain groups remain significantly underrepresented in scientific literature, despite their ecological and evolutionary importance. Determining whether this limitation arises from a lack of studies or rather a genuine scarcity of AMPs is crucial. Four phyla are explored (Nematoda, Placozoa, Platyhelminthes, and Porifera) highlighting their known AMPs (Table S 14) and addressing the challenges involved in studying these organisms.

#### 3.4.8.1. Nematoda

The phylum Nematoda comprises over 28,000 species of roundworms divided into two classes, Chromadorea and Enoplea [265,377]. While AMPs in the terrestrial model

organism *Caenorhabditis elegans* are well-studied, few works have explored AMPs in aquatic nematodes [547]. A notable exception is the discovery of anisaxins, cecropin-like helical AMPs from the marine parasite *Anisakis*, the etiological agent of anisakiasis. These AMPs show potent bactericidal activity against Gram-negative bacteria with minimal cytotoxicity to human cells [548]. Recently, an integrated homology computational methodology was applied for the discovery of new helminth (both Nematoda and Platyhelminthes) AMPs. This approach identified thousands of putative AMPs and out of the selected few synthetized, four (nAMP-LP-18, nAMP-LP-104, nAMP-LP-249, and nAMP-LP-298) revealed antibacterial activity (<100 μg/mL) against at least one bacterial species [549].

3.4.8.2. Placozoa

Placozoa is an ancient phylum of highly simplified multicellular organisms, considered among the most basal metazoans [550]. Although only four species are currently described, phylogenomic and gene content analyses suggest much greater diversity within the phylum [551,552]. Trichoplaxins, α-helical peptides from *Trichoplax adhaerens*, are the sole known AMP family in Placozoa, showing strong antimicrobial activity against bacteria and fungi. Additionally, the synthetic analog Trichoplaxin-2A shows minimal cytotoxicity to mammalian cells and high anticancer activity and selectivity [553,554].

3.4.8.3. Platyhelminthes

Platyhelminthes, or flatworms, are a diverse phylum that includes parasitic species like *Schistosoma mansoni*, the causative agent of schistosomiasis, a disease affecting millions globally [377,555]. A peptide from *S. mansoni*, SmDLP, shares high homology with dermaseptin 3.1 from the frog *Agalychnis annae*. SmDLP demonstrates strong antimicrobial and hemolytic activities, which may help the parasite survive and evade the host immune system [556]. Additionally, an in silico study using an innovative algorithm and homology searches found that flatworms lack traditional AMPs, instead possessing a repertoire of unique, mostly cysteine-rich AMP-like peptides. Two novel AMP-like peptides, fAMP-LP-5 and fAMP-LP-17, showed antibacterial activity (<100 μg mL−1), highlighting their potential as novel antimicrobials [549].

3.4.8.4. Porifera

The phylum Porifera, the oldest living metazoan group, comprises over 9300 species across four classes, with Demospongiae representing over 80% of sponge diversity [265]. Sponges form symbiotic relationships with microorganisms that produce chemical

compounds previously thought synthesized by the sponges themselves [296]. Nearly half of all marine natural products discovered from invertebrates between 1990 and 2019 originated from sponges [557]. However, only one known AMP has been extracted from sponges: ASABF_SUBDO, a defensin-like cysteine-stabilized αβ motif from *Suberites domuncula*. Identified through EST database screening, ASABF_SUBDO shares high similarity with ASABF, a cysteine-rich AMP from terrestrial nematodes [329]. The ancient lineage of sponges may explain their advanced chemical defense mechanisms, with their broad peptide arsenal likely reducing the need for traditional short, linear AMPs. This suggests that sponges have evolved multiple defense strategies, including antimicrobial cyclic peptides [558–560].

### 3.4.9. "Non-Classical AMPs" Derived from Aquatic Invertebrates

While most AMPs derived from aquatic invertebrates are traditionally described as short, linear peptides composed of basic amino acids and directly involved in host defense, a broader category of "non-classical AMPs" is also found within these organisms. These molecules diverge significantly from the classical definition and demonstrate remarkable diversity in structure, origin, and function. This group encompasses a wide array of bioactive compounds, including cyclic peptides, antimicrobial enzymes, neuropeptides, pattern recognition proteins, and various cryptides with distinct origins (Fig. 11, Table S 15).

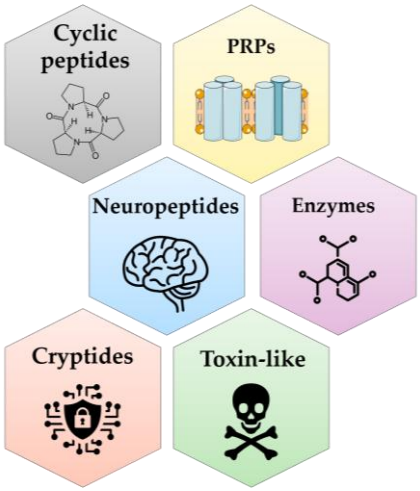


**Fig. 11** Key categories of "non-classical" AMPs.

#### 3.4.9.1. Cyclic Peptides

Among several chemical modifications used to enhance peptide-based therapeutics, including N-methylation, PEGylation, glycosylation, lipidation, peptide stapling, and the incorporation of unnatural amino acids and disulfide bonds, cyclization of linear peptides

is particularly promising [561–565]. Cyclization restricts peptide flexibility, improving bioavailability, stability, and specificity, while enhancing resistance to exopeptidase hydrolysis due to the absence of terminal ends, and protecting against endopeptidase cleavage with its rigid ring structure. These structural changes also improve membrane interactions, which may contribute to the enhanced antimicrobial properties by increasing the peptide's ability to penetrate microbial membranes and maintain its functional conformation in the presence of environmental stressors such as changes in pH, temperature fluctuations, high salt concentrations, and the presence of proteases [566–568]. Cyclized peptides can be homodetic (with only peptide bonds) or heterodetic (containing non-peptide bonds like isopeptides, disulfides, or esters), with cyclic depsipeptides being a subtype that incorporates ester bonds by replacing amino acids with hydroxy acids [569,570]. Over 50 cyclic peptide drugs have been FDA-approved, making up nearly half of all approved peptide drugs, with many more in clinical trials [568,571]. However, only one originates from a marine organism: ziconotide, a synthetic version of ω-conotoxin MVIIA from the marine snail *Conus magus* [572]. Most antimicrobial cyclic peptides and depsipeptides from aquatic invertebrates have been isolated from marine sponges, with some exceptions from ascidians and sea slugs. These compounds primarily exhibit antibacterial activity, with some showing antifungal, antiviral, and antiparasitic properties. Although none have been approved, several, including Celebesides, Dolastatins, Homophymine A, Mirabamides, Theonellamides, and Theopapuamides, are being evaluated by pharmaceutical and nutraceutical industries [573].

3.4.9.2. Antimicrobial Enzymes

Enzymes, primarily proteins or RNA molecules, act as biological catalysts to accelerate chemical reactions. In aquatic invertebrates, lysozymes and chitinases are key enzymes involved in innate immunity. Lysozymes, first identified by Alexander Fleming when he observed nasal mucus dissolving bacteria [574], hydrolyze glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid in bacterial peptidoglycans, resulting in cell lysis [575]. Beyond bacteriolysis, they enhance immune defenses by activating the complement pathway, promoting phagocytosis, and modulating immune responses, while also exhibiting anti-inflammatory, antitumor, and digestive functions [576]. Although they display antimicrobial activity, lysozymes are not classified as classical AMPs due to their enzymatic action. Based on their sequence, structure, and properties, lysozymes are categorized into three types: chicken-type (c-type), goose-type (g-type), and invertebrate-type (i-type) [577]. C-type lysozymes are common in both vertebrates and invertebrates, g-type lysozymes, initially discovered in goose egg whites, are also

present in some invertebrates, and i-type lysozymes are primarily found in invertebrates. In aquatic invertebrates, i-type lysozymes are reported in Annelida, Arthropoda, Echinodermata, Mollusca, and Porifera [578–582], c-type lysozymes in Arthropoda and Mollusca [583,584], and g-type lysozymes in Arthropoda, Chordata, Mollusca, and Placozoa [585–588]. Chitinases, another class of immune-related enzymes, are glycosyl hydrolases that catalyze the degradation of chitin, a structural polysaccharide found in arthropod exoskeletons, fungal cell walls, and crustacean shells [589]. These enzymes are classified into two main families—GH18 and GH19—which differ significantly in their catalytic mechanisms and structural features. GH18 chitinases typically require two catalytic residues: a glutamic acid to break down the chitin substrate and an acidic residue that stabilizes the reaction. In contrast, GH19 chitinases rely on a single catalytic residue, usually glutamate, for their activity. The active site and overall structure of GH19 chitinases, which are characterized by a higher content in α-helices, contrast with the triose-phosphate isomerase (TIM) barrel fold seen in GH18 enzymes. Additionally, GH19 chitinases exhibit higher specificity for chitin and enhanced stability under acidic conditions, while GH18 chitinases are more versatile, and capable of degrading both chitin and peptidoglycan. Beyond their direct antimicrobial activity, chitinases play important roles in immune regulation, tissue remodeling, and wound healing [590]. In aquatic invertebrates, the antimicrobial activity of chitinases has been reported only in arthropods, particularly prawns and crabs [591–593].

#### 3.4.9.3. Neuropeptides

Neuropeptides, highly conserved peptide-based neuromodulators released by neurons, regulate physiological processes, neural signaling, and behavioral plasticity, with some also displaying antimicrobial activity. Among these, tachykinin-related peptides (TRPs) are a prominent neuropeptide family characterized by a conserved C-terminal region. They have been identified in various aquatic invertebrates, including arthropods [594], mollusks [256], and annelids [595]. Notably, urechistachykinins, TRPs isolated from the echiuroid worm *Urechis unicinctus*, exhibit broad-spectrum antimicrobial activity against bacteria and fungi without cytotoxicity to human erythrocytes [595]. In addition, other neuropeptides with antimicrobial properties include peptide B, derived from proenkephalin A in the leech *T. tessulatum*, and nda-1, hym-357, hym-370, and rfamide III, secreted by sensory and ganglion neurons in the ectodermal epithelium of *Hydra* [596].

3.4.9.4. Pattern Recognition Proteins

The innate immune response relies on the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition proteins (PRPs). PAMPs are conserved pathogen structures, such as LPS, β-1,3-glucans, and peptidoglycans, which trigger immune responses including phagocytosis, encapsulation, proteinase cascades, and AMP synthesis [597–599]. PRPs include Toll-like receptors (TLRs), lectins, peptidoglycan recognition proteins (PGRPs), lipopolysaccharide-binding proteins (LBPs), bactericidal permeability-increasing proteins (BPIs), β-glucan binding proteins (βGBPs), and lipopolysaccharide- and β-1,3-glucan-binding proteins (LGBPs). TLRs are the most extensively studied in aquatic invertebrates. They play a crucial role in both innate and adaptive immunity by recognizing diverse PAMPs, including bacterial DNA, LPS, mannose, and peptidoglycans [600,601]. TLRs have been functionally characterized in numerous aquatic invertebrate phyla, such as Annelida [602], Arthropoda [603], Chordata [604], Echinodermata [605], and Mollusca [606]. Although the functional roles of LBPs and BPIs in invertebrates are unclear, these LBP/BPI proteins have been identified in Echinodermata [607], Mollusca [608], and Nematoda [609]. Similarly, LGBPs, which bind both LPS and β-1,3-glucans, have been characterized in Arthropoda [610] and Mollusca [611]. PRPs have also inspired AMPs' development, such as HDH-LGBP-A1 and HDH-LGBP-A2, derived from the polysaccharide-binding motif of *Haliotis discus hannai* LGBP. These AMPs show antibacterial, antifungal, and anticancer activities with minimal toxicity to human cells [612].

3.4.9.5. Cryptides from Regulatory Proteins

Cryptides are bioactive peptides derived from larger parent proteins or precursor peptides that serve a primary function. These cryptides are "hidden" within the parent protein, become functional once cleaved, and often exhibit activities distinct from their parent protein's primary role, such as immunomodulatory, hormone-like, or antimicrobial [613]. Histones, essential proteins for DNA packaging in eukaryotic cells and regulating gene expression, also hide cryptides with antimicrobial properties such as Buforin I derived from histone H2A in *Bufo gargarizans* [614]. In aquatic invertebrates, several histone-derived cryptides with antibacterial and antifungal effects have been reported in Arthropoda [615] and Mollusca [616]. Ubiquitin, a small, highly conserved protein found in all eukaryotes is classified into two groups: type-I and type-II ubiquitin-like proteins. Type-I ubiquitin contains a conserved Gly-Gly sequence at its C-terminus, enabling cleavage and attachment to target proteins through a post-translational modification (PTM) known as ubiquitination, which commonly leads to protein degradation, protein

activity regulation, or removal of damaged proteins [617]. In contrast, type-II ubiquitin-like proteins lack the glycine motif and do not undergo cleavage; instead, they regulate protein interactions and cellular pathways [618]. Ubiquitin also plays roles in DNA repair, cell signaling, and immune response [619]. In aquatic invertebrates, ubiquitin-derived cryptides with antimicrobial activities have been found in Mollusca, including cgUbiquitin from *C. gigas* [620], and RpUbi from *R. philippinarum* [621]. Additionally, paracentrin, a cationic cryptide derived from a β-thymosin of *Paracentrotus lividus* displays antibacterial and antibiofilm properties [622,623]. There are also synthetic peptides designed based on portions of proteins, such as schistocins. Their design was inspired by the C-terminal of *S. mansoni* Kunitz Inhibitor SmKI-1. Synthetized schistocins undergo a membrane-induced conformational change from random coil to α-helix and their antimicrobial activities are variable with some having interesting effects against bacteria and fungi [624].

3.4.9.6. Cryptides from Respiratory Proteins

Respiratory proteins primarily function to bind and transport oxygen, but some cryptides derived from them also contribute to immune responses. Hemerythrin, an ancient non-heme oxygen-binding protein family, is found across all three domains of life and is most diverse in Annelida [625–628]. In aquatic invertebrates, the cryptide MsHemerycin, derived from the N-terminus of hemerythrin, was isolated from the lugworm *Marphysa sanguinea*. This peptide, characterized by N-terminal acetylation and an unordered structure, demonstrates unique mechanisms of action and shows antimicrobial activity against *B. subtilis* [629]. Hemocyanin, a binuclear type 3 copper protein responsible for oxygen transport in the hemolymph of many arthropods and mollusks, is also a source of cryptides with antimicrobial properties [630]. Cryptides derived from C-terminal fragments of shrimp hemocyanin, including PsHCt1, PsHCt2, PvHCt, FCHc-C1, and FCHc-C2, exhibit diverse antimicrobial activities [631,632]. Additionally, haliotisin, a cryptide from the functional unit E of abalone *Haliotis tuberculata* hemocyanin, has been used as a template for synthetic peptides with demonstrated antibacterial properties [633]. Furthermore, five synthetic peptides (L1, L2, L8, L10, and L12) predicted from the large subunit of *P. vannamei* hemocyanin also show antibacterial effects [634].

3.4.9.7. Cryptides from Ribosomal Proteins

Ribosomal proteins, traditionally known for their role in protein synthesis, can also generate cryptides involved in host defense. In aquatic invertebrates, CgRPL29, a cryptide derived from the *C. gigas* 60S ribosomal protein L29, has a predicted unordered, non-amphipathic structure with two partial α-helical regions and demonstrates

antibacterial activity [635]. Additionally, BjRPS23, a cryptide from the *B. japonicum* ribosomal protein RPS23, functions as both a pattern recognition receptor and an antimicrobial effector. It interacts with bacterial membranes and promotes the generation of reactive oxygen species within bacterial cells, leading to their death [636].

3.4.9.8. Toxin-like Proteins

Toxin-like proteins play a vital role in prey capture and defense in various organisms, especially in cnidarians. These proteins, released through specialized cells called nematocysts, immobilize or kill prey by disrupting cellular membranes, interfering with signaling pathways, or causing tissue damage, facilitating prey capture. In defense, they deter predators by inducing pain, inflammation, or paralysis, thus reducing predation risk. Evolutionarily, these proteins are optimized to balance prey acquisition and protection against a variety of ecological threats, ensuring survival in competitive marine environments [24].

Beyond their role in prey capture and defense, toxin-like proteins have evolved to target and neutralize microbial pathogens [637]. Cytolysins from the sea anemone *Stichodactyla helianthus*, sticolisins, demonstrate antiparasitic activity against *Giardia duodenalis* with minimal toxicity to human erythrocytes [638]. Neurotoxin 2 from *Anemonia sulcata* demonstrates antibacterial effects against Gram-positive *M. luteus* [639], while the cysteine-rich Ueq 12-1 from *Urticina eques* displays moderate activity against Gram-positive bacteria and enhances transient receptor potential ankyrin 1 channel activity [311]. Additionally, transcriptomic analysis of *Epiactis japonicus* tentacles identified toxin-like AMPs, some of which display weak antibacterial activity [640].

## 3.5. Applications of Aquatic Invertebrate AMPs in Aquaculture

The intensification of aquaculture has led to increased stress on farmed species, weakening their immune systems and leading to the overuse of antibiotics. This overuse has exacerbated the rise of AMR, posing significant risks to both aquaculture and public health [218]. Effective strategies to mitigate AMR include adopting good aquaculture and biosecurity practices, implementing disease prevention measures, and minimizing antibiotic use by exploring alternative solutions [641,642]. Research has identified multiple antibiotic-resistance genes in aquaculture, including those related to β-lactams, tetracyclines, macrolides, quinolones, and sulfonamides [643]. To combat AMR, global regulations should enforce stricter controls on antibiotic use and ban commonly misused antibiotics, following the good examples of FDA-banned nitrofurans and chloramphenicol

[641]. Currently, only four antibiotics are FDA-approved for aquaculture use, namely florfenicol, oxytetracycline, sulfadimethoxine/ormetoprim, and sulfamerazine [644]. Several alternatives to antibiotics have been explored, including vaccines, probiotics, prebiotics, synbiotics, bacteriophages, chicken egg yolk immunoglobulin (IgY), medicinal plants, bacteriocins, and AMPs [643]. AMPs are particularly promising due to their immediate and direct antimicrobial action, broad-spectrum activity against diverse pathogens (Gram-positive and Gram-negative bacteria, viruses, fungi, and parasites), and low risk of AMR development. Additionally, some AMPs possess immunomodulatory properties that enhance the host's innate immunity. AMPs offer practical advantages, including relatively low production costs, easy and long storage in bulk, and rapid availability after an infection [645]. In aquaculture, AMPs have been studied as feed additives and therapeutic drugs and through genetic engineering, provide a potential alternative to antibiotics and support the health and productivity of farmed species. However, their application faces several challenges, including the high cost and difficulty of scaling up production, as natural extraction often yields low quantities. AMPs are susceptible to degradation by proteases, and their stability can be affected by environmental factors such as pH, salinity, and temperature. Moreover, some AMPs may exhibit cytotoxicity at higher concentrations and may potentially impact non-target organisms. Delivering AMPs effectively in aquatic environments is challenging due to dilution and dispersion, necessitating optimized delivery systems. Furthermore, stringent regulatory requirements for safety and efficacy must be met before AMPs can be approved for use in aquaculture [646].

### 3.5.1. AMPs as Aquaculture Feed Additives and Therapeutic Drugs

Several studies have explored the use of AMPs as dietary supplements in aquaculture, focusing primarily on fish, but also extending to shrimp and shellfish [647–649]. Wang *et al.* [650] analyzed the literature results on the application of AMPs as dietary supplements and concluded that AMPs can significantly enhance growth performance, enzymatic activity, and disease resistance of cultured species. Furthermore, although research on this topic is still limited, AMPs have also demonstrated a positive impact on the gut microbiomes of fish. However, none of the AMPs reviewed were derived from aquatic invertebrates. Studies on the application of aquatic invertebrates' AMPs as feed additives are indeed very scarce. The AMP octopromycin derived from the proline-rich protein 5 of *O. minor* was *in vivo* tested in zebrafish infected with *A. baumannii* and showed to cause a significant survival increase in fish [536]. Additionally, 6His-tatritin, derived from the AMP tatritin with six added histidines, revealed an improvement in

immune responses and intestinal microbiota in the grass carp *Ctenopharyngodon idellus* challenged with *A. hydrophila* [407].

### 3.5.2. Genetic Engineering and Transgenesis of AMP Genes in Fish

CRISPR/Cas9 gene editing is a powerful tool for enhancing fish immunity by introducing vector-engineered antimicrobial peptide genes and other immune-related genes. Studies reviewed by Wang and Cheng [651] show that AMP gene transgenesis in aquaculture fish reduces bacterial loads, improves survival rates after infection, and modulates the expression of immune and AMP genes. AMP genes often work synergistically with other immune-related genes to strengthen defenses, though their effectiveness varies by fish species, pathogen targeted, and the AMP gene used. While highly effective against bacterial infections, immune defense by AMP gene transgenesis against viral and parasitic infections is limited. Additionally, CRISPR/Cas9-based transgenesis can also combine immune improvements with trait enhancements like rapid growth, sterility, and improved fatty acid profiles. While CRISPR/Cas9 technology holds great promise for introducing AMP genes into farmed species, its practical application faces several challenges. These include the high costs of gene editing, difficulties in scaling these methods for commercial use, limited genomic knowledge of many farmed species, challenges in achieving stable AMP gene expression across generations, and significant regulatory and ethical concerns. Currently, there are no works on the application of aquatic invertebrates AMPs gene transgenesis, and further work should be done to explore the significant potential CRISPR/Cas9 gene editing has to offer for sustainable and efficient aquaculture production.

## 3.6. Conclusions

The rapid intensification of aquaculture has resulted in higher population densities of farmed species, increasing their vulnerability to disease outbreaks, while the overreliance on traditional antibiotics has exacerbated the global AMR crisis. This underscores the urgent need for alternative strategies to manage pathogens and ensure the sustainability of aquaculture systems, with AMPs emerging as a promising solution. As natural components of the humoral defense systems of many organisms, AMPs play a critical role in protecting hosts from pathogenic threats. These molecules possess several unique advantages, including immediate and direct antimicrobial action, broad-spectrum activity against bacteria, viruses, fungi, and parasites, and a low risk of promoting AMR. Additionally, AMPs exhibit additional bioactivities such as immunomodulatory, anticancer, and antifouling properties, making them versatile tools

in biotechnology. Practical benefits of AMPs include cost-effective production, long-term bulk storage, and rapid availability for deployment during infections.

Marine-derived AMPs are especially promising due to evolutionary adaptations to high-salinity environments, resulting in improved stability, enhanced electrostatic interactions, and a broader antimicrobial spectrum—traits that are particularly suited for aquaculture applications, where salinity fluctuations are common. Despite their potential, research on AMPs from aquatic invertebrates, particularly from phyla like Cnidaria, Platyhelminthes, and Porifera, remains underdeveloped. Existing knowledge is fragmented, with limited information in AMP databases, and dispersed in literature. Around 350 AMPs from 10 invertebrate phyla have been identified, with some demonstrating promising *in vitro* results, including activity against 85 pathogens relevant to aquaculture. However, the lack of *in vivo* studies hinders the application of these peptides in aquaculture. Addressing this gap could open new opportunities for using AMPs from aquatic invertebrates in aquaculture, including their applications as feed additives, therapeutic agents, and in genetic engineering approaches, such as the transgenesis of AMP genes, in line with recent successful efforts involving AMPs from other sources.

The application of AMPs in aquaculture faces several technical challenges, including efficacy, availability, and stability in aquatic environments, as well as ecological concerns, such as potential impacts on non-target organisms. To overcome these hurdles, the development of peptidomimetics—synthetically designed molecules inspired by natural AMPs—presents a promising alternative [652,653]. However, challenges such as production scalability, regulatory compliance, and other ecological impacts remain. To fully exploit the potential of AMPs, future research should focus on expanding the understanding of their natural diversity, particularly regarding their structures, mechanisms of action, and bioactivities. Comprehensive *in vivo* assessments are essential to evaluate their efficacy and safety as feed additives and therapeutic agents in relevant aquaculture species. Furthermore, advances in genetic engineering, particularly CRISPR/Cas9-mediated transgenesis of AMP genes into fish genomes, hold significant promise for developing enhanced aquaculture species. These advancements can strengthen the immune system of farmed species while also improving key physical and compositional traits, making them more resilient and ultimately a better aquaculture product. Exploring the biotechnological potential of AMPs can revolutionize aquaculture, fostering sustainability and resilience, safeguarding ecosystem health and public well-being, and curbing antibiotic dependency while tackling the escalating threat of AMR.

# Chapter 4.

# Proteomic Diversity of the Sea Anemone *Actinia fragacea*: Comparative Analysis of Nematocyst Venom, Mucus, and Tissue-Specific Profiles

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## Abstract

Sea anemones (Actiniaria, Cnidaria) are promising targets for biomedical research, as they produce unique bioactive compounds, including toxins and antimicrobial peptides (AMPs). However, the diversity and mechanisms underlying their chemical defenses remain poorly understood. In this study, we investigate the proteomic profiles of the unexplored sea anemone *Actinia fragacea* by analyzing its venom nematocyst extract, tissues, and mucus secretion. A total of 4011 different proteins were identified, clustered into 3383 protein groups. Among the 83 putative toxins detected, actinoporins, neurotoxins, and phospholipase A2 were uncovered, as well as two novel zinc metalloproteinases with two specific domains (ShK) associated with potassium channel inhibition. Common Gene Ontology (GO) terms were related to immune responses, cell adhesion, protease inhibition, and tissue regeneration. Furthermore, 1406 of the 13,276 distinct peptides identified were predicted as potential AMPs, including a putative Aurelin-like AMP localized within the nematocysts. This discovery highlights and strengthens the evidence for a cnidarian-exclusive Aurelin peptide family. Several other bioactive compounds with distinctive defense functions were also detected, including enzymes, pattern recognition proteins (PRPs), and neuropeptides. This study provides the first proteome map of *A. fragacea*, offering a critical foundation for exploring novel bioactive compounds and valuable insights into its molecular complexity.

**Keywords**: sea anemones, cnidaria, proteomics, toxins, antimicrobial peptides (AMPs)

## 4.1. Introduction

The phylum Cnidaria, recognized as the sister group of Bilateria [654], represents the oldest extant lineage of venomous animals [655–658], encompassing approximately 11,000 described species [659]. This group exhibits remarkable diversity in morphology, lifecycles, ecology, and development processes, being established by three major clades – the sessile Anthozoa (e.g., sea anemones, stony corals, and sea fans), the free-living Medusozoa (e.g., jellyfish and Hydrozoa), and the microscopical endoparasites Myxozoa [17,77,660]. These animals possess specialized stinging cells distributed along their body, named cnidocytes, with harpoon-like large secretory organelles – nematocysts – that have an established role in venom delivery, being discharged upon contact for prey capture and defense against predators [6,655,661]. Cnidaria venom is constituted by a combination of several peptides and other bioactive molecules, collectively referred to as toxins. Known peptide toxins from Cnidaria include enzymes (e.g., phospholipase A2, metalloproteinases), membrane-active toxins (e.g., jellyfish toxins, actinoporins and hydralysins), neurotoxins (e.g., sodium and potassium-channel inhibitory toxins and SCRiPs – small cysteine rich proteins) and protease inhibitors [24,662–665]. Moreover, cnidarians rely heavily on their innate immune systems through the production of AMPs, as they thrived in challenging environments for hundreds of millions of years with high microbial and viral loads (approximately $10^6$ bacteria/mL and $10^9$ viruses/mL of seawater [666]) and without adaptative immune responses [300,469,667]. Some uncovered AMPs include Aurelin [492] from the scyphoid jellyfish *Aurelia aurita* and Damicornin [487] from the stony coral *Pocillopora damicornis*, with potent antibacterial and antifungal properties.

Sea anemones (order Actiniaria) are generally large, solitary soft-bodied polyps usually attached to rocks or other suitable substrata, with approximately 1350 species estimated to exist worldwide [668]. They are distributed from tropical to polar regions, occupying several habitats from the intertidal oceanic zones to depths of more than 10,000 m [661]. Sea anemones possess vital roles in marine ecosystems. They act as predators of several organisms, including crustaceans, fish, and mollusks, which are caught with tentacles, paralyzed and secured by the cnidae, and carried to the mouth. Mutualistic interactions are also common with hermit crabs and fish of the genus *Amphiprion* (clownfish) [668]. Sea anemones account for most of the toxins uncovered in Cnidaria, which have been identified to date by traditional protein analyses [662]. Also, their ecological function can be inferred by their location in the sea anemone tissues. Neurotoxins and membrane-active toxins are more common in the tentacles and

epidermis, supporting prey immobilization and predator deterrence. Enzymatic toxins support digestive roles in the gastrodermis and mesenteric filaments [656,669–671]. Sea anemones also have additional structures from other anthozoans. Acrorhagi are found within the Actiniidae family, inflatable organs located below the tentacles with roles in intraspecific aggression. Acrorhagins from *Actinia equina* were the first described toxins from the acrorhagi, showing lethality in crabs [672]. Additionally, acontia is a structure unique to specific lineages of the superfamily Metridioidea, constituted from long thread-like structures formed from the mesenterial filaments with a possible role in defense [673]. Depending on the species, their toxins may be localized in both nematocysts and ectodermal gland cells [674]. Interestingly, certain neurotoxins produced by sea anemones exhibit antimicrobial properties, highlighting a dual role in their survival. These toxins facilitate prey capture and defend against bacterial infections that could result from injuries to their tentacles [490,639].

The strawberry anemone *A. fragacea* [675], with yellow or green spots displayed in its column, is genetically distinct from the most abundant *A. equina* in the northern coasts of Portugal, which shows a high degree of polymorphism. Compared with *A. equina*, *A. fragacea* lacks viviparity, is larger (up to 10cm in diameter), and is mainly found solitary [676,677]. Five pore-forming toxins (actinoporins) causing hemolysis are known from *A. fragacea*, corresponding to the Fragaceatoxins [677,678].

Recent advances in omics technologies—including genomics, transcriptomics, proteomics, and metabolomics—have significantly deepened our understanding of marine organisms' ecology, distribution, and defense mechanisms and the ability to uncover novel bioactive compounds for biotechnological applications. Despite these developments, the chemical arsenal and protein composition of *A. fragacea* remains poorly characterized, with only 111 proteins currently documented in the NCBI database. In this study, we present a comprehensive proteomic profile of the nematocyst venom extract, four different tissues and the mucus secretion of the sea anemone *A. fragacea*, addressing this knowledge gap (Fig. 12). To the best of our knowledge, this research represents the first proteomic characterization of this species.

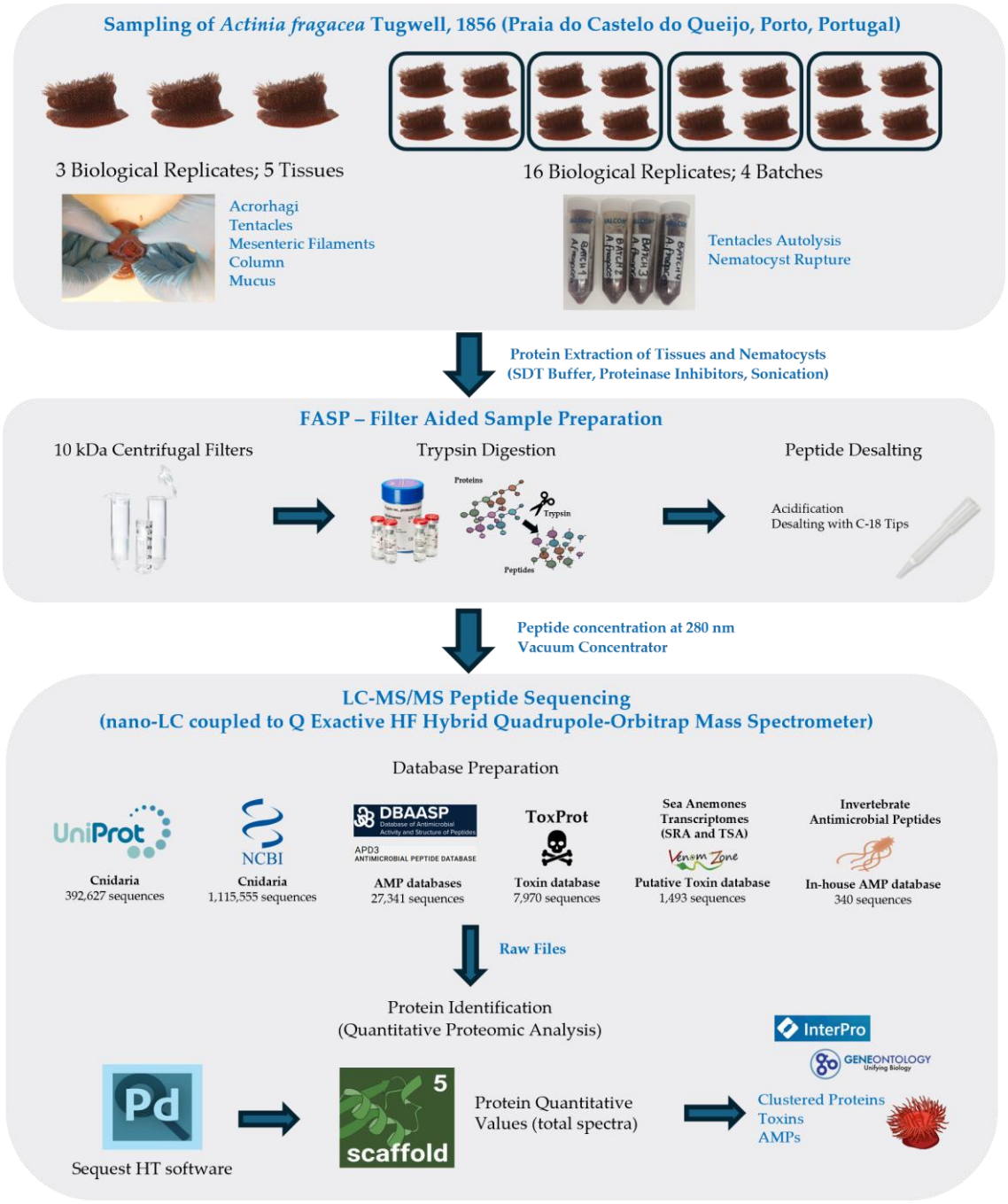



**Fig. 12** Methodological workflow for proteomic profiling of nematocysts and tissues in *A. fragacea*.

## 4.2. Results

### 4.2.1. LC-MS/MS Analyses and Protein Identification

Specimens of *A. fragacea* collected from Praia do Castelo do Queijo, Porto, Portugal, were analyzed to assess their protein and peptide content. The study encompassed the nematocyst venom extract, four different tissues, and the mucus secretion, providing a comprehensive overview of the species' proteomic profile (Fig. 13).

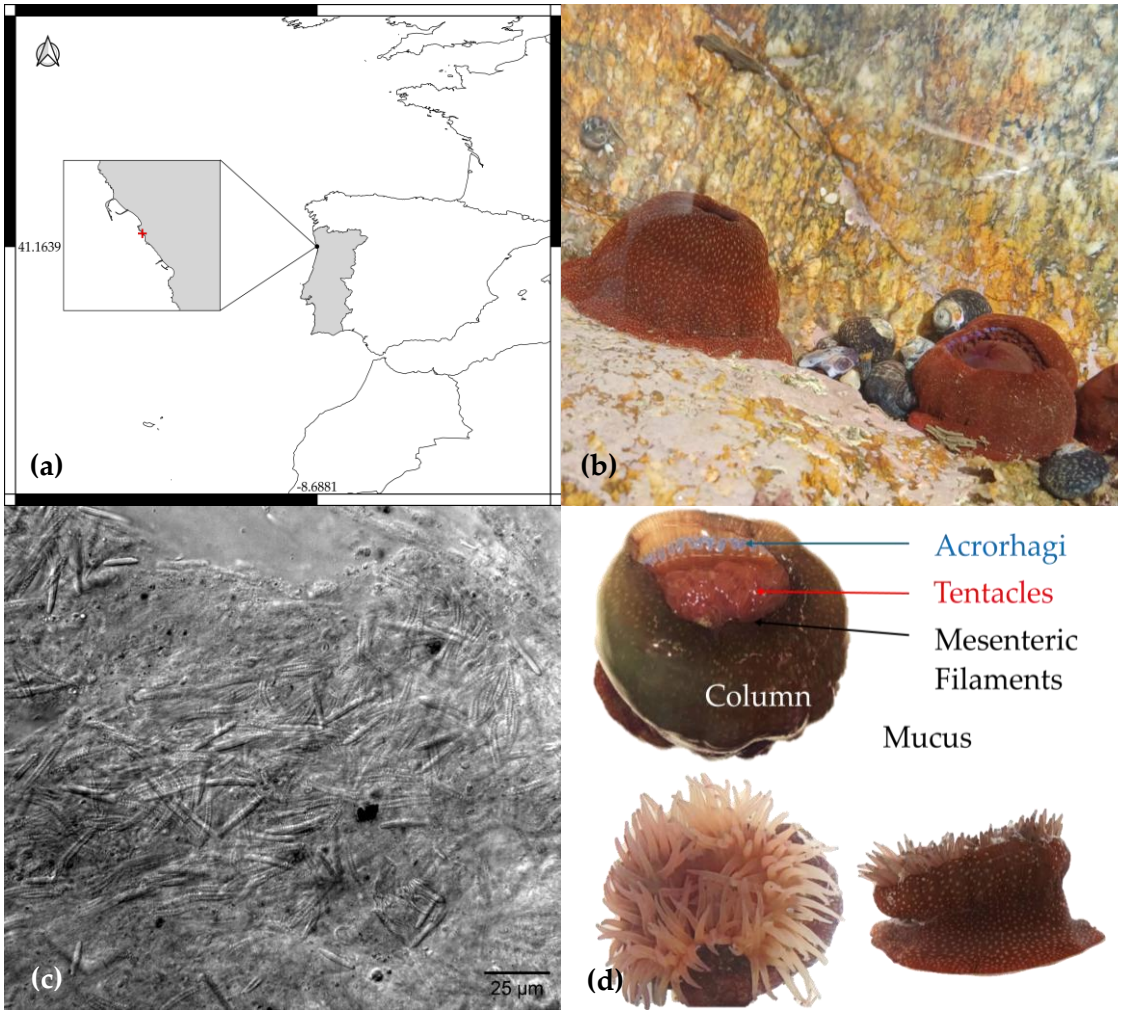



**Fig. 13** Overview of sampling location, specimens, and experimental approach for *A. fragacea*. (a) Map showing the geographic context of the sampling location, centered on Praia do Castelo do Queijo, Porto, Portugal. (b) Photograph of *A. fragacea* in its natural habitat, a tide pool at the sampling site, showcasing the species' distinctive spots in the column. (c) Microscopic image of nematocysts isolated from *A. fragacea* tentacles, with a scale bar of 25 µm. (d) Photographs illustrating the location of the dissected tissues of *A. fragacea*, including top and side views of the specimen.

A total of 4011 different proteins, clustered into 3383 protein groups, were identified in *A. fragacea* across all analyzed samples (Table S 16). Detailed sample composition statistics are presented in Table 5.

**Table 5** Summary of results from *A. fragacea* protein profiling experiments by LC-MS/MS.

| Sample Category | Proteins (mean ± SD) | Protein Groups (mean ± SD) | Total Proteins | Total Protein Groups |
|---|---|---|---|---|
| Acrorhagi | 1358 ± 275 | 1086 ± 217 | 1958 | 1561 |
| Column | 1241 ± 203 | 966 ± 158 | 1821 | 1416 |
| M. Filaments | 1523 ± 180 | 1181 ± 129 | 2078 | 1580 |
| Mucus | 866 ± 334 | 637 ± 273 | 1523 | 1150 |
| Nematocyst | 2156 ± 113 | 1936 ± 107 | 3009 | 2769 |
| Tentacle | 1237 ± 186 | 980 ± 154 | 1660 | 1322 |

"Proteins (mean ± SD)" and Protein Groups (mean ± SD)" indicate the average number and respective standard deviation of unique proteins and protein groups identified per sample category. "Total Proteins" and "Total Protein Groups" show the cumulative counts of unique proteins and protein groups identified across all samples within each category.

In total, 891 proteins were ubiquitous and identified across all sample categories (Fig. 14). Nematocysts were the samples with more exclusively identified proteins (1440), followed by mesenteric filaments (147), acrorhagi (50), mucus (35), and tentacles (16). Interestingly, no proteins were exclusive from column samples. Acrorhagi, column, and mesenteric filaments shared the highest number of proteins (1735), while mucus, nematocysts, and tentacles shared the lowest (Table 6).

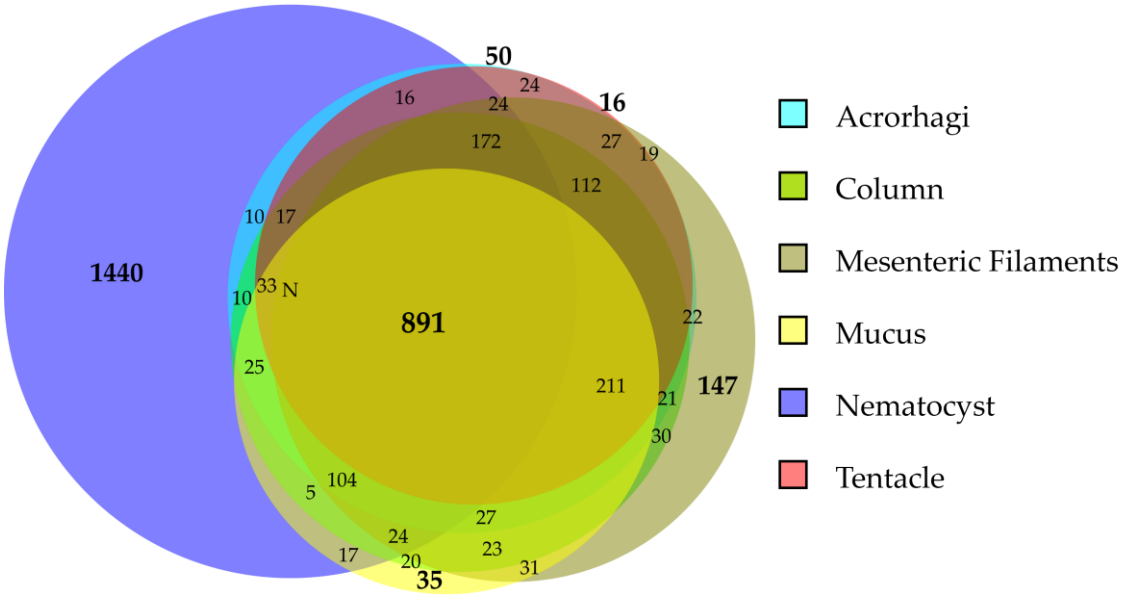



**Fig. 14** Area-proportional Venn diagram illustrating the overlap of identified proteins across six sample categories, including nematocysts, tissues and mucus secretions of *A. fragacea*. Generated using DeepVenn (https://www.deepvenn.com, accessed on 10 January 2025).

**Table 6** Pairwise comparison of shared proteins across sample categories, highlighted by gradient coloring (red shades indicate higher protein overlap, while blue shades indicate lower protein overlap)

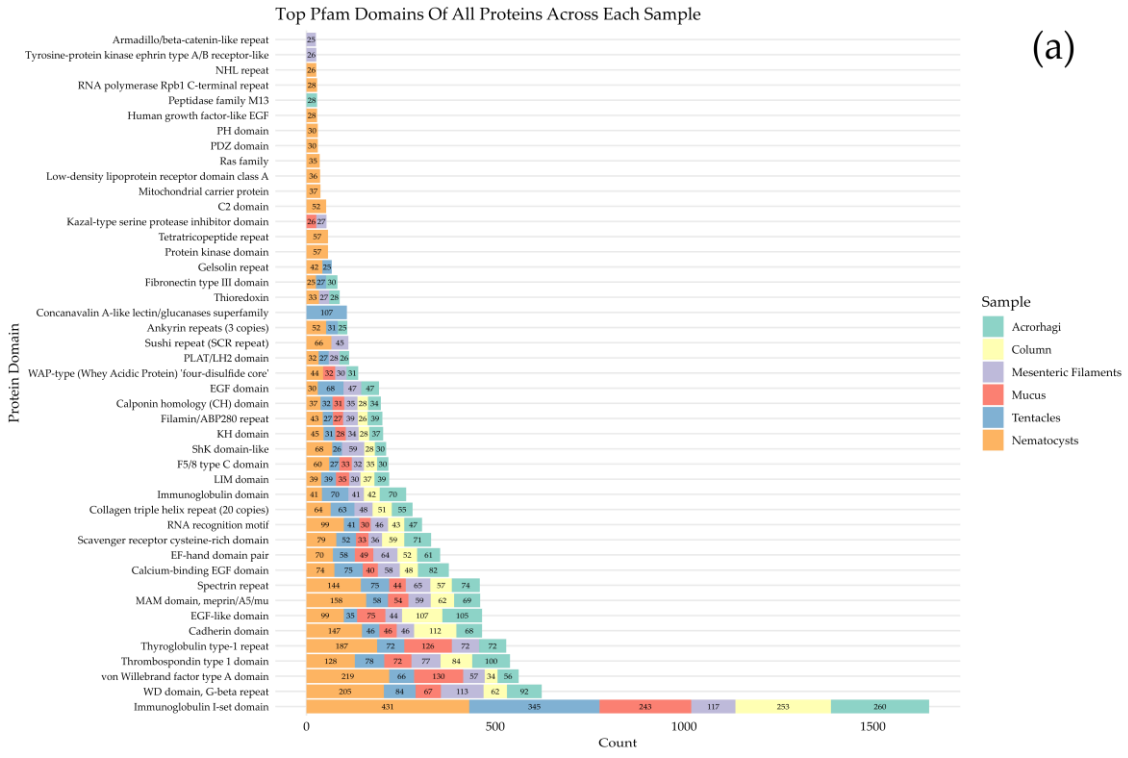
| | A | C | F | M | T | N |
|---|---|---|---|---|---|---|
| **A** | | 1735 | 1715 | 1424 | 1616 | 1398 |
| **C** | 1735 | | 1714 | 1446 | 1556 | 1346 |
| **F** | 1715 | 1714 | | 1446 | 1574 | 1407 |
| **M** | 1424 | 1446 | 1446 | | 1254 | 1203 |
| **T** | 1616 | 1556 | 1574 | 1254 | | 1232 |
| **N** | 1398 | 1346 | 1407 | 1203 | 1232 | |

A: Acrorhagi; C: Column; F: Mesenteric filaments; T: Tentacle; N: Nematocysts.

### 4.2.2. Functional Annotation

A comprehensive functional analysis was conducted to gain insight into the biological roles of the identified proteins. InterProScan was used to predict Pfam protein domains

and key functional sites, with the analysis performed separately for each sample category to identify functional differences. InterProScan was run independently for each sample category to all validated proteins and a subset of uncharacterized proteins (e.g., hypothetical, predicted without annotation, unnamed protein product) (Fig. 15). The results revealed that the Immunoglobulin-I set domain is the most abundant across all sample types. Nematocyst samples contained the most identified protein domains, including several exclusively found in them. Interestingly, the Concanavalin A-like lectin/glucanases superfamily was the second most abundant domain in tentacle samples but was absent in all the other categories. Also, the Thyroglobulin type-1 repeat domain was highly present in all sample types except the column. Among uncharacterized proteins, the most common domains across all sample types included Thyroglobulin type-1 repeat, von Willebrand factor type A, Thrombospondin type 1, and EGF domains. These are often associated with protease inhibition, cell adhesion, tissue remodeling, and cell growth. Complete InterProScan results are provided in Table S 17.



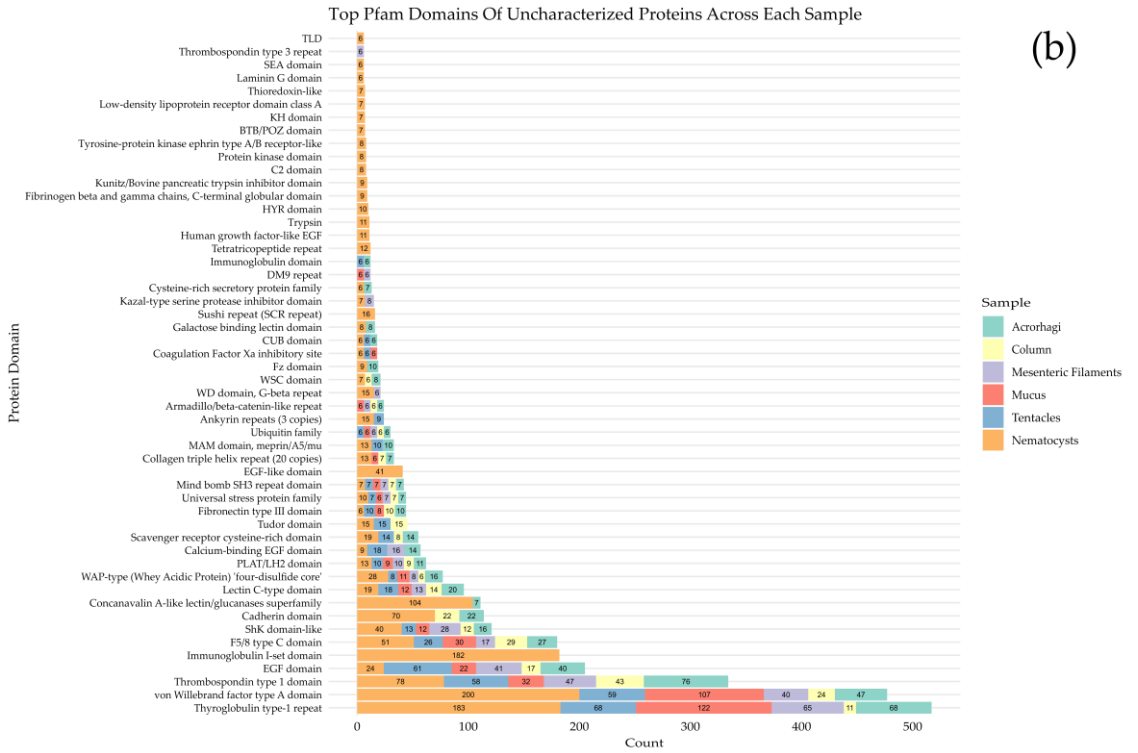
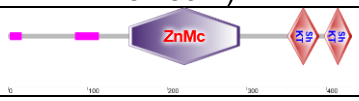
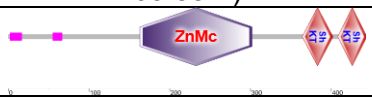



**Fig. 15** Top Pfam domains identified in *A. fragacea* samples: (a) all proteins (minimum 25 copies), (b) uncharacterized proteins (minimum six copies).

A common toxic domain found in potassium channel inhibitors of sea anemones, the Stichodactyla toxin (ShK) domain-like, was detected with high abundance across all samples. Among the 17 uncharacterized proteins containing this domain, two proteins from the transcriptomes of *Anthopleura elegantissima* (Aelegantissima_304099_TSA) and *Anemonia viridis* (Avirdis_207505_SRA) identified in nematocysts, showed high percentage identity with zinc metalloproteinases containing ShK and astacin domains from other sea anemones, suggesting potential toxin functionality (Table 7). Additionally, several other major domains associated with the nematocyst proteome that may contain toxic functions include Kazal-type serine protease inhibitor domains, the cysteine-rich secretory protein family (CAP/CRISP), and Kunitz/bovine pancreatic trypsin inhibitor domains. Ubiquitin family domains were detected in all samples, with their peptides potentially possessing immune function. Detailed functional annotation results are available in Tables S 17 and S 18.

**Table 7** Identification of a zinc-metalloproteinase (ZnMc) domain and two ShK domains in two proteins identified in the nematocyst proteome of *A. fragacea*.

| Protein | Aelegantissima_304099_TSA | Avirdis_207505_SRA |
|---|---|---|
| Domain 1 | ZnMc (152–291; $5.88 \times 10^{-52}$) | ZnMc (163–302; $2.19 \times 10^{-51}$) |
| Domain 2 | ShK (351–391; $1.96 \times 10^{-3}$) | ShK (364–402; $2.16 \times 10^{-5}$) |
| Domain 3 | ShK (397–432; $6.46 \times 10^{-4}$) | ShK (408–444; $5.24 \times 10^{-4}$) |

| | | |
|---|---|---|
| BLASTp Results | VFU02998.1 (0; 73.77%)<br>XP_031553445.1 (0; 72.31%)<br>VFU02997.1 ($2.0 \times 10^{-160}$; 57.89%) | VFU02998.1 (0; 72.65%)<br>XP_031553445.1 (0; 72.54%)<br>VFU02997.1 ($2.0 \times 10^{-150}$; 63.88%) |
| SMART Domains |  |  |

These proteins are homologous to transcripts from *A. elegantissima* and *A. viridis* obtained from the putative toxin-tailored database. The homologous sequences include VFU02998.1 (mRNA, *Oulactis* sp. MM-2018), XP_031553445.1 (zinc metalloproteinase nas-15-like, *Actinia tenebrosa*), and VFU02997.1 (mRNA, *Oulactis* sp. MM-2018). Domain start–end positions and E-values are shown in parentheses. BLASTp results show homologous sequences, along with their E-values and percentage identities. Protein domain representations were obtained using the SMART database [679].

Proteins annotated by InterProScan were further blasted for Gene Ontology (GO) mapping and annotation using Blast2GO software (implemented in the OmicsBox platform version 3.4.0). The annotation process followed standard GO configuration parameters, and redundancy was eliminated through GO validation. Proteins were categorized into the three main GO domains at Ontology level 2: cellular component (CC), biological process (BP), and molecular function (MF) (Fig. 16). A total of 11,740 GO annotations were assigned, with an average GO level of 6.65 and a standard deviation of 2.39 (Fig. S 7). The annotation results indicate that the identified proteins are predominantly localized in the cytoplasm, primarily involved in binding functions, and participate in biological processes such as proteolysis.

Additionally, the annotated proteins were categorized according to their enzymatic functions by mapping the corresponding Enzyme Commission (EC) codes to their respective annotations (Fig. S 8 and S 9).

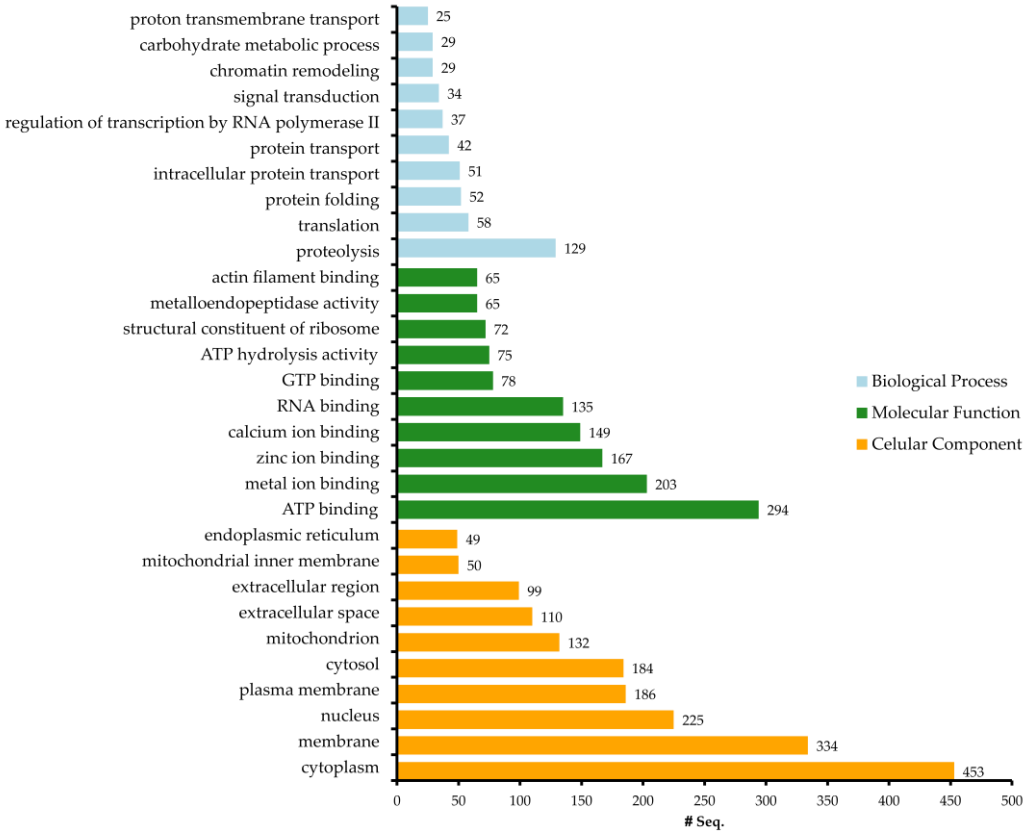



**Fig. 16** The most represented GO terms of the *A. fragacea* samples in the three domains of biological process, molecular function, and cellular component.

### 4.2.3. Putative Venom Proteins Identified in *A. fragacea*

Overall, 83 putative toxins were detected (Fig. 17). Of these, 32 constitute well-characterized toxins, while 51 may be related to venom-like functions (Fig. 18).

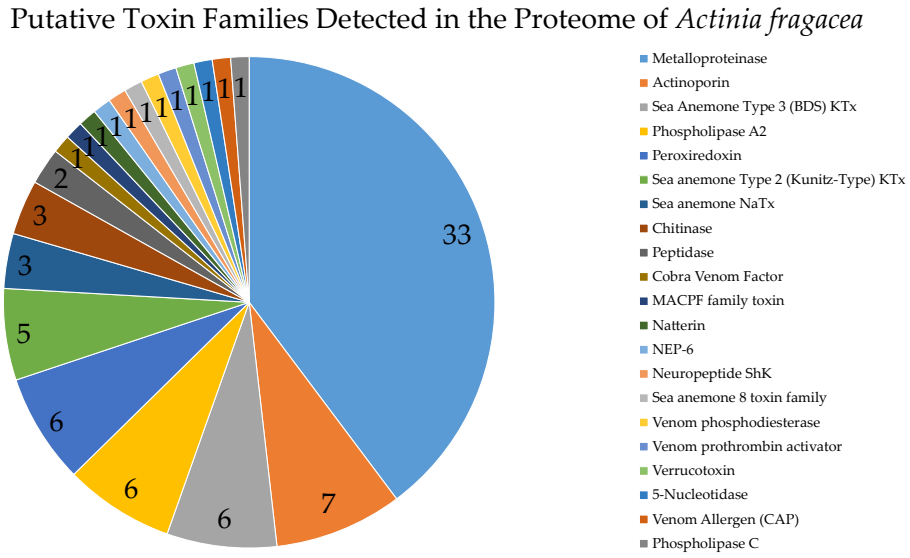



**Fig. 17** Overview of toxin family types detected in the proteome of the sea anemone *A. fragacea*, including nematocysts, tissues, and mucus secretions. KTx – potassium-channel inhibitory toxins; NaTx – sodium-channel inhibitory toxins. In

the legend, toxin families are arranged in descending order of abundance. Comprehensive details of the detected toxins associated with each family can be found in Table S 19.

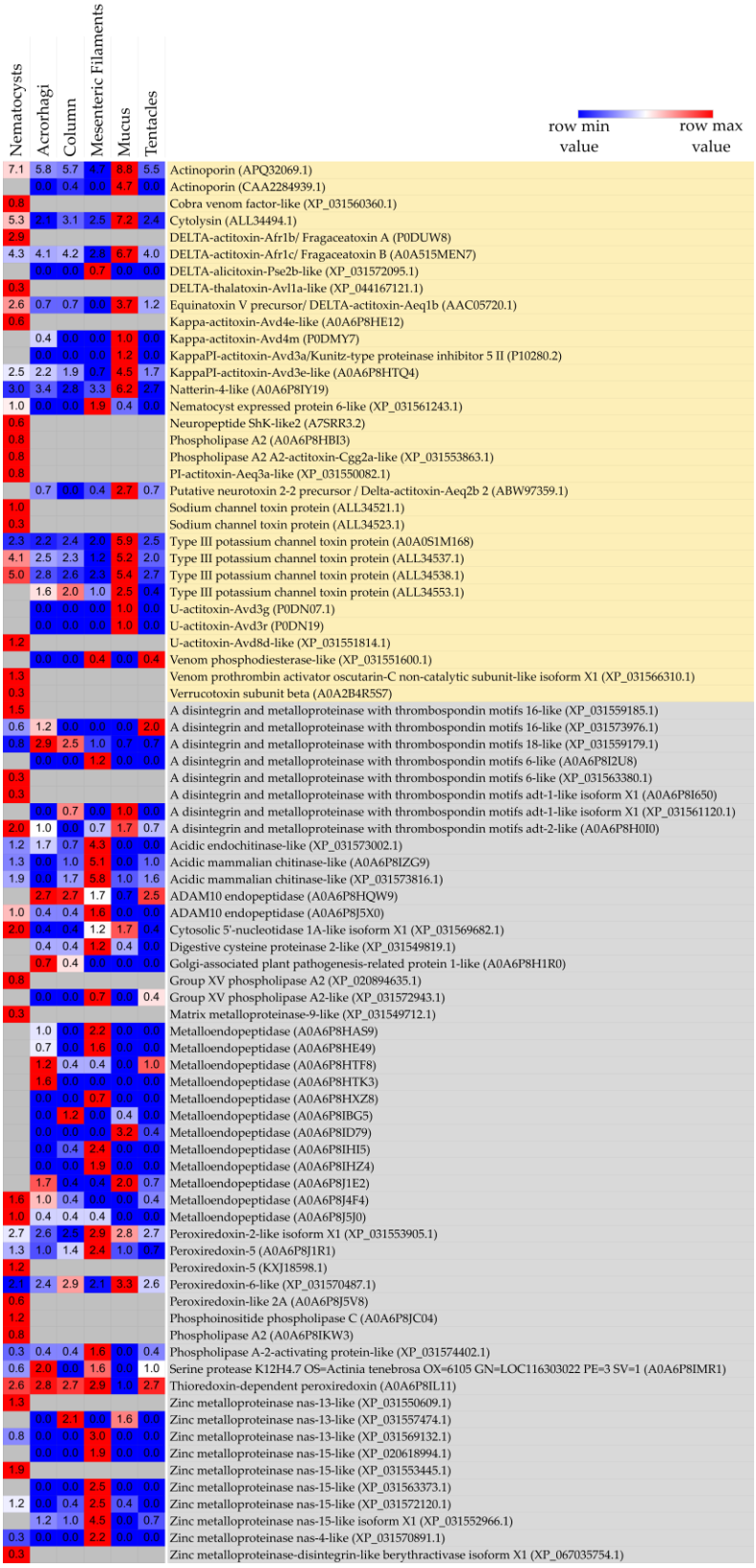



**Fig. 18** Heatmap of the venom protein expression across nematocysts, tissues, and mucus secretion samples in *A. fragacea*. Proteins highlighted in yellow constitute well-characterized toxins, while those highlighted in blue are related to

venom-like functions. The expression levels of each protein across sample types were log-transformed using the formula $\log_2$ (mean of the normalized total spectrum counts from the three biological replicates +1). The heatmap was constructed using Morpheus https://software.broadinstitute.org/morpheus (accessed on 7 January 2025).

Most toxins identified showed close identity with those from *Actinia* (59/83), followed by *Anemonia sulcata* (8/83), *A. equina* (3/83) and *A. viridis* (3/83). Two common actinoporins from *A. fragacea* were detected in the proteomic data: DELTA-actitoxin-Afr1b/Fragaceatoxin A (P0DUW8), which was exclusively expressed in the nematocysts, and DELTA-actitoxin-Afr1c/Fragaceatoxin B (A0A515MEN7), which was expressed in all sample types but exhibited higher expression in the mucus. Among the toxins detected, most were identified as putative metalloproteinases (33/83), followed by actinoporins (7/83), members of the sea anemone type 3 (BDS) potassium channel toxin family (6/83), and phospholipases A2 (6/83). XP_031553863.1 and XP_031553799.1 from *A. tenebrosa* were classified as toxins based on the presence of the phospholipase A2 domain (Table S 17), high sequence similarity to other PLA2s from other sea anemones and snakes, and the presence of conserved catalytic residues (Fig. 19).

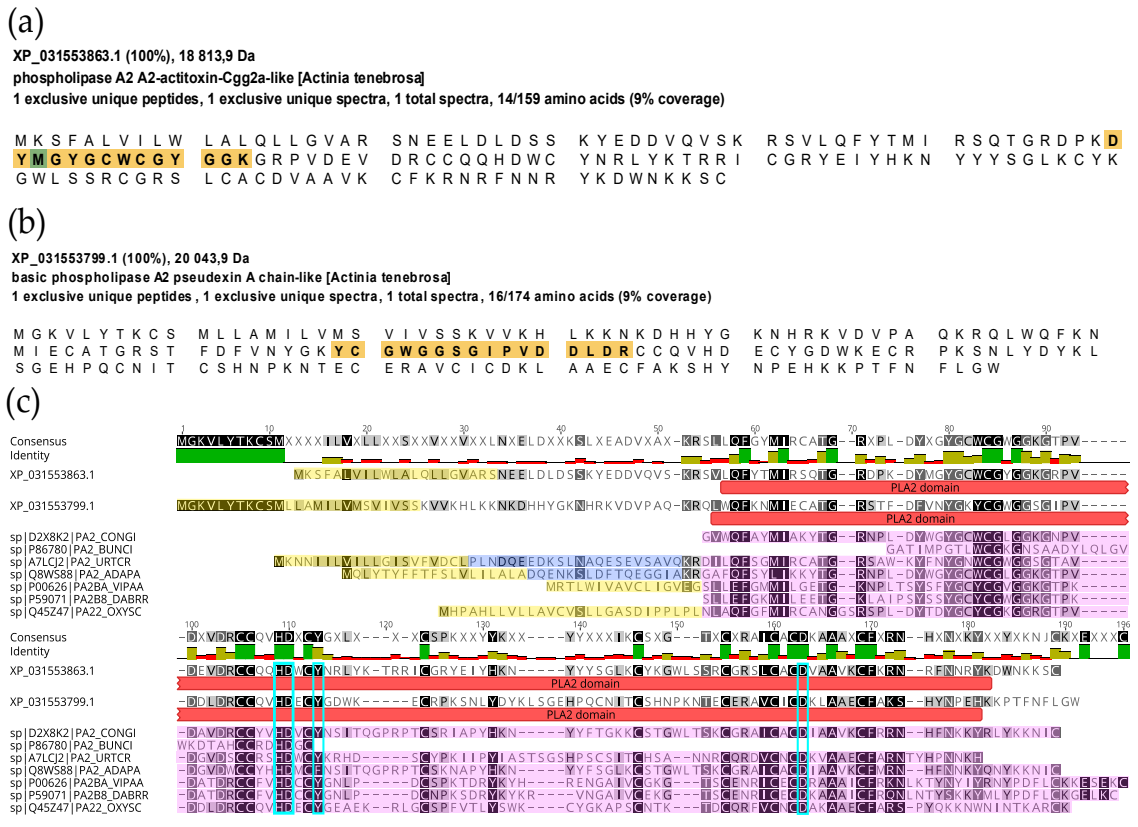



**Fig. 19** Identification and alignment of the phospholipase A2 (PLA2). The amino acid sequence of the two identified PLA2 domains in the nematocyst proteome of *A. fragacea* are represented in (a) XP_031553863.1 and (b) XP_031553799.1, both derived from the genome of *A. tenebrosa*. (c) Multiple sequence alignment of the identified PLA2 with others from sea anemones (*Condylactis gigantea* [CONGI], *Bunodosoma caissarum* [BUNCI], *Urticina crassicornis* [URTCR], *Adamsia palliata* [ADAPA] and snakes (*Vipera ammodytes* [VIPAA], *Daboia russelii* [DABRR], and *Oxyuranus scutellatus* [OXYSC]). The signal peptides are highlighted in yellow, the propeptide in dark blue, and mature peptide in pink. The

PLA2 domain detected in the identified proteins is marked in red. The catalytic center characteristic of PLA2 is highlighted in light blue. Generated using Geneious software (version 11.1.5).

Other neurotoxins commonly found in sea anemones were also detected, including five sea anemone type-2 potassium channel toxins/Kunitz-type proteinase inhibitors, two sea anemone sodium-channel inhibitory toxin families, and one sea anemone 8 toxin family. Other interesting compounds include the detection of Natterin-4-like expressed in all sample categories but with higher expression in the mucus and the Verrucotoxin subunit beta, detected exclusively in nematocysts. These are common toxins from the venom glands of *Thalassophryne nattereri* and *Synanceia verrucosa* fish. Furthermore, two toxins typically found in the sea anemone *Nematostella vectensis* were identified: the metalloprotease nematocyst expressed protein 6-like, present in all tissues, with higher expression in the column, and the neuropeptide ShK-like2, which was detected exclusively in the nematocysts (Fig. 20). All information is available in Table S 19.

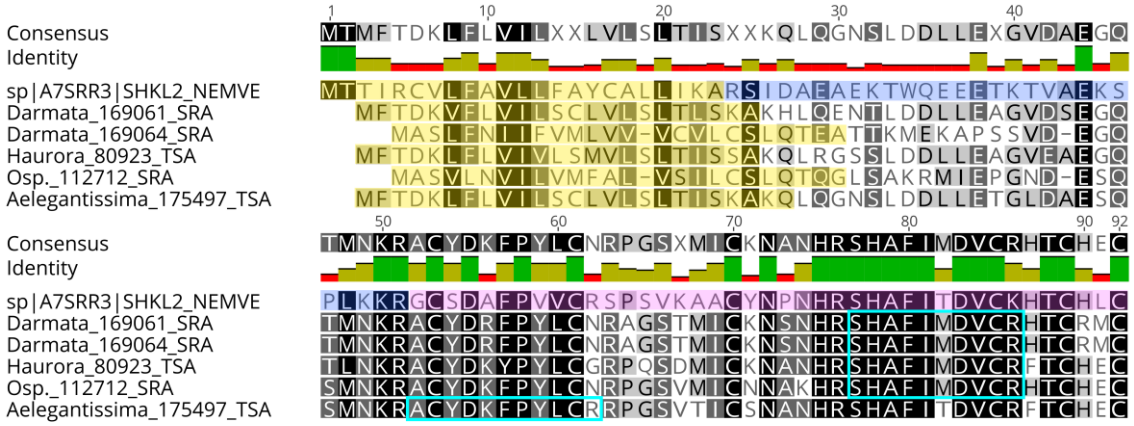



**Fig. 20** Multiple sequence alignment of ShK-like neuropeptides detected in the nematocyst proteome of *A. fragacea*. These are derived from the transcriptomes of the sea anemones *Dofleinia armata* (Darmata_169061_SRA, Darmata_169064_SRA), *Heteractis aurora* (Haurora_80923_TSA), *Oulactis* sp. (Osp._112712_SRA), and *A. elegantissima* (Aelegantissima_175497_TSA) and had high sequence identity (E-value $1 \times 10^{-13} – 4 \times 10^{-10}$) with the neuropeptide Shk-like2 from the sea anemone *N. vectensis* (A7SRR3). The signal peptides are highlighted in yellow, the propeptide in dark blue and mature peptide in pink. The peptides identified in the proteome of *A. fragacea* are highlighted in light blue. Generated using Geneious software (version 11.1.5).

In addition, a whole animal sample was included as a preliminary test, and interestingly, this analysis revealed the presence of two exclusively identified cephalotoxin-like proteins, which are known for their neurotoxic properties and potential bioactivity (Fig. 21).

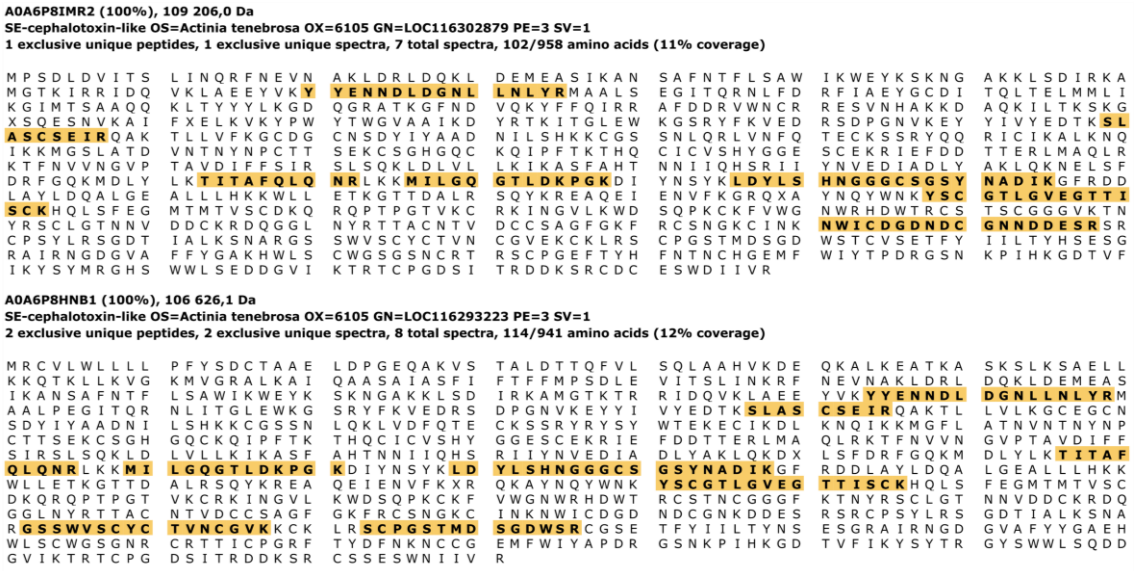



**Fig. 21** Amino acid sequences of the two identified cephalotoxin-like proteins, with the detected peptides highlighted.

## 4.2.4. Putative Peptides with Antimicrobial Activity Identified in *A. fragacea*

Several bioactive compounds with potential immune-related and antimicrobial functions were identified, providing valuable insights into the organism's defense mechanisms. Among these, AMPs such as Aurelin-like peptide and Myticin-A were detected (Fig. 22). Additionally, immune-related molecules, including neuropeptides like RFamide, enzymes such as peroxiredoxins, and PRPs like lectins were also detected (Table S 20). These findings highlight the organism's diverse biochemical repertoire for pathogen defense and immune regulation.

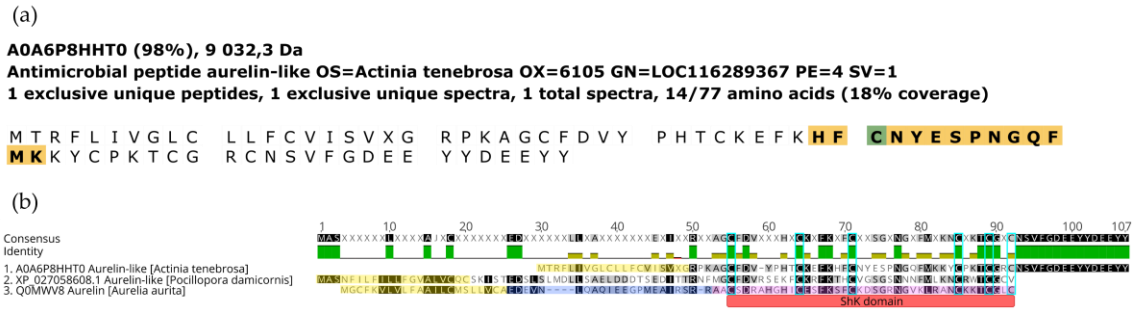



**Fig. 22** Identification and alignment of the Aurelin-like antimicrobial peptide. (a) Amino acid sequence of the identified Aurelin-like antimicrobial peptide, with peptides detected in this study highlighted. (b) Multiple sequence alignment of Aurelin from *A. aurita* and Aurelin-like antimicrobial peptides from *A. tenebrosa* and *P. damicornis*. The signal peptides are highlighted in yellow, the propeptide in dark blue and mature peptide in pink. The ShK domain is marked in red, while the six cysteine residues forming the conserved motif are highlighted in light blue. Generated using Geneious software (version 11.1.5).

To further explore the antimicrobial potential of the identified trypsinized peptides, peptide predictions were performed using the Collection of Anti-microbial Peptides

(CAMPR4) https://camp.bicnirrh.res.in/ (accessed on 20 January 2025), and the Antimicrobial Peptide Scanner vr.2 https://www.dveltri.com/ascan/v2/ascan.html (accessed on 20 January 2025). The CAMPR4 tool predicted a total of 2950 AMPs, while the Antimicrobial Peptide Scanner vr.2 identified 2073 with antimicrobial potential (Table S 21). Upon comparison, 1037 peptides were predicted as AMPs by both tools, which represents approximately 5% of the 20,353 distinct peptides identified in the entire dataset. Importantly, only nine peptides from the total dataset achieved a prediction probability greater than 90% in both tools, indicating a relatively high level of confidence in their antimicrobial potential (Table 8).

**Table 8** Peptides predicted to be AMPs with over 90% probability according to both CAMPR4 and Anti-microbial Peptide Scanner vr.2.

| ID | Sequence | Protein Name | Accessions | Domains | GO Terms |
|---|---|---|---|---|---|
| 1280 | ASLGQWPAGSYCILASGGCPK | Uncharacterized protein LOC116288220 isoform X2 | XP_031550846 XP_031550847 | Periplasmic Binding Protein Type-2 (IPR010068) C-terminal domain of apextrin (IPR031569) | |
| 6378 | GIAFPTCISVNNCVCHCSPLK | Proliferation-associated protein 2G4-like | XP_031550715 | Metallopeptidase family M24 (IPR000994) | |
| 6420 | GILFAVPGAFTPGCSK | Peroxiredoxin-5 | A0A6P8J1R1 | Thioredoxin (IPR013766) | GO:0034599 GO:0016491 GO:0008379 |
| 6581 | GLGPSVISAVIPLYGK | Uncharacterized protein LOC116295095 | A0A6P8I1A2 | GG-type lectin domain profile | GO:0005515 |
| 7224 | GVVIIGPATVGGIKPGCFK | ATP-citrate synthase | XP_031568246 | Citrate synthase Integrated (IPR036969) Succinyl-CoA synthetase domains Integrated (IPR016102) | GO:0006085 GO:0006101 GO:0003824 GO:0046912 GO:0003878 |
| 8437 | ILGLPGFSVPPAAFVPVCK | Uncharacterized protein LOC116307005 | A0A6P8J4T9 | Thyroglobulin_1 (IPR000716) | GO:0030414 GO:0005515 GO:0004867 |

| | | | | | |
|---|---|---|---|---|---|
| 17784 | VCLLGCGISTGYGAAINTAK | S-(hydroxymethyl)glutathione dehydrogenase | A0A8B7E018<br>T2MHJ5<br>XP_065652493 | Class III alcohol dehydrogenases (IPR014183) | GO:0046294<br>GO:0008270<br>GO:0051903<br>GO:0016491 |
| 18406 | VLDALFPCVQGGTTAIPGAFGCGK | H(+)-transporting two-sector ATPase | KAK3738516<br>XP_020913463<br>A0A6P8IB06 | P-loop containing nucleotide triphosphate hydrolases (IPR027417) | GO:0046034<br>GO:1902600<br>GO:0005524<br>GO:0046961<br>GO:0016887<br>GO:0033180 |

## 4.3. Discussion

### 4.3.1. Comparative Analysis of Protein Content and Distribution Across Sample Categories

Total protein content varied significantly across the different sample categories. Nematocysts exhibited the highest protein content, followed by mesenteric filaments and acrorhagi, while mucus samples showed the lowest. These differences may reflect the distinct nature of the samples and variations in the sample preparation protocols. Notably, nematocyst samples were processed using a different method than the other sample types, as explained in the methods section. The standard deviation for proteins and protein groups was particularly high in the mucus samples, potentially reflecting the inherent variability of mucus composition or challenges associated with sample collection. Nematocyst samples were the samples with the most distinctive content. Internal tissues (mesenteric filaments and column) show stronger overlap, likely due to shared metabolic or structural proteins, whereas mucus and nematocyst samples have distinct protein profiles due to their more specialized functions.

### 4.3.2. Functional Annotation of Identified Proteins

The most common Pfam domains identified in the proteome of *A. fragacea* were associated with housekeeping functions. Immunoglobulin I-set domains, which are involved in immune defense mechanisms in antibodies or cell surface receptors, were also receptors. However, like other marine invertebrates, sea anemones lack adaptive immune responses and instead rely on their innate immune systems, including PRRs—pattern recognition receptors—that recognize intracellular and extracellular molecular patterns associated with microorganisms or cellular damage [680] The high presence of this domain in the proteome of *A. fragacea* underscores their critical role in survival in high microbial marine environments. Several immunity-related domains were also

identified in the immunotranscriptome of other Anthozoans [681]. These domains may be associated with cell adhesion molecules (CAMs), which mediate cell interactions in the nervous system and potentially support nerve regrowth and tissue organization in sea anemones [682]. Additionally, other commonly found domains in the proteome of *A. fragacea* included the von Willebrand A (VWA), Thrombospondin Type 1 (TSP1), Thyroglobulin type-1 Repeat, and Epidermal Growth Factor (EGF) domains. The VWA domain is known to be involved in cell adhesion, extracellular matrix proteins, and integrin receptors [683]. TSP1 proteins are multidomain glycoproteins involved in tissue homeostasis and remodeling. Notably, the phyla Cnidaria and Porifera encode a broader diversity of TSP superfamily members than vertebrates [684]. For instance, a TSP from *Hydra magnipapillata* was identified as a major component of the cnidarian mesoglea, where it plays a crucial role in head regeneration [205]. Similarly, a TSP from the sea anemone *N. vectensis* was found to be upregulated during regeneration, being present in neuron-like cells within the mesoglea of the retractor muscles and the pharynx [685]. Thyroglobulin type-1 repeat is often associated with protease inhibition. For instance, Equistatin, detected in the mesenteric filaments of *A. fragacea*, is a protein found in *A. equina* that contains three of these domains and acts as an inhibitor of papain-like cysteine proteinases and the aspartic proteinase cathepsin D [686] (Table S 19). EGF domains, which are associated with cell growth, proliferation, and differentiation, were also notably abundant. Additionally, an EGF-like toxin, Gigantoxin I (UniProt: Q76CA1), isolated from the nematocysts of the sea anemone *Stichodactyla gigantea*, shows 31-33% sequence identity with mammalian EGF. This toxin exhibits EGF-like activity by inducing cell rounding in human epidermoid carcinoma A431 cells and promoting tyrosine phosphorylation of the EGF receptor while also displaying potent paralytic activity in crabs [687].

Several toxin-like domains were also detected in the proteome of *A. fragacea*. ShK domains were found in mucin-like glycoproteins from nematodes, which are implicated in parasite immune invasion [688], and in cysteine-rich secretory proteins (CRISPs), found in the mammalian male reproductive tract and in the venom of reptiles [689]. In sea anemones, these domains are associated with potassium channel toxin inhibitors, including BgK from *Bunodosoma granulifera* [690], and ShK from *Stichodactyla helianthus* [691]. In the current study, two proteins with zinc metaloproteinases and ShK domains, homologous to other sea anemone metalloproteinases, were detected, suggesting a potential role in toxin function. Similar findings were reported in the jellyfish *Pelagia noctiluca* and the sea anemone *N. vectensis* [692] Domains commonly associated with protease inhibitors were also detected in the nematocyst proteome of *A.*

*fragacea*, including Kunitz/bovine pancreatic trypsin inhibitor domains, commonly found in other venomous organisms, and Kazal-type serine protease inhibitors, previously detected in the venom of other sea anemones [693].

Additionally, several ubiquitin domains were also identified. While ubiquitin is primarily recognized for its role as a post-translational modification that targets proteins for proteasomal degradation, ubiquitin-derived peptides are known to contain antimicrobial activity [694], making them an interesting target for biomedicine in cnidaria proteomic studies.

### 4.3.3. Identification of Putative Venom Proteins in *A. fragacea Reveals a Diverse Toxin Arsenal*

Sea anemones are known to produce a diverse range of toxins with mixed functionalities. To understand their chemical arsenal, several proteomics studies have been made, including for *Bunodactis verrucosa* [695], *B. caissarum* [696], *Bunodosoma cangicum* [697], *Entacmaea quadricolor* [698], *Heteractis magnifica* [699] and *Stichodactyla haddoni* [700].

Here, we detected different toxin types. We detected seven actinoporins, common pore-forming toxins specific to sea anemones that interact with sphingomyelin from animal cell membranes to form oligomeric pores [701]. Here, we detected two out of the five fragaceatoxins previously isolated from *A. fragacea* itself: FraA and FraB. We may have only detected these because they are known to be the most thermally stable isoforms. Additionally, they are the most potent toxins causing hemolysis (HC50 of 0.4 and 0.3, respectively) of sheep red blood cells [678]. Both are expressed in the nematocysts of *A. fragacea*, but curiously, only FraB was detected in the tissues and mucus secretion, with higher expression in mucus, which could mean distinct intracellular locations and ecological functions. Additionally, Equinatoxin-V (DELTA-actitoxin-Aeq1b) from *A. equina* [702] was also detected in all sample types (with higher expression in nematocysts and mucus), as well as four other homologous actinoporins from the sea anemones *A. equina* (APQ32069.1), *Oulactis* sp. (CAA2284939.1), and *A. sulcata* (ALL34494.1), and the stony coral *Acropora millepora* (XP_044167121.1).This provides evidence that the *A. fragacea* membrane-active toxin arsenal may be more diversified.

We also detected some neurotoxins. Of the sea anemone potassium channel toxin family, we found five toxins of type II (venom Kunitz-type family) and six of type III (BDS) with close identity to others from *A. viridis*, *A. sulcata* and *A. tenebrosa*. Type-II KTx are homologous to Kunitz-type inhibitors of serine proteases, possessing dual functionality by blockage of Kv activity, namely Kv1.2 channels, and also by inhibiting rapid

degradation of the venom by endogenous enzymes of prey [663]. On the other hand, type-III KTx include BDS-I and II from *A. viridis* [703] and APETx1 from *A. elegantissima* [704] and are involved in blocking of Kv3 subunits or ERG (ether-a-go-go, Kv11.1) channels [663]. We also detected two sea anemone sodium-channel toxins, including Delta-actitoxin-Aeq2b 2 from *A. equina*.

Previously, metalloprotease NEP-6 was detected in the nematocytes and pharyngeal glands in later life stages of the sea anemone *N. vectensis* [705]. Here, we had a similar pattern, where a NEP-6 like toxin is expressed in both nematocysts and mesenteric filaments and may be a common toxin expressed in all sea anemones that may aid in digestion. Another interesting compound from *N. vectensis*, the toxin-like Neuropeptide ShK-like2, was also detected in the nematocysts of *A. fragacea*. This neuropeptide, together with the neurotoxin ShK-like1, have a contraction paralysis effect on zebrafish and body contraction on *Nematostella* polyps [706]. As previously mentioned, the ShK-like2 peptide is conserved throughout sea anemone phylogeny [706]. In the present work, homologous genes to this peptide were detected in the transcriptomes of *A. elegantissima*, *D. armata*, *H. aurora*, and *Oulactis* sp., further supporting this assumption. Additionally, we detected two interesting PLA2 domains (XP_031553863.1 and XP_031553799.1, both derived from the genome of *A. tenebrosa*) with close identity to others from sea anemones and snakes, with the conserved catalytic domain. PLA2 has been convergently recruited into the venom of several taxa, including cephalopods, cnidarians, insects, arachnids, and reptiles [707].

Other interesting toxins found in the proteome of *A. fragacea* included a toxin homologous to the Verrucotoxin subunit beta (A0A2B4R5S7) [708], which has hemolytic, cytolytic, hypotensive, and calcium-channel-modulation activities, and Natterin-4-like (A0A6P8IY19), a class of toxins from stonefish that demonstrates nociceptive, edema-inducing and kininogenase activities [709]. This is evidence of convergent evolution of toxins across the animal kingdom, and sea anemones may have ancestral toxin-like genes that suffered neofunctionalizations.

### 4.3.4. *A. fragacea* Reveals Peptides With Putative Antimicrobial Activity

Sea anemones are known to produce several bioactive compounds that contribute to their innate immune defense system. Among these, AMPs are particularly understudied, with only two examples reported to date: Crassicorin from *U. crassicornis* [490] and Equinin from *A. equina* [491]. In this work, two putative AMPs were identified: Aurelin-like peptide and Myticin. Aurelin, the first AMP described in cnidarians, was initially isolated from the moon jellyfish *A. aurita* [492]; a putative Aurelin homolog was predicted

from the transcriptome of the coral *P. damicornis* (XR_003447557), while another potential homolog, referred to as Aurelin-like peptide, was identified in *A. tenebrosa* (A0A6P8HHT0). This peptide has a 6-cysteine residue motif and a ShK domain characteristic of Aurelin. The Aurelin-like peptide identified in *A. fragacea* in the present work may represent another member of a cnidarian-exclusive Aurelin peptide family. Given its evidence in the proteome of a sea anemone, evolutionary studies targeting this family should be performed to understand the origin and phylogenetic distribution of Aurelin-like peptides. Myticin, on the other hand, is a well-characterized AMP that has been exclusively identified in mussels [518]. Its detection in *A. fragacea* samples is likely attributable to cross-contamination, given the strong evidence supporting its mussel-specific origin. This is further supported by the presence of mussels (*Mytilus* spp.) in the tide pools where the sea anemones were collected, which could explain the detection of Myticin in the samples. This finding underscore both the importance of Myticin in *Mytilus* defense system and the need for caution when interpreting AMP data from environmental samples.

Putative peptides with antimicrobial activity include not only AMPs but also other bioactive compounds with antimicrobial properties. Among these are RFamide peptides, a class of neuropeptides defined by a C-terminal arginine and an amidated phenylalanine motif. While their primary function involves neuroregulation, such as cell signaling within the nervous system [710], RFamide III identified in the cnidarian *Hydra*, also exhibits antimicrobial activity [596]. In the present work, an Antho-RFamide neuropeptide type-1-like isoform X2 was detected in all sample types analyzed except for the mesenteric filaments. Peroxiredoxins are a conserved family of antioxidant enzymes present across all domains of life that use a highly reactive cysteine residue to neutralize hydroperoxides [711]. Their activity relies on a recycling mechanism mediated by thioredoxin, which restores their catalytic function by reducing oxidized cysteine residues in their active sites. Peroxiredoxins are classified into six subfamilies, four of which are represented in cnidarians (Prx1-AhpC, BCP-PrxQ, Prx5, and Prx6) [712]. A key portion of the (Prx5) peroxiredoxin-5 active site (**AFTPGCSK**THL) has been identified. Moreover, peroxiredoxins also perform functions such as molecular chaperone and phospholipase activities, contributing to the host antimicrobial defenses.

The innate immune response is mediated by the recognition of pathogen-associated molecular patterns (PAMPs) through pattern recognition proteins (PRPs). Lectins, which are carbohydrate-binding proteins, play essential roles in immune responses, cell signaling, and pathogen recognition [713]. By binding to PAMPs such as lipopolysaccharides in Gram-negative bacteria or mannose in fungi, certain lectins can

trigger innate immune responses, including processes like phagocytosis and the activation of signaling pathways involved in inflammation and immune defense [714]. In this study, Galectin, L-rhamnose-binding lectin ELEL-1-like, and Malectin-A-like lectins were directly identified from the Scaffold report, while C-type lectin domain-containing protein and SUEL-type lectin domain-containing protein were detected through GO annotations. Additionally, one uncharacterized protein was manually identified based on its GG-type lectin domain profile, further emphasizing the diverse lectin repertoire present in *A. fragacea*. Additional proteins associated with pattern recognition processes were identified, including Toll-interacting protein-like, and a variety of scavenger receptors (SRs). SRs represent a highly diverse superfamily of innate immunity genes that are essential for microbial ligands recognition and facilitating phagocytosis of pathogens [715]. Among the identified SRs were macrophage SR types I and II-like, SR cysteine-rich type-1 protein M130 and M130-like, and SR cysteine-rich domain superfamily protein-like.

## 4.4. Materials and Methods

### 4.4.1. Sample Collection and Preparation

A total of 19 specimens of the sea anemone *A. fragacea* were collected in April 2024 from Praia do Castelo do Queijo, Porto, Portugal (41.1639341, −8.6880545) during low tide (Fig. 13). The specimens were maintained in aerated aquaria under starvation conditions for two days to eliminate potential impurities, such as food particles and excretions. The samples were divided into two groups. The first group consisted of four batches, each containing four biological replicates ($n$ = 16). Tentacles were dissected from the specimens and placed in distilled water at 4 °C to induce osmotic lysis of nematocytes, leading to the release of the nematocysts. The resulting suspension was filtered using Falcon cell strainers with a 100 µm mesh size and centrifuged at 10,000× $g$ for 15 min at 4 °C to remove debris and concentrate the nematocysts. The isolation of nematocysts was confirmed through electron microscopy using a Leica DMi8 widefield microscope equipped with a Hamamatsu ORCA Flash v3 camera, and the samples were stored at –80 °C until protein extraction. The second group comprised three specimens ($n$ = 3). Mucus and four tissue types—acrorhagi, tentacles, mesenteric filaments, and column—were dissected and stored at –80 °C for subsequent analyses.

### 4.4.2. Protein Extraction

Nematocyst and tissue samples were thawed on ice and incubated in SDT buffer (2% SDS, 0.1M Tris/HCl pH 7.6, and 0.1 M dithiothreitol) at a ratio of 0.5 g fresh weight/mL.

Protease inhibitors (Halt PI Cocktail, Cat No. 78429, Thermo Fisher Scientific, Waltham, MA, USA) were added at a 1:100 dilution. Samples were kept in the dark at room temperature (RT, 21 °C) for 20 min. Tissue samples were homogenized for 1 min in a cold room (0 °C) using a benchtop homogenizer (Kinematica Polytron Model PT MR 2100). All samples were sonicated for 1 min, with 10 s intervals, at a pulse amplitude of 15 µm (MSE Soniprep 150). Samples were heated to 95 °C for 3 min, followed by centrifugation at 16,000× g for 1 h at RT. The resulting supernatants were transferred to new Eppendorf tubes, and total protein concentration was measured by absorbance at 280 nm using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Extracted protein samples were stored at –20 °C until further use.

### 4.4.3. Filter Aided Sample Preparation for LC-MS Analyses

The Filter-Aided Sample Preparation (FASP) method was performed as described by Wiśniewski et al. (2009) [716] with modifications. Protein samples were diluted to a concentration of 1 µg/µL, in SDT buffer. A total of 30 µL of diluted protein sample was mixed with 200 µL of UA buffer (8 M urea in 0.1 M Tris/HCl, pH 8.5). The mixture was transferred to pre-washed filter units (Merck Millipore Amicon Ultra 0.5 mL, Ultracel 10K, Cat. No. UFC501096, Merck Millipore, Darmstadt, Germany) and centrifuged at 14,000× *g* for 20 min. The flow-through was discarded, and iodoacetamide solution (100 µL, 0.05 M iodoacetamide in UA buffer) was added to the filter, mixed at 600 rpm for 1 min using a Thermomixer, and incubated in the dark at RT for 20 min. Filters were then centrifuged at 14,000× *g* for 20 min. Three washes were performed with 100 µL UA buffer for sample washing, centrifuging at 14,000× *g* for 15 min each. Three additional washes were carried out using 100 µL of 0.05 M ammonium bicarbonate, centrifuging at 14,000× *g* for 10 min each. Peptide digestion was performed by adding trypsin (Roche, recombinant, proteomics grade, Cat No. 3708985001, Mannheim, Germany) at a 1:100 enzyme-to-protein ratio in 0.05 M ammonium bicarbonate. Samples were mixed at 600 rpm for 1 min in a Thermomixer and incubated in a wet chamber at 37 °C for 16h. Digested peptides were eluted into new collection tubes by centrifugation at 14,000× *g* for 10 min. A second elution step was performed using 0.5 M NaCl, followed by centrifugation at 14,000× *g* for 10 min. Peptides were acidified with trifluoroacetic acid (TFA, 10% *v/v*) to achieve a pH between 2 and 3. For peptide desalting, C18 columns (Thermo Fisher Scientific Pierce C18 Tips, 100 µL, Cat. No. 87784, Thermo Fisher Scientific, Waltham, MA, USA) were conditioned with 80% acetonitrile (*v/v*) and 0.1% formic acid (*v/v*). Acidified samples were loaded onto the columns and washed with 0.1% formic acid (v/v) and eluted with 80% acetonitrile (*v/v*) and 0.1% formic acid (*v/v*) into new tubes. Peptide

concentration was measured at 280 nm, and samples were dried using a vacuum concentrator (Eppendorf Concentrator Plus, Eppendorf, Hamburg, Germany). Dried peptides were stored at −20 °C until further processing.

### 4.4.4. LC-MS Analysis of Protein Samples

The LC-MS/MS was carried out using a nano-LC coupled to Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Peptide separation was performed by reverse-phase chromatography using an EASY-Spray C18 reversed-phase nano-LC column (PepMap RSLC C18, 2 µm, 100A 75 µm × 25 cm, Thermo Fisher Scientific) with a gradient of 0.1% formic acid in water (A) and 0.1% formic acid in 80% acetonitrile (B) as follows: from 6% B to 30% B in 65 min; from 30% B to 100% B in 20 min; and 100% B from 85 to 90 min at a flow rate of 0.3 µL/min. Separated peptides were electrosprayed and analyzed using a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), operated in positive polarity in a data-dependent mode. Full scans were performed at 120,000 resolutions at a range of 380–1400 *m/z*. The top 15 most intense multiple charged ions were isolated (1.2 *m/z* isolation window) and fragmented at a resolution of 30,000 with a dynamic exclusion of 30.0 s.

### 4.4.5. Protein Identification and Quantification

The generated RAW files were analyzed using Sequest HT software package in Proteome Discoverer (v. 2.4.0.305, Thermo Fisher Scientific, San Jose, CA, USA). The Sequest HT search to identify proteins in *A. fragacea* was conducted against a custom database that integrated protein sequences from multiple sources: Cnidaria sequences retrieved from The National Center for Biotechnology Information (NCBI) (1,115,555 sequences, accessed on 13 December 2024) [266], Cnidaria sequences from the Universal Protein knowledgebase (UniProt) (392,627 sequences, accessed on 13 December 2024) [267], sequences from Database of Antimicrobial Activity and Structure of Peptides (DBAASP) (22,272 sequences, accessed on 13 December 2024) [260], sequences from the Animal toxin annotation project (ToxProt) (7970 sequences, accessed on 13 December 2024) [717], sequences from the Antimicrobial Peptide Database (APD3) (5069 sequences, accessed on 13 December 2024) [258], putative toxin sequences identified through Hidden Markov Models of cnidarian toxin families from VenomZone available online https://venomzone.expasy.org/ (accessed on 13 December 2024), BLASTp searches against ToxProt on sea anemones transcriptomes (1493 sequences) [718], and sequences from invertebrate antimicrobial peptides (340 sequences) [23].

Sequest was configured to use a fragment ion mass tolerance of 0.1 Da and a parent ion tolerance of 10 ppm; trypsin was selected for protein cleavage, allowing for one missed cleavage. Carbamidomethylation of cysteine was specified as a fixed modification, and oxidation of methionine was specified as a variable modification. MS/MS-based peptide and protein identifications were validated using Scaffold (version Scaffold_5.3.3, Proteome Software Inc., Portland, Oregon, USA). Peptides were accepted if they met a 0.1% false discovery rate (FDR) threshold, and proteins were accepted with a 1% FDR threshold and required at least one unique identified peptide. Protein probabilities were assigned using the Protein Prophet algorithm [719]. Peptide quantification was based on quantitative values (total spectra). Proteins with similar peptide evidence that could not be distinguished based solely on MS/MS data were grouped to satisfy the principle of parsimony. Only proteins with significant peptide evidence were grouped into clusters and included in further analyses. Venn diagrams were used to identify the shared proteins between different tissues using DeepVenn available online: https://www.deepvenn.com (accessed on 10 January 2025).

### 4.4.6. Functional Annotation and Expression Profiles

Functional annotation was performed using the BLAST2GO suite [720], implemented through the OmicsBox platform version 3.4.0 available at https://www.biobam.com/omicsbox (accessed on 17 January 2025). This process incorporated InterProScan [721], GO mapping, and GO annotation [720]. Proteins were first categorized into families, and functional domains were predicted using InterProScan. The graphical representation of InterProScan results was generated using R software (version 2024.12.0), *ggplot* package [722]. Potential GO terms were then mapped based on sequence similarity and domain information. Subsequently, GO annotations were refined and validated using experimental data and curated databases, providing a comprehensive characterization of the molecular functions, biological processes, and cellular components associated with the identified proteins.

The expression profiles of putative toxins across different sample categories were visualized using a heatmap, created with the online software Morpheus (Broad Institute, Cambridge, MA, USA, https://software.broadinstitute.org/morpheus, accessed on 14 January 2025). Necessary alignments were performed using Geneious software 11.1.5 (Biomatters Ltd., Auckland, New Zealand; https://www.geneious.com/) (accessed on 19 January 2025).

Additionally, identified peptides were scanned for their potential to be antimicrobial using the Collection of Anti-microbial Peptides (CAMPR4) [259], which employs a Random

Forest (RF) algorithm, and the Antimicrobial Peptide Scanner vr.2 [723], which uses a Support Vector Machines (SVM) algorithm. These tools were used to predict peptides from the proteomic dataset that could potentially exhibit antimicrobial activity, based on their sequence characteristics and structural features associated with known AMPs.

## 4.5. Conclusions

This study represents the first comprehensive proteomic analysis of the sea anemone *A. fragacea*, comparing the protein profiles of its nematocysts, mucus and tissues. A total of 4011 proteins were identified in the whole proteome, providing a valuable reference for further research on sea anemone biology. Functional bioinformatic analysis revealed that these proteins play key roles in immune responses, cell adhesion, protease inhibition and tissue regeneration, highlighting the complexity of the proteome. Moreover, some potential toxins were identified, including neurotoxins, membrane-active actinoporins, PLA2 enzymes similar to those found in other sea anemones and snakes, metalloproteinases with ShK domains, and putative cephalotoxins—emphasizing the intricate venom composition used for both prey capture and defense. The study also uncovered AMPs, including an Aurelin-like peptide and nine peptides with predicted strong antimicrobial potential, suggesting an important role in pathogen defense. Additionally, immune-related molecules, such as PRPs, antioxidants, and neuropeptides, were detected. Despite the limitations of not having a transcriptomic dataset for the analyzed specimens, we ensured the accuracy of the results by using a robust cnidarian-specific database, which included sea anemones from the same genus and other closely related species, providing a reliable foundation for our analysis. Our findings provide new insights into the survival strategies of *A. fragacea* and identify bioactive compounds that could have promising applications in biomedical research.

# Chapter 5.

# Unveiling the Bioactive Potential of the Invasive Jellyfish *Phyllorhiza punctata* Through Integrative Transcriptomic and Proteomic Analyses

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## Abstract

The white-spotted jellyfish, *Phyllorhiza punctata*, is an invasive species with significant ecological and economic relevance spreading across various regions. While its ecological impact is well-documented, its molecular and biochemical characteristics remain poorly understood. In this study, we integrate proteomic data generated by LC-MS/MS with publicly available transcriptomic information to characterize *P. punctata*, analyzing differential protein expression across three distinct tissues: oral arms, mantle, and gonads. A total of 2764 proteins and 25,045 peptides were identified, including several venom components such as jellyfish toxins (JFTs) and phospholipase A2 (PLA2), which were further investigated and compared to toxins from other species. Enrichment analyses revealed clear tissue-specific functions. Additionally, deep learning and machine learning tools identified 274 promising AMP candidates, including the α-helical, β-sheet, and αβ-motif peptides. This dataset provides new insights into the protein composition of *P. punctata* and highlights strong AMP candidates for further characterization, underscoring the biotechnological potential of underexplored cnidarian species.

**Keywords**: Phyllorhiza punctata, jellyfish, proteomics, antimicrobial peptides (AMPs), toxins, venom, bioactive compounds, transcriptomics, invasive species, cnidaria, marine biology, deep learning

## 5.1. Introduction

Jellyfish (phylum Cnidaria) are an ancient and diverse group of gelatinous zooplankton predominantly found in marine ecosystems, where they play important ecological roles [724,725]. Their ability to sting through the deployment of specialized stinging cells called nematocysts [6] and their association with jellyfish blooms are their most well-known characteristics. These blooms are sudden massive population increases that can disrupt ecosystems by altering nutrient dynamics, outcompeting native species, and interfering with local food chains. Moreover, they negatively impact human industries such as fishing, tourism, and aquaculture [12,15,725]. Despite these issues, jellyfish remain essential components of marine food webs, playing a crucial role in the dynamics of these ecosystems. They serve as predators of planktonic organisms, crustaceans, small fish, and the eggs and larvae of various marine species [724,726]. Conversely, they are prey for fish, sea turtles, sea slugs, and birds [727,728]. Jellyfish also contribute to nutrient cycling, as they assimilate nutrients during feeding and release inorganic nutrients through excretion and decomposition [11,729]. Given their versatility and ecological importance, jellyfish are increasingly recognized as valuable bioresources. They are used as human food [730], as aquafeed for fish and crustaceans [72], as attractions in zoos and aquariums [731], and as sources of bioactive compounds for biotechnological applications in pharmaceutical, nutraceuticals, and cosmeceuticals [210].

*Phyllorhiza punctata* von Lendenfeld, 1884 (Cnidaria: Rhizostomeae: Mastigiidae), commonly known as the Australian white-spotted jellyfish, has become a subject of concern due to its invasive nature and expanding distribution. Originally from the South Pacific Ocean, this species has spread to various regions, including the Indian Ocean [732], Caribbean Sea [733], Gulf of Mexico [734], Mediterranean Sea [735], and Northeastern Atlantic Ocean [197]. The rise in sightings of this jellyfish correlates with global changes such as ocean acidification, global warming, and salinity fluctuations [736]. Research on *P. punctata* has largely concentrated on its ecological aspects (e.g. life cycle, distribution, feeding behavior and invasiveness) [737]. However, there is a gap in studies focusing on its molecular and biochemical characteristics. Some research has examined its lipid composition and extract properties [738]. Moreover, the venom composition of the jellyfish remains poorly understood, with only one article analyzing a crude protein extract using High-Performance Liquid Chromatography (HPLC) [739].

Investigating the proteomic profiles of different tissues, such as the gonads, mantle, and oral arms, could reveal valuable insights into the general protein content of the species, as well as the reveal interesting bioactive compounds.

Recent advancements in computational models have significantly impacted biological sequence analysis, particularly in AMP prediction. These models use various machine learning and deep learning algorithms to analyze peptide sequences and predict their antimicrobial properties. Traditional machine learning methods, based on algorithms such as random forests (RF), support vector machines (SVM), and artificial neural networks (ANNs), have been widely used to predict AMPs based on their physicochemical properties, amino acid composition, and sequence motifs, as exemplified by resources like CAMPR4 [259]. More recently, deep learning methodologies, including convolutional neural networks (CNNs), recurrent neural networks (RNNs), and transformer-based models, have improved AMP prediction by effectively capturing complex sequence patterns and structural features, as illustrated by platforms such as AI4AMP [740], AMPScanner vr.2 [722], and PepNet [741]. These computational tools accelerate the discovery of AMPs and help identify potential candidates for therapeutic applications in areas like aquaculture, human medicine, and biotechnology [742]. Despite their advantages, these predictive models show variability in their accuracy, sensitivity, and generalization capabilities, highlighting the need for comparative performance studies to evaluate their reliability [743].

Thus, this study aims to combine transcriptomic and proteomic data, together with computational tools, to explore the bioactive potential of *P. punctata.* By utilizing publicly accessible RNA-seq data, proteomic analysis by mass spectrometry, and computational prediction tools, this research identifies interesting bioactive compounds, including putative toxins and candidate peptides with potential antimicrobial properties that could serve different biotechnological applications.

## 5.2. Materials and Methods

### 5.2.1. Sample Collection and Tissue Preparation

Three adult specimens of the jellyfish *P. punctata* were provided and collected in March 2022 from the Oceanário de Lisboa (Fig. 23). They were maintained in aerated, circulating aquariums at 25 °C, with salinity of 33.5–34.0, pH of 7.9–8.10, ammonia of 0–0.10 mg/L, nitrites of 0.050–0.150 mg/L, and nitrates of 1.0–10.0 mg/L. These water quality parameters follow established aquaculture guidelines, under routine water exchanges, as outlined in the Jellyfish Care Manual [744]. Oral arms, gonads, and

mantle tissues were dissected on-site and immediately placed on dry ice for transport to the laboratory, where they were stored at –80 °C for subsequent analyses.

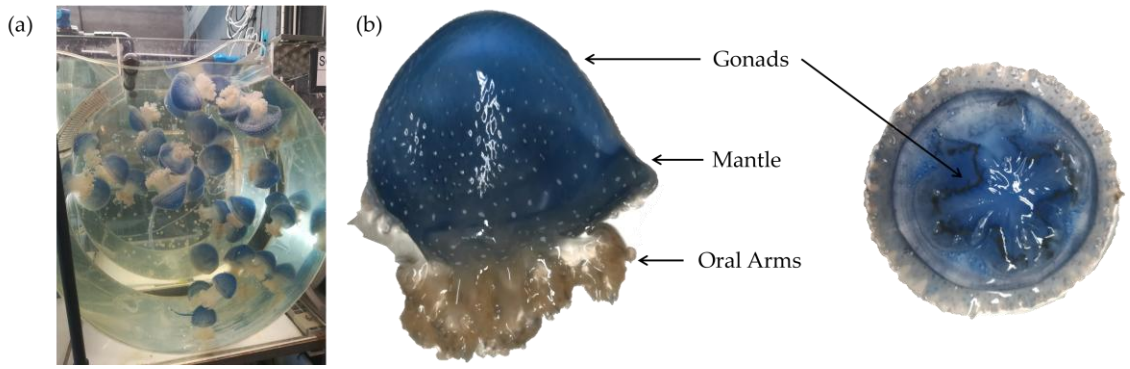



**Fig. 23** Overview of *P. punctata* specimens and sampled tissues. (a) Live specimens maintained in an aerated, circulating aquarium. (b) Frontal (left) and dorsal (right) views of a single specimen, illustrating the sampled tissues and highlighting the characteristic x-shaped gonads.

### 5.2.2. Protein Extraction and Filter-Aided Sample Preparation

Samples were kept on ice to thaw and then incubated in SDT buffer (2% SDS, 0.1 M Tris/HCl pH 7.6, and 0.1 M dithiothreitol) at a ratio of 0.5 g fresh weight per mL. A protease inhibitor cocktail (Halt PI Cocktail, Cat No. 78429, Thermo Fisher Scientific, Waltham, MA, USA) was added at a 1:100 dilution. The mixture was incubated in the dark at room temperature for 20 min. Following incubation, samples were subjected to sonication for a total of 1 min, with 10-s pulses at an amplitude of 15 µm, using an MSE Soniprep 150 sonicator. Subsequently, the samples were heated to 95 °C for 3 min and then centrifuged at 16,000× *g* for 1 h at room temperature. The supernatants were collected and transferred into new Eppendorf tubes, and the total protein concentration was quantified by absorbance at 280 nm using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The extracted protein samples were stored at –20 °C until further use.

The Filter-Aided Sample Preparation (FASP) method was performed as described by Wiśniewski et al. (2009) [716] with modifications. Protein samples were diluted to 1 µg/µL in SDT buffer. A total of 30 µL of the diluted protein sample was mixed with 200 µL of UA buffer (8 M urea in 0.1 M Tris/HCl, pH 8.5) and transferred to pre-washed filter units (Merck Millipore Amicon Ultra 0.5 mL, Ultracel 10K, Cat. No. UFC501096, Merck Millipore, Darmstadt, Germany). The mixture was centrifuged at 14,000× *g* for 20 min. The flow-through was discarded, and 100 µL of iodoacetamide solution (0.05 M iodoacetamide in UA buffer) was added, mixed at 600 rpm for 1 min using a Thermomixer, and incubated in the dark at room temperature for 20 min. The filters were then centrifuged at 14,000× *g* for 20 min. Three washes were performed with 100 µL UA

buffer, then centrifuging conducted at 14,000× *g* for 15 min each. Additional washes were performed using 100 µL of 0.05 M ammonium bicarbonate, then centrifuging conducted at 14,000× *g* for 10 min each. Peptide digestion was carried out by adding trypsin (Roche, recombinant, proteomics grade, Cat No. 3708985001, Mannheim, Germany) at a 1:100 enzyme-to-protein ratio in 0.05 M ammonium bicarbonate. Samples were mixed at 600 rpm for 1 min in a Thermomixer and incubated in a wet chamber at 37 °C for 16 h. Digested peptides were eluted into new collection tubes by centrifugation at 14,000× *g* for 10 min, and a second elution step was performed using 0.5 M NaCl, followed by centrifugation at 14,000× g for 10 min. The peptides were acidified with trifluoroacetic acid (TFA, 10% *v/v*) to a pH of 2–3. Peptides were desalted using C18 columns (Thermo Fisher Scientific Pierce C18 Tips, 100 µL, Cat. No. 87784, Thermo Fisher Scientific, Waltham, MA, USA), and conditioned with 80% acetonitrile (*v/v*) and 0.1% formic acid (*v/v*). The acidified samples were loaded onto the columns, washed with 0.1% formic acid (*v/v*), and eluted with 80% acetonitrile (*v/v*) and 0.1% formic acid (*v/v*) into new tubes. Peptide concentration was measured at 280 nm, and samples were dried using a vacuum concentrator (Eppendorf Concentrator Plus, Eppendorf, Hamburg, Germany). Dried peptides were stored at −20 °C until further analysis.

### 5.2.3. LC-MS Analysis of Protein Samples

LC-MS/MS analysis was performed using a nano-LC coupled to Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Peptides were separated by reverse-phase chromatography on an EASY-Spray C18 reversed-phase nano-LC column (PepMap RSLC C18, 2 µm, 100A 75 µm × 25 cm, Thermo Fisher Scientific) with a gradient of 0.1% formic acid in water (A) and 0.1% formic acid in 80% acetonitrile (B) as follows: from 6% B to 30% B in 65 min; from 30% B to 100% B in 20 min; and 100% B from 85 to 90 min, at a flow rate of 0.3 µL/min. Separated peptides were electrosprayed and analyzed on a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) in a positive polarity, data-dependent mode. Full scans were performed at a resolution of 120,000 over the 380–1400 *m/z* range. The top 15 most intense multiple charged ions were isolated (1.2 *m/z* isolation window) and fragmented at a resolution of 30,000, with a dynamic exclusion set to 30.0 s.

### 5.2.4. Transcriptome Assembly, Quantification, and Annotation

Raw RNA-seq data for *P. punctata* were obtained from a publicly available Sequence Read Archive (SRA) under accession ERR14056194, originating from Biosample SAMEA114771597. Quality assessment was performed using FastQC [745], followed by

the trimming of adapter sequences and low-quality bases using Trimmomatic (version 0.39) [746]. Cleaned reads were assembled into contigs using Trinity (version 2.2.1) [747] with default parameters. Potential coding sequences were predicted using TransDecoder (version 5.7.1) [748], also with default settings. Transcript abundance quantification was performed using Kallisto (version 0.46.1) with default settings to obtain Transcripts Per Million (TPM) values. The coding sequence predictions were annotated using DIAMOND BLAST (version 2.1.9) [749] against a custom protein database previously described in Barroso et al. (2025) [750]. This integrative database includes curated sequences from Cnidaria, antimicrobial peptide (AMP), and toxin-related databases, and manually annotated venom components. Transcriptome completeness was assessed using BUSCO (version 6.0.0) with the metazoa_odb10 lineage dataset, yielding 88.8% complete BUSCOs (67.0% single-copy, 21.8% duplicated), 6.7% fragmented, and 4.5% missing out of the 954 BUSCO groups searched.

### 5.2.5. LC-MS/MS Data Processing and Protein Identification

The RAW files generated were analyzed using the Sequest HT software package within Proteome Discoverer (v. 2.4.0.305, Thermo Fisher Scientific, San Jose, CA, USA). Protein identification was performed using Sequest HT against the obtained annotated transcriptome. The search parameters included a fragment ion mass tolerance of 0.1 Da and a parent ion mass tolerance of 10 ppm. Trypsin was specified as the digestion enzyme, allowing for up to two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification, while methionine oxidation was specified as a variable modification. Peptide and protein identifications were validated with Scaffold (version 5.3.3, Proteome Software Inc., Portland, Oregon, USA), applying a 0.1% false discovery rate (FDR) for peptides and a 1% FDR for proteins, with a minimum requirement of one uniquely identified peptide per protein. Protein probabilities were calculated using the Protein Prophet algorithm [719]. Peptide quantification was based on total spectral counts. Proteins supported by identification metrics that met these thresholds were considered to have significant peptide evidence and were retained for downstream analyses. Proteins with shared peptide evidence that could not be differentiated based solely on MS/MS data were grouped according to the principle of parsimony. Shared proteins between different tissues were visualized using Venn diagrams created with DeepVenn, available online: https://www.deepvenn.com (accessed on 24 January 2025).

### 5.2.6. Functional Annotation and Enrichment Analyses

Protein functional annotation was performed using InterProScan associated with Gene Ontology (GO) [721]. This analysis was carried out on both the complete set of validated proteins and a subset comprising uncharacterized proteins (e.g., hypothetical, predicted without annotation, or unnamed protein products).

In parallel, GO enrichment analysis was conducted. GO annotations were obtained through EggNOG-mapper, and enrichment testing was performed using the topGO R package (version 5.3.3) with the classic Fisher's exact test. To enhance biological interpretability, only GO terms with more than three annotated proteins and at least two significant hits were retained for visualization. The most-enriched terms (top six per ontology: Biological Process, Molecular Function, and Cellular Component) were selected based on the highest $-\log_{10}(p\text{-value})$ scores. The GO.db package was used to retrieve ontology classifications. Results were visualized as horizontal bar plots using ggplot2 (version 3.5.2), with terms grouped and color-coded by ontology. Data preprocessing was conducted using the dplyr (version 1.1.4) and tidyr (version 1.3.1) packages.

Additionally, KEGG pathway enrichment analysis was performed to explore the pathway-level functional specializations among tissue-specific proteins. KEGG Orthology (KO) assignments obtained via EggNOG-mapper were used to associate proteins with pathways. Enrichment analysis was carried out using a custom R pipeline based on Fisher's exact test, comparing the frequency of each KEGG pathway in each tissue-specific dataset (gonads, mantle, and oral arms) against the complete protein set. KEGG pathway descriptions were retrieved from the KEGG REST API. *p*-values were adjusted using the FDR method, and significantly enriched pathways ($p < 0.05$) were visualized using ggplot2. The enrichment plot displayed tissue-specific pathway enrichment based on $-\log_{10}(p\text{-value})$, with the dot size proportional to the number of tissue-associated proteins.

The identification of putative protein toxins was based on their functional annotations, comparative analysis, and bibliographic support.

### 5.2.7. Identification of Putative Toxins and Phylogenetic Analysis of Jellyfish Toxins (JFTs)

Putative protein toxins were identified based on functional annotations, domain predictions, comparative sequence analyses, and bibliographic evidence. Due to the presence of incomplete or redundant transcript isoforms and inconsistencies between transcriptomic and proteomic datasets, protein sequences detected in the *P. punctata*

proteome were mapped to their closest full-length homologs in public databases (UniProt and NCBI). This approach improved annotation confidence and enabled reliable downstream analyses. Notably, the transcriptomic and proteomic data originated from different individuals, further justifying the use of curated reference sequences for functional and phylogenetic inference.

Amino acid multiple sequence alignments (MSAs) were performed for jellyfish toxins (JFTs), incorporating *P. punctata* matched peptides, along with 12 additional JFT sequences from Scyphozoa and Cubozoa. Three-domain Cry toxins (3d-Cry) from *Bacillus thuringiensis* were used as outgroup sequences.

Alignments were generated using Geneious v11.1.5. and the MAFFT algorithm via the GUIDANCE2 webserver (https://guidance.tau.ac.il/, accessed on 31 January 2025), with 100 bootstrap replicates. Alignment columns with fewer than 17.65% informative residues were trimmed to improve phylogenetic reliability.

The refined MSA was submitted to ProtTest3 v3.4.2 [751] to determine the best-fitting amino acid substitution model based on the corrected Bayesian Information Criterion (BIC). Phylogenetic inference was conducted using the maximum-likelihood (ML) algorithm implemented in IQ-TREE v2.0.7 (http://www.iqtree.org/, accessed on 31 January 2025) under the WAG substitution model. Node support was assessed using 10,000 ultrafast bootstrap replicates, 10,000 replicates of the Shimodaira–Hasegawa approximate-likelihood ratio test (SH-aLRT), and an approximate Bayes test (aBayes).

### 5.2.8. Peptide characterization and Antimicrobial Peptide (AMP) Prediction

The peptides identified in the dataset were evaluated for their potential to function as AMPs. Key physicochemical properties, including peptide length, net charge, isoelectric point (pI), molecular weight (MW), average hydropathicity (GRAVY), and Boman index, were calculated using an in-house Python script integrated with the ProtParam tool from the Expasy Proteomics Server [275].

To assess the AMP potential of each peptide, a combination of machine learning- and deep learning-based AMP prediction tools was employed, including AMPScanner vr.2 [723], PepNet [741], AI4AMP [740], and CAMPR4 with Random Forest algorithm [259]. Although a precision-weighted average of AMP probability scores could improve prediction reliability by accounting for inter-model variability, reported precision values for these models vary considerably across studies [752−754]. Due to this inconsistency, we used a simple unweighted average of the probabilities from all models, acknowledging this as a limitation in the prediction approach.

Candidate peptides were filtered based on thresholds derived from the average physicochemical and predictive characteristics of AMPs reported in aquatic invertebrates, as described in our previous study [22]. Peptides were retained for further analysis if they met the following criteria:

- A positive net charge;
- A GRAVY value between −1.5 and +1.5;
- An average AMP prediction probability ≥ 0.7.

For structural characterization, the tertiary structures of the selected AMP candidates were modeled using two complementary tools: ColabFold [280], based on the AlphaFold2 algorithm [257], and PEP-FOLD4 [755], a tool specifically designed for the structural prediction of short linear peptides. AlphaFold2-based predictions were generated using five independent models per peptide, with 20 recycles to enhance structural accuracy. Predicted local distance difference test (pLDDT) scores and predicted template modeling (pTM) scores were retrieved to assess model confidence. PEP-FOLD4 was employed in parallel under default parameters for cross-validating structural predictions. All predicted structures from both methods were visualized and analyzed using ChimeraX [281] to identify secondary structural features. Structural patterns such as α-helices, β-sheets, and coil regions were annotated and compared with known AMPs to infer potential functional relevance. These structural models provide a basis for further studies on the mechanism of action and therapeutic potential of the predicted AMPs.

## 5.3. Results

### 5.3.1. Quantitative Correlation Between Transcriptomic and Proteomic Data

The assembly produced 126,144 contigs (File S 1), with 45,011 predicted coding sequences. There were 3160 transcript counts identified in the oral arms (out of 3184 total transcripts), 3064 in the gonads (out of 3086), and 2836 in the mantle (out of 2861). To assess the relationship between transcript expression and protein abundance, TPM values were correlated with two mass spectrometry-derived metrics from oral arm samples: Total Spectrum Matches (TSMs) and Exclusively Unique Spectrum Counts (EUSCs). $Log_{10}$-transformed TPM, TSM, and EUSC values were used after adjusting by +1 to avoid undefined values. The results indicated moderate positive correlations, with Pearson's correlation coefficients of $R$ = 0.35 for TPM vs. TSMs and $R$ = 0.33 for TPM vs. EUSCs (Fig. 24).

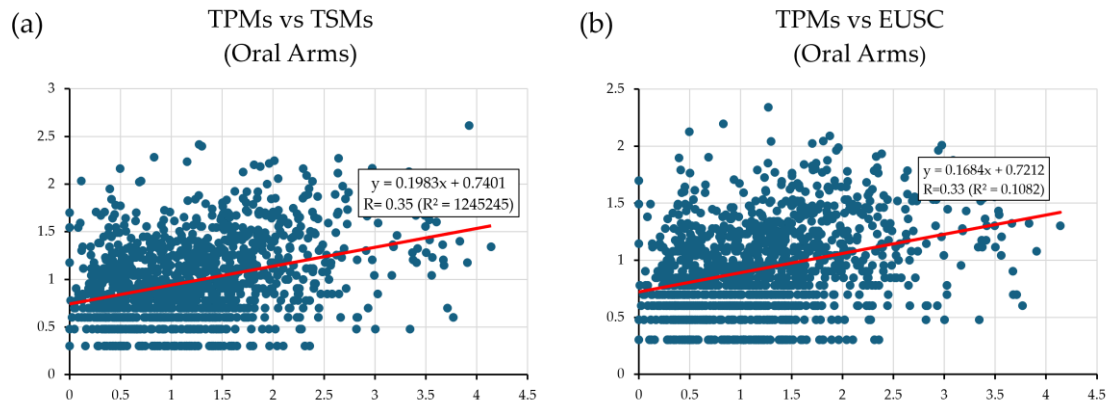



**Fig. 24** Scatter plots showing the correlation between transcript expression and protein abundance in oral arm samples. (a) Correlation between log10-transformed TPM values and TSMs, adjusted by adding +1. (b) Correlation between log10-transformed TPM values and EUSCs, also adjusted by +1.

### 5.3.2. Overview of Proteomic Data from *P. punctata*

A total of 2764 proteins and 25,045 peptides were identified in the proteome of *P. punctata* (Table S 22). Among these, 2180 proteins and 9207 peptides were present across all sample categories (Fig. 25). Results show that oral arms contained the highest total number of proteins (2614) and peptides (18,058), followed by the gonads (2547 proteins and 16,459 peptides) and mantle (2385 proteins and 15,437 peptides). The oral arms also had the highest number of uniquely identified proteins (83) and peptides (4088), followed by the mantle (49 and 2935), and the gonads (30 and 2320).

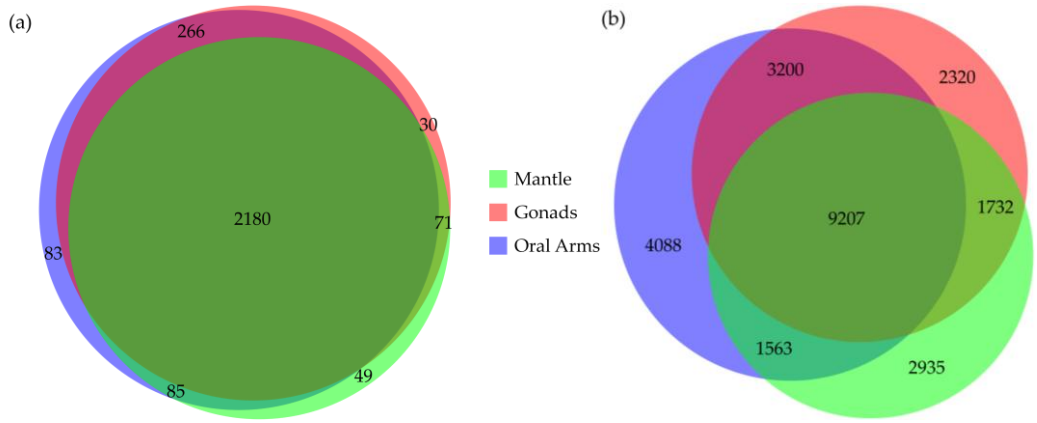



**Fig. 25** The area-proportional Venn diagram depicting the overlap of identified (a) proteins and (b) peptides across the three sample categories: mantle, gonads, and oral arms of *P. punctata*. The diagrams were generated using DeepVenn (https://www.deepvenn.com, accessed on 25 January 2025).

The most abundant proteins identified were primarily housekeeping proteins, which are responsible for basic cellular functions (Table 9).

**Table 9** Most abundant proteins identified in the proteome of *P. punctata*.

| Accession | Protein Name | G | M | O | T |
|---|---|---|---|---|---|
| XP_065065861.1 | myosin heavy chain, striated muscle-like | 623 | 1724 | 706 | 3053 |
| XP_065067169.1 | filamin-A-like isoform X5 | 432 | 871 | 436 | 1739 |
| ADR10434.1 | non-muscle actin II | 461 | 621 | 410 | 1492 |
| XP_065065923.1 | myosin-10-like isoform X1 | 367 | 272 | 400 | 1039 |
| XP_065676232.1 | tubulin beta chain | 221 | 259 | 254 | 734 |
| CAH3037106.1 | unnamed protein product (actin) | 190 | 323 | 170 | 683 |
| XP_065054978.1 | clathrin heavy chain 1-like | 226 | 152 | 259 | 637 |
| XP_065051959.1 | probable glutathione S-transferase 8 | 235 | 193 | 202 | 630 |
| XP_065060176.1 | alpha-1-macroglobulin-like isoform X4 | 179 | 265 | 169 | 613 |
| ACT_HYDVU | Actin, non-muscle 6.2 | 185 | 239 | 186 | 610 |
| XP_065052735.1 | uncharacterized protein LOC135681977 | 170 | 258 | 134 | 562 |
| XP_065052612.1 | ATP synthase subunit beta | 146 | 282 | 131 | 559 |
| XP_065065546.1 | alpha-actinin-like | 168 | 145 | 233 | 546 |
| CAH3152489.1 | unnamed protein product | 193 | 80 | 248 | 521 |
| XP_065065852.1 | myosin heavy chain-like isoform X1 | 262 | 44 | 195 | 501 |
| XP_065054022.1 | collagen alpha-1(I) chain-like isoform X1 | 178 | 129 | 191 | 498 |
| XP_065063720.1 | heat shock protein HSP 90-alpha-like | 184 | 150 | 154 | 488 |
| XP_065051708.1 | myosin light chain kinase, smooth muscle-like isoform X2 | 103 | 273 | 106 | 482 |
| XP_065059929.1 | ATP synthase subunit alpha, mitochondrial-like | 120 | 250 | 108 | 478 |

G: Gonads, M: Mantle, O: Oral arms, T: Total

### 5.3.3. Functional Annotation and Enrichment Analysis of Tissue-Specific Proteins Identified in *P. punctata*

To elucidate the biological functions of tissue-specific proteins in *P. punctata*, functional annotation was performed using InterProScan associated with GO terms, revealing a diverse array of protein domains and functional sites (Fig. 26). Notably, many previously uncharacterized proteins were found to contain conserved domains and associated GO terms, suggesting involvement in enzymatic activity, structural roles, and signal transduction. This analysis not only confirmed known cnidarian protein signatures but also uncovered potential novel functions among less-characterized sequences. The full InterProScan annotation dataset is provided in Table S 23.

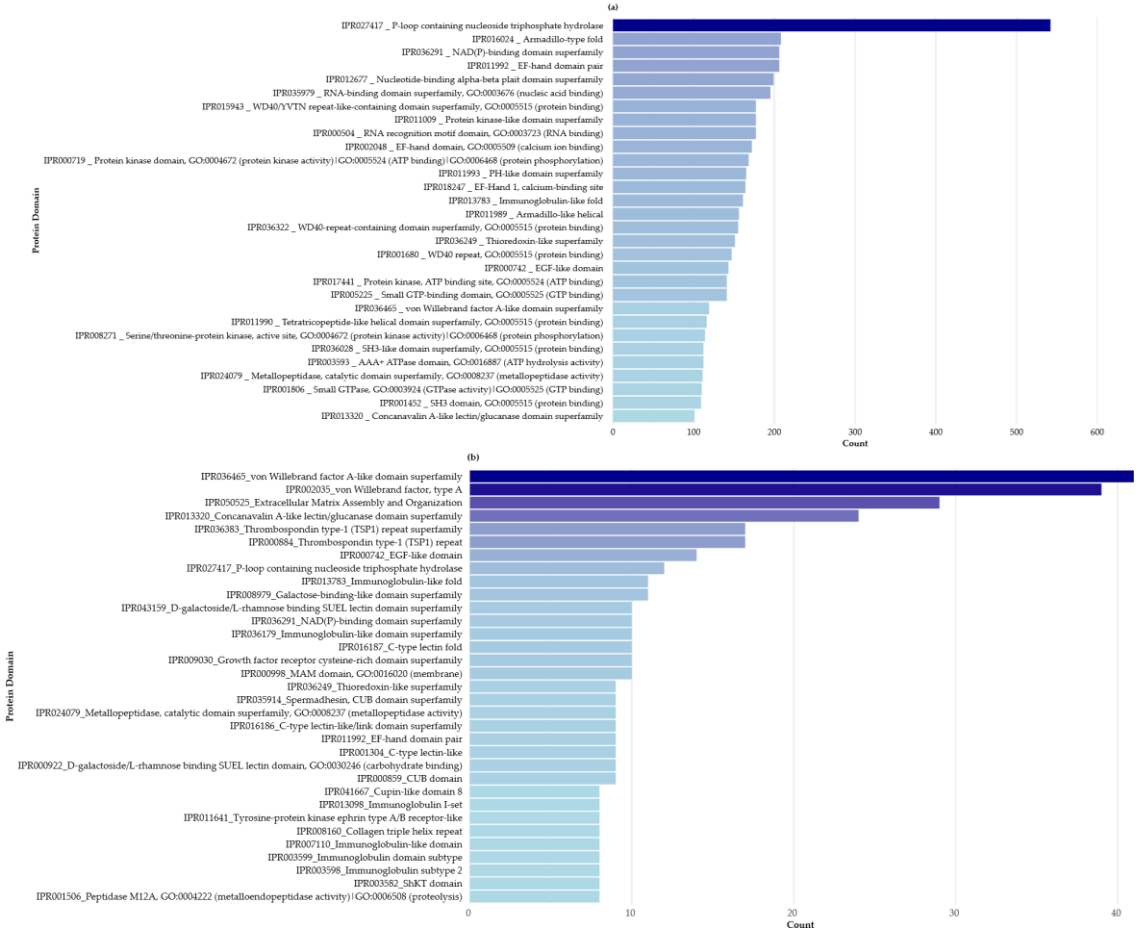



**Fig. 26** Most frequently identified InterPro domains in the *P. punctata* proteome with associated GO terms and descriptions: (a) top 30 domains across all identified proteins; (b) domains found in uncharacterized proteins only (minimum of 8 occurrences).

Subsequent GO enrichment analysis revealed the significant overrepresentation of specific functional categories within the tissue-specific protein sets of the oral arms, gonads, and mantle. The analysis covered the three GO domains, Biological Process (BP), Molecular Function (MF), and Cellular Component (CC), revealing clear functional differentiation across tissues (Fig. 27). In the BP domain, gonads were enriched in terms related to mRNA processing and ion homeostasis; in the oral arms, for responses to mechanical and chemical stimuli, skeletal development, and angiogenesis; and in the mantle, for metabolic, catabolic, and oxidative stress-related processes. In MF, gonads were enriched in nucleotide hydrolysis and protein modification; the oral arms showed enrichment in transcriptional regulation, signaling, and proteolytic activity; and the mantle in catalytic functions, metal ion binding, and amino acid metabolism. For the CC domain, gonads were associated with RNA processing complexes and membrane-related structures; the oral arms with dendritic components and neuromuscular junctions; and

the mantle with mitochondrial components and protein kinase complexes. The full GO enrichment dataset is provided in Table S 24.

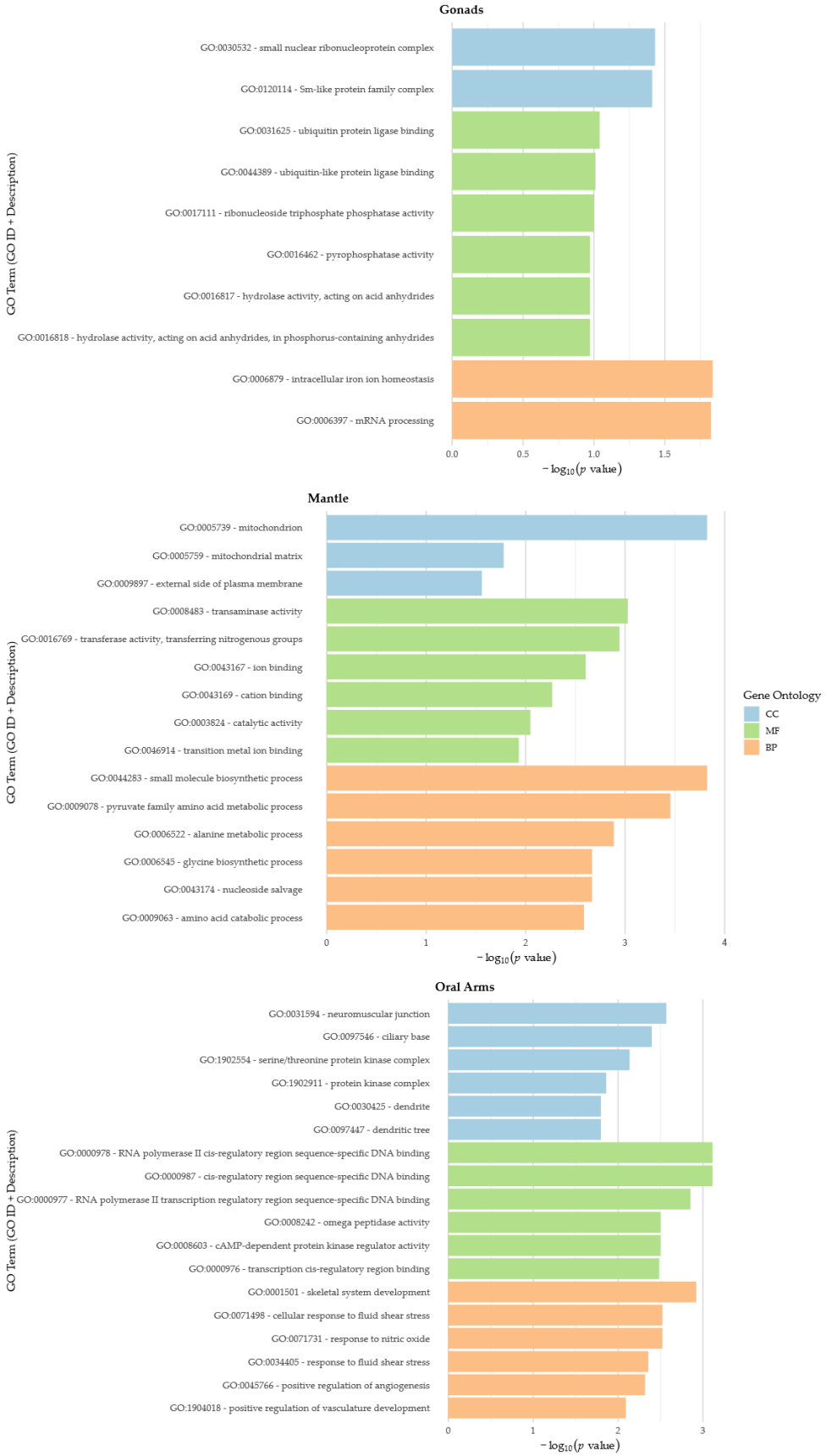


**Fig. 27** GO enrichment analysis of tissue-specific proteins in *P. punctata*. Bar plots show the top six enriched GO terms in each ontology, Biological Process (BP), Molecular Function (MF), and Cellular Component (CC), identified among proteins exclusive to the gonads, mantle, and oral arms. Enrichment significance is represented as $-\log_{10}(p\text{-value})$, and terms are color-coded by ontology. Only GO terms with at least three annotated proteins and two significant hits were included.

To complement GO-based insights, KEGG pathway enrichment analysis was carried out using KO annotations to identify pathway-level functional specializations. Several pathways were significantly enriched in a tissue-specific manner, supporting the presence of metabolic and functional compartmentalization among the tissues (Fig. 28). In the oral arms, enriched pathways included TNF signaling, GnRH signaling, Notch signaling, glycosaminoglycan degradation, and apelin signaling. Gonads showed enrichment in protein digestion and absorption, ovarian steroidogenesis, and circadian rhythm. In the mantle, enriched pathways included cholesterol metabolism; cysteine and methionine metabolism; steroid biosynthesis; valine, leucine, and isoleucine degradation; metabolic pathways; as well as malaria and TGF-beta signaling pathways. The complete KEGG enrichment results are provided in Table S 25.

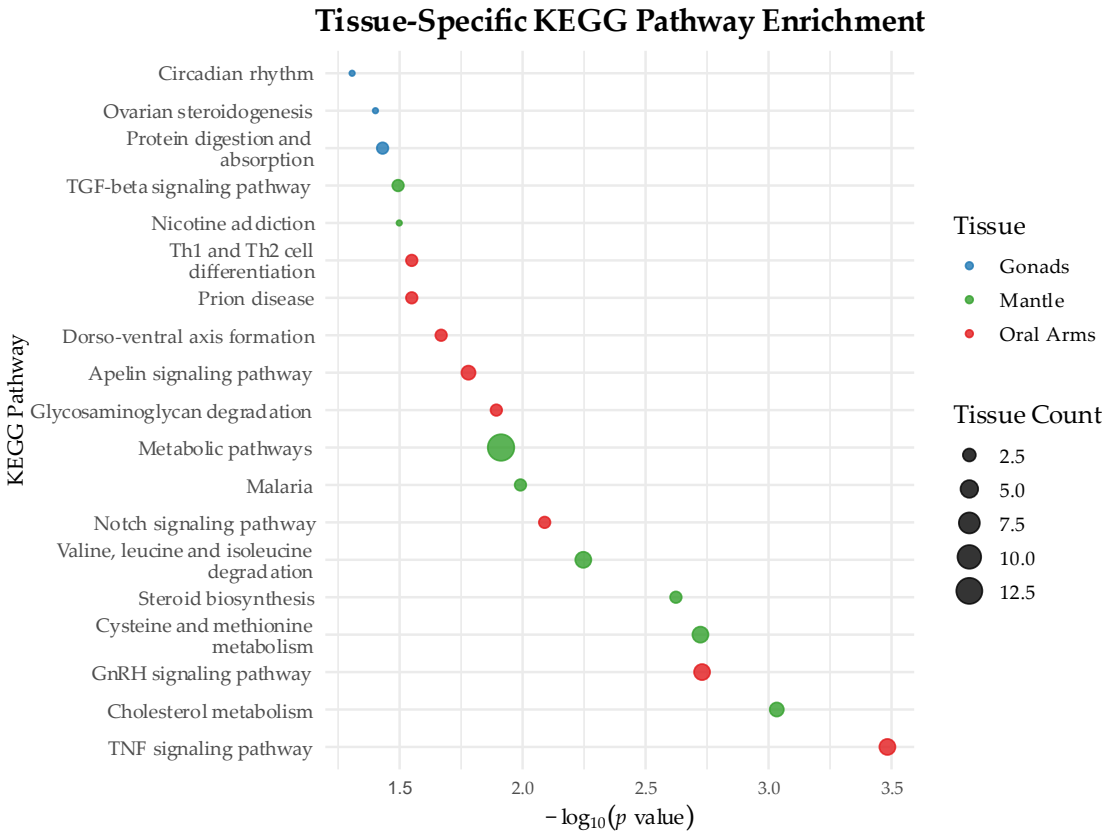



**Fig. 28** KEGG pathway enrichment analysis of tissue-specific proteins in *P. punctata*. Dot plot showing significantly enriched KEGG pathways (Fisher's exact test, FDR-adjusted $p < 0.05$) among proteins exclusive to the gonads, mantle, and oral arms. The *x*-axis represents enrichment significance as $-\log_{10}(p\text{-value})$. Dot size indicates the number of proteins

associated with each pathway, and colors distinguish the tissue of origin. Pathway descriptions were retrieved via the KEGG REST API.

### 5.3.4. Identification and Comparative Analysis of Venom Components Across *P. punctata* Tissues

A total of 15 high-confidence toxin-related proteins were identified in the proteome of *P. punctata*, supported by consistent annotations across multiple databases (e.g., UniProt, Tox-Prot), the presence of conserved toxin-like domains, and homology to known venom proteins from other cnidarians (Table 10). An extended list, which also includes lower-confidence candidates with partial toxin signatures, is provided in Table S 26.

**Table 10** Major toxins identified in *P. punctata* and their corresponding total spectrum counts across sample categories (G: gonads, M: mantle; O: oral arms, T: sum of total spectrum counts). Underlined: jellyfish toxins (JFTs).

| Accession | Toxin Protein Name | G | M | O | T |
|---|---|---|---|---|---|
| DAC80636 | TPA_exp: toxin a | 121 | 23 | 200 | 344 |
| XP_065065639 | ras-related C3 botulinum toxin substrate 1 | 84 | 84 | 112 | 280 |
| XP_065067572 | venom factor-like | 50 | 45 | 24 | 119 |
| XP_065064594 | conodipine-P3-like | 34 | 25 | 45 | 104 |
| XP_065067570 | LOW-QUALITY PROTEIN: venom factor-like | 37 | 18 | 19 | 74 |
| XP_065054632 | aflatoxin B1 aldehyde reductase member 2-like | 22 | 30 | 10 | 62 |
| CAB3985090 | agrin-like | 33 | 15 | 6 | 54 |
| A0A7M5UUY9 | BPTI/Kunitz inhibitor domain-containing protein | 19 | 28 | 7 | 54 |
| XP_065070484 | toxin CfTX-A-like | 18 | 2 | 28 | 48 |
| XP_065055594 | plancitoxin-1-like | 10 | 11 | 13 | 34 |
| QNH72454 | toxin candidate | 19 | 7 | 7 | 33 |
| AFK76348 | toxin TX1 | 5 | 12 | 15 | 32 |
| XP_065063396 | ADAM 17-like protease | 10 | 9 | 13 | 32 |
| XP_065065308 | snake venom 5'-nucleotidase-like | 4 | 7 | 10 | 21 |
| XP_065071254 | zinc metalloproteinase-disintegrin-like MTP8 | | | 3 | 3 |

G: gonads, M: mantle, O: oral arms, T: total.

Among the high-confidence toxins, three were classified as JFTs: toxin TX1 from *A. aurita* (AFK76348); TPA_exp: toxin a from *Cassiopea xamachana* (DAC80636); and CfTX-A-like from *Rhopilema esculentum* (XP_065070484.1), which was not detected in the mantle tissue (Fig. 29).

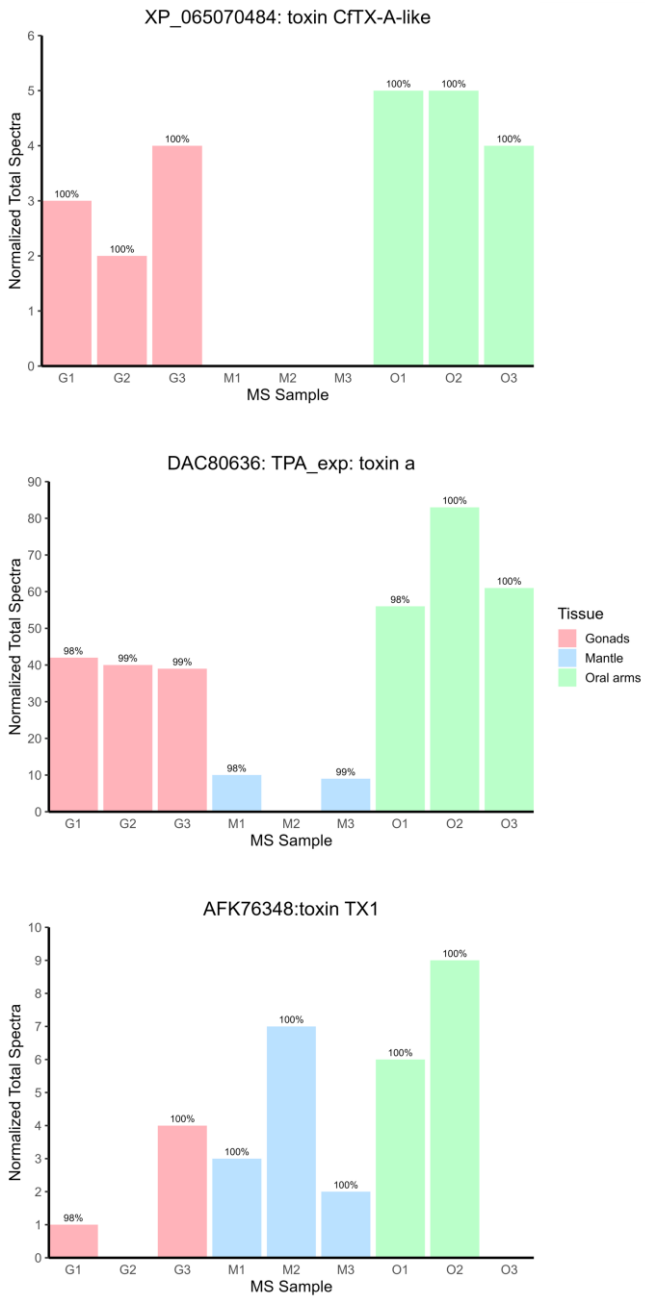



**Fig. 29** Normalized spectral abundance of JFT proteins identified in the *P. punctata* proteome. Bars represent normalized total spectra counts across different MS samples (oral arms, gonads, mantle). Only proteins with a minimum identification probability of 95% (as determined by the PeptideProphet algorithm in Scaffold) were included to ensure high-confidence identifications. TPA_exp: toxin a (DAC80636) was detected across multiple transcript entries; spectral values were aggregated and normalized accordingly. CfTX-A-like and TX1 toxins were each detected from a single unique transcript.

These JFTs, which belong to Scyphozoa, are phylogenetically distinct from their Cubozoan counterparts (Fig. 30). The corresponding multiple sequence alignment is available in Supplementary Material (File S 2). Additionally, the identified phospholipase $A_2$ conodipine-P3-like toxin (XP_065064594) exhibited conserved sequence motifs when aligned with the original conodipines from *Conus purpurascens* (Fig. 31).

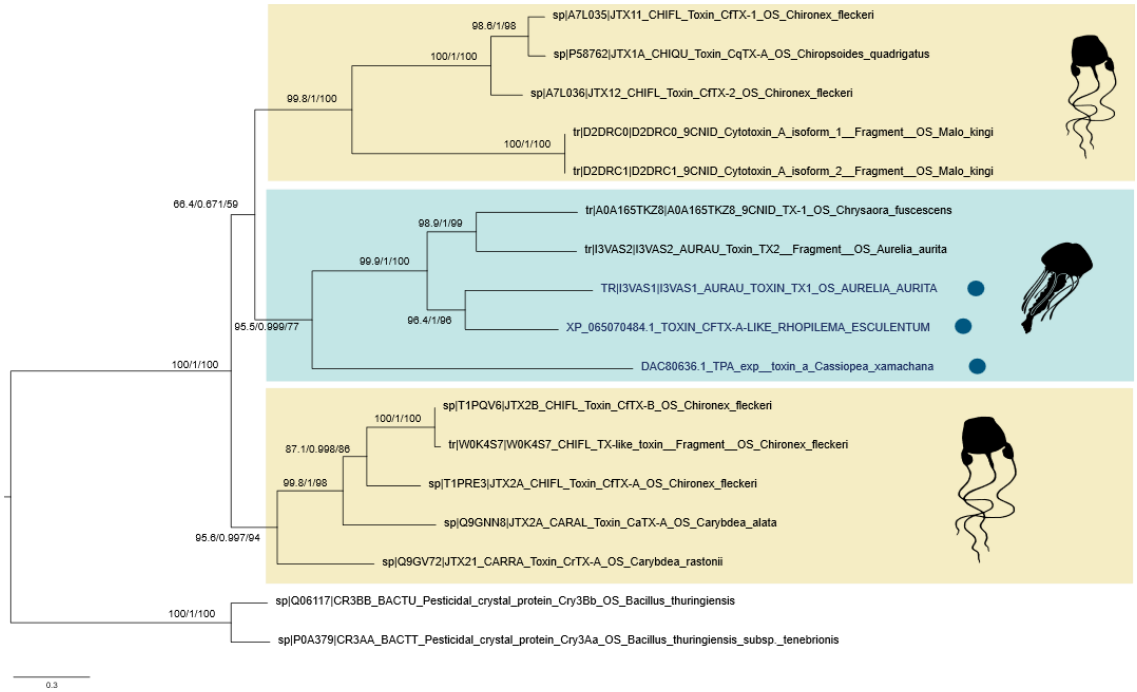



**Fig. 30** Maximum-likelihood (ML) phylogenetic tree of jellyfish toxins (JFTs). The tree was constructed using IQ-TREE based on amino acid sequences of best-matching reference proteins from UniProt and NCBI, with detected peptides from the *P. punctata* proteome mapped to these sequences. Node support values are shown for Shimodaira–Hasegawa approximate-likelihood ratio test (SH-aLRT), approximate Bayes test (aBayes), and ultrafast bootstraps. Colored dots indicate JFTs detected in the *P. punctata* proteome. Cubomedusae sequences are highlighted in yellow, and Scyphozoan sequences are highlighted in blue. Three-domain Cry (3d-Cry) toxins from *Bacillus thuringiensis* were included as outgroup sequences.

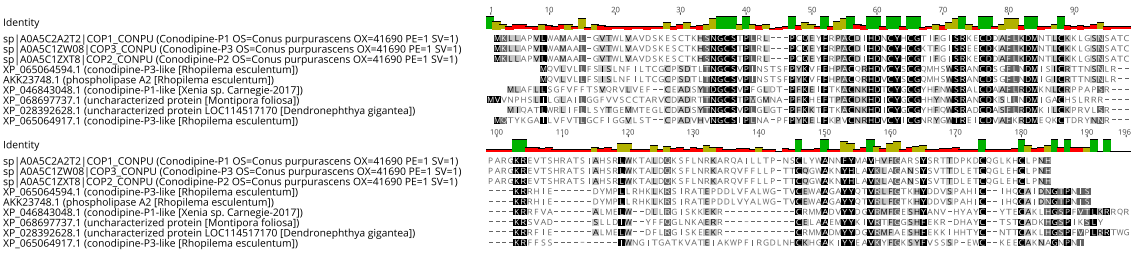



**Fig. 31** Multiple sequence alignment of a phospholipase $A_2$ ($PLA_2$) protein identified in the *P. punctata* proteome, aligned with conodipine-P3 sequences from *Conus purpurascens* (A0A5C2A2T2, A0A5C1ZW08 A0A5C1ZXT8), and top BLASTp hits from other cnidarian species. The *P. punctata* sequence corresponds to the best-matching reference protein from *Rhopilema esculentum* (XP_065064917.1). Conserved $PLA_2$ motifs are highlighted.

## 5.3.5. Peptide Characterization and AMP Prediction

A total of 25,045 peptides were identified in the *P. punctata* proteome, with sequence lengths ranging from 7 to 44 amino acids. Their physicochemical properties varied as follows: net charge (−15 to +3), isoelectric point (pI) (4 to 12), molecular weight (MW)

(730 to 4610 Da), GRAVY (−3.3 to +2.8), Boman index (0.02 to 0.15), and average predicted AMP probability of 24%.

Based on selection thresholds derived from aquatic invertebrate AMPs [22], a total of 274 unique AMP candidates were retained, with 81 shared across all sample types, 29 exclusive to the gonads, 52 to the mantle, and 47 to the oral arms.

AMP potential was assessed using multiple machine learning and deep learning models (AMPScanner vr.2, PepNet, AI4AMP, and CAMPR4), and the final score for each peptide was computed as a precision-weighted average across all predictors.

The tertiary structures of all AMP candidates were predicted using both ColabFold with the AlphaFold2 model and PEP-FOLD4. While AlphaFold2 offers state-of-the-art protein structure prediction, it has recognized limitations for short, linear peptides, often resulting in a high number of disordered (random coil) predictions [257]. PEP-FOLD4, a tool specialized for de novo modeling of short peptides, was used to cross-validate these predictions. The predictions revealed notable differences between the two tools (Table 11). AlphaFold2 classified a high proportion (130/274) of peptides as random coils, whereas PEP-FOLD4 yielded fewer (45/274) in this category. Conversely, PEP-FOLD4 predicted a greater number of α-helical structures (203 vs. 140). Consistent predictions across both tools were found in 113 α-helical peptides, 1 β-sheet, and 28 random coils. These results suggest that AlphaFold2 may overestimate disorder in short peptides, reinforcing the importance of complementary prediction approaches.

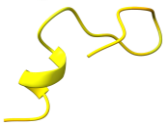
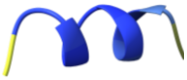
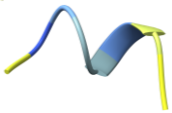
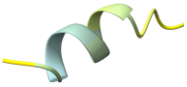
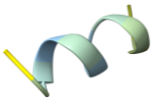
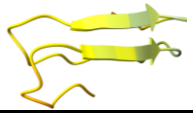
**Table 11** Summary table comparing AlphaFold2 and PEP-FOLD4 results.

| Structure | No. Peptides (AlphaFold2) | No. Peptides (PEP-FOLD4) | Consistent Predictions (Both Tools) |
|---|---|---|---|
| α-helix | 140 | 203 | 113 |
| β-sheet | 4 | 23 | 1 |
| αβ-motifs | 0 | 3 | 0 |
| Random coil | 130 | 45 | 28 |

To represent potential promising candidates, the top 10 AMPs were selected based on their physicochemical properties, the unweighted average of AMP probability across all models, and the structural consistency using both predictors (Table 12). The complete dataset, including AMP scores and structural predictions, is available in the Supplementary Material (Table S 27 and File S 3).

**Table 12** Top 10 AMP candidates filtered by physicochemical properties, AMP prediction scores, and structural consistency.

| Protein Accession | Sequence | Predicted Structure (Alphafold2) | C Score | Avg pred. | O | M | G |
|---|---|---|---|---|---|---|---|

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| XP_065064294 | QLGWCSTVKQAMKALCEK |  | 0.47 | 0.95 | | X | |
| XP_065069205 | VCLIGAGNWGSAIAK |  | 0.40 | 0.92 | X | X | |
| XP_065058610 | IGTKVLLKIYK |  | 0.55 | 0.91 | | | X |
| XP_065058870 | IPTHAPYVIIGGGTASHAACR |  | 0.53 | 0.90 | | X | |
| XP_065062238 | LPSSVIGSLIGK |  | 0.57 | 0.89 | | | X |
| XP_065055185 | GIRPAINVGLSVSR |  | 0.52 | 0.89 | | X | |
| XP_065059349 | KPIGLCCIAPVLAAK |  | 0.47 | 0.88 | X | X | X |
| XP_065062330 | LPVVTNQICSILNR |  | 0.46 | 0.88 | | X | X |
| XP_065055150 | GIQCLISVGLGTR |  | 0.51 | 0.88 | | | X |
| XP_065051697 | DVMIIGPATVGGIKPGCFK |  | 0.56 | 0.86 | | | X |

The table includes the protein accession number, peptide sequence, predicted structure, composite score (C Score), average AMP prediction probability (Avg pred.), and occurrence across the different sample types: oral arms (O), mantle (M), and gonads (G). C Score was calculated as (0.6 × N pLDDT + 0.4 × pTM) to balance local and global structure confidence.

## 5.4. Discussion

### 5.4.1. Proteomic Analysis of *P. punctata* Tissues

The proteomic profile of *P. punctata* was constructed using the only available transcriptome of *P. punctata* as of January 2025. The dataset is labeled as “tentacle” but likely corresponds to oral arm tissue, since *P. punctata*, being a Rhizostomeae jellyfish, lacks marginal tentacles [756]. This discrepancy should be considered when analyzing the data, as the oral arms transcriptome is expected to provide a more accurate representation of the proteome for this specific tissue. Despite this, the approach still yielded significantly more and higher-quality results than using a general cnidarian-based database (results not shown), underscoring the need to improve the availability and quality of omics data for better species-specific analysis.

Oral arm samples indeed revealed a greater number of proteins and peptides identified. Interestingly, while the total protein coverage from the oral arms accounts for 95% of the total proteins identified, this value decreases to 72% when considering peptides instead. This difference could very likely be attributed to post-translational modifications (PTMs). The most abundant proteins identified in the proteome of *P. punctata* were unsurprisingly mostly those with essential cellular functions: structural proteins such as myosin, filamin, actin, tubulin, and collagen, which contribute to maintaining cell shape, motility, and structural integrity [757,758]; proteins involved in energy production, like ATP synthase subunits alpha and beta, which play a fundamental role in cellular respiration and ATP synthesis for cellular metabolism [759]; proteins related to protein folding and stabilization, such as heat shock protein HSP 90-alpha-like, that ensure proper protein conformation and function, particularly under stress conditions, which is crucial for maintaining cellular homeostasis in response to environmental changes [760]; and proteins associated with vesicular trafficking, namely clathrin, which is vital for endocytosis and intracellular transport [761]. Additionally, there were proteins associated with cellular stress responses, like glutathione S-transferase 8 that participates in detoxification processes and protection against oxidative damage [762], and immune-related proteins such as macroglobulin, known for their role as broad-spectrum protease inhibitors [763]. Some of the most abundant identified proteins exhibit remarkable properties and are already widely applied across various fields. A notable example is collagen, which serves as a natural alternative to mammalian-derived collagen. Traditionally used in industries such as cosmetics and food production, mammalian collagen has faced growing concerns due to the risk of diseases like bovine spongiform encephalopathy and religious restrictions [764]. The highly conserved structure and sequence of fibrillar collagen make jellyfish-derived collagen a promising biomaterial, valued for its low immunogenicity and high biocompatibility [765].

### 5.4.2. Interpretation of Transcriptome–Proteome Correlation

The weak positive correlation between transcript and protein abundance suggests that while transcript abundance partially reflect protein detection, other biological and technical factors likely influence this relationship. This discrepancy is common, as proteomic datasets are often incomplete when compared to transcriptomic datasets [766]. The issue is possibly aggravated by the fact that transcriptomic and proteomic data were obtained from different specimens, introducing potential genetic and physiological variability. Additionally, environmental factors may contribute to these differences as well, as the transcriptomic data were derived from a wild specimen,

whereas the proteomic data originated from aquarium-maintained individuals. This variation in habitat could lead to differences in gene and protein expression patterns [767,768]. Moreover, it is important to acknowledge the main factors of protein expression regulation, including post-transcriptional, translational and protein-degradation processes [769]. Studies have shown that translation-related sequence features alone can account for up to 26% of the total variation of transcript–protein correlations [770]. Despite the moderate correlation observed, these findings provide valuable insights into the complexity of transcript–protein relationships, underscoring the importance of integrative approaches in transcriptomic and proteomic studies.

### 5.4.3. Tissue-Specific Functional Enrichment Reveals Metabolic and Regulatory Specialization

GO and KEGG enrichment analyses of *Phyllorhiza punctata* revealed distinct tissue-specific molecular profiles in the oral arms, gonads, and mantle, reflecting their specialized functions and highlighting the species' ecological resilience and biotechnological potential.

In the gonads, enrichment was linked to RNA metabolism and nucleotide processing, supporting the high transcription and translation demands of gametogenesis in this fast-proliferating species [771]. Small nuclear ribonucleoprotein (snRNP) terms indicated active pre-mRNA splicing typical of dividing cells [772]. Pathways for ovarian steroidogenesis and circadian rhythm were also enriched, emphasizing hormone production and the temporal regulation of reproduction [773]. Metal regulation likely reveals the mechanisms of germ cells' protection from oxidative stress [774].

The oral arms were enriched in signal transduction, transcriptional regulation, and mechanosensory response, consistent with their roles in prey capture and environmental sensing, which require neuromuscular coordination [775]. Immune and developmental pathways, including Th1/Th2 cell differentiation, TNF, apelin, and dorso-ventral axis formation, indicate immune defense and regenerative functions, aligning with their interaction with microbes and potential AMP production.

The mantle showed enrichment in mitochondrial components and metabolic pathways, supporting energy production and adaptation to environmental stress, traits likely contributing to the species' invasive success. Metal ion binding and catalytic activity suggest roles in detoxification and microbial defense, as seen in other marine invertebrates [776].

### 5.4.3. *P. punctata* Venom Composition

Given that the diagnostic feature of cnidarians is the presence of cnidocytes, specialized cells containing cnidae, among which nematocysts are the venom-bearing type [6], it is important to acknowledge that all cnidarians have the potential for toxicity. This includes species not considered a threat to humans, such as the case of *P. punctata*. Therefore, analyzing the species' venom proteome is crucial for gaining a comprehensive understanding of their full toxicological profile.

Among other toxins, three JFTs were identified in the proteome of *P. punctata*: toxin CfTX-A-like (XP_065070484) originally predicted from a genomic sequence of the rhizostomeaen jellyfish *R. esculentum*; toxin TX1 (AFK76348), previously identified from mRNA of *A. aurita* polyps; and TPA_exp: toxin a (GenBank ID: DAC80636.1), verified through proteomic analysis of the stinging-cell structures (cassiosomes) released in the mucus of *Cassiopea xamachana* [777]. These toxins are homologous to the JFTs, potent cytolytic toxins that disrupt cell membranes through pore creation. These were isolated initially from several species of box jellyfish and were linked with harmful stinging reactions to humans. For example, the toxins CfTX-1/2 and CfTX-A/B from the Australian box jellyfish *Chironex fleckeri* are highly cardiotoxic and hemolytic, respectively [778]. JFTs have also been identified in the tentacle and stinging-cell proteomes of Scyphozoa [214,777]; in the hydrozoan *Hydractinia symbiolongicarpus*, where their expression in nematocysts was confirmed at the proteomic level using immunohistochemistry [779]; and in Anthozoa, within the transcriptomes of Ceriantharia [780], though these findings still require validation through proteomic analysis. In the phylogenetic tree, the three JFTs are detected in the proteome of *P. punctata* clustered with other JFTs from Scyphozoa, forming a distinct clade separate from the two Cubozoan-specific clades. This phylogenetic pattern suggests that each group may have arisen through independent gene duplication and neofunctionalization events, potentially linked to lineage-specific differences in venom composition and toxic potency. A similar phylogenetic structure was observed in a broader study analyzing JFT distribution across 20 species representing all major medusozoan groups, but with more extensive taxon sampling [781]. Further studies will be required to understand the phylogenetic distribution of JFTs within scyphozoans and other cnidarians.

Additionally, a conodipine-P3-like derived from a genomic sequence of the scyphozoan *R. esculentum* was detected in the proteome of *P. punctata*. Conodipines are PLA2 toxins characterized from cone snail venoms [782]. However, similar toxins may be present in other venomous organisms, given that PLA2 enzymes have been convergently recruited into multiple insect orders, cephalopods, arachnids, reptiles, and

cnidarians [707]. As revealed by the sequence alignment (Fig. 29), several putative PLA2 toxins in Cnidaria with high homology to conodipines are known, containing the conserved catalytic domain residues, including the Asp/His dyad. Indeed, PLA2 activity has previously been detected in tissue homogenates of anthozoans, hydrozoans, scyphozoans, and cubozoans [783].

### 5.4.4. Potential AMPs Identified in *P. punctata*

Our previous study on the AMPs derived from aquatic invertebrates [22] provided valuable insights that enabled a more informed selection of candidate AMPs based on their most common physicochemical properties.

As a result, while the identification of both α-helical and β-like peptides among the candidates is an intriguing finding, it is not entirely unexpected. Generally, most AMPs adopt cationic amphipathic helices, a characteristic commonly associated with antimicrobial activity [242]. This trend is also observed in cnidarian AMPs, with peptides such as Aurelin from *A. aurita* [492], Arminin 1a from *Hydra* [494], Damicornin from *Pocillopora damicornis* [487], and AmAMP1 from *Acropora millepora* [488] all sharing cationic α-helices. However, some cnidarian AMPs adopt β-like structures instead, including defensin BDS-I from *Anemonia sulcata* [311], and Pd-AMP1 from the *Phyllogorgia dilatata* [489].

Additionally, no significant differences were found between tissues when comparing either the total number of peptides with the number of putative AMPs identified in each tissue or the number of exclusive peptides from each tissue with the number of exclusive putative AMPs identified in each tissue, which may suggest that *P. punctata* AMPs are not tissue-specific.

Furthermore, our integrative structure prediction approach revealed discrepancies between AlphaFold2 and PEP-FOLD4 outputs, particularly in the prediction of disordered regions. While AlphaFold2 tended to overpredict random coil structures, PEP-FOLD4 provided a more α-helix-rich profile. The overlap between methods in identifying consistent α-helical AMPs supports the utility of a dual-tool strategy for short-peptide modeling and strengthens confidence in the top candidate structures. Taken together, these findings support the presence of diverse and potentially bioactive AMP candidates in *P. punctata*, with structural traits aligned with known cnidarian antimicrobial peptides. This lays a strong foundation for future functional validation and potential biotechnological exploration.

## 5.5. Conclusions

Our findings highlight the largely untapped potential of cnidarians, particularly understudied species like *P. punctata*, as sources of toxins and AMPs. While *P. punctata* may not possess the potent toxin arsenal found in other jellyfish species, it still exhibits inherent toxicity. Moreover, as an invasive species, *P. punctata* demonstrates key features that favor its biotechnological exploration, including its adaptability to diverse environmental conditions, suggesting the production of bioactive compounds in response to environmental stressors, and its high reproductive capacity, which enables rapid biomass production for compound extraction. In this study, we identified several protein toxins in the species' proteome, including JFTs and PLA2. Furthermore, using a proteome-wide and peptidome-integrated approach, we applied precision-informed AMP prediction combined with structural validation via AlphaFold2 and PEP-FOLD4. This pipeline enabled the identification of 274 candidate AMPs, including structurally diverse α-helical and β-sheet peptides. Importantly, no strong tissue-specific distribution of AMPs was observed, implying a systemic expression of these peptides in *P. punctata*. These findings lay a strong foundation for future studies, including in vitro assays to validate antimicrobial activity efficacy, assess cytotoxicity, and evaluate peptide stability and bioavailability. Together, these efforts will advance *P. punctata* as a suitable and versatile source of bioactive compounds for potential biotechnological applications.

# Chapter 6.

## Concluding Remarks

## Concluding Remarks

This thesis provides a broad exploration of cnidarian diversity, AMPs from aquatic invertebrates, and the proteomics of two distinct cnidarian species, offering significant contributions to our understanding of these marine organisms and their ecological significance and biotechnological potential.

The research provides an extensive overview of Medusozoa diversity and distribution in the Iberian region, identifying 593 species across the four classes, with hydrozoans being the most prevalent and species such as *P. physalis* being the most recorded. Holoplanktonic and free-swimming species exhibit higher dispersal and diversity, with the Northeast Atlantic part of the region harboring greater medusozoan diversity than the Mediterranean part. This study also underlines invasive, introduced, bloom-forming, and edible species. A critical gap in available molecular data was identified, underscoring the need for further omics-based research to fully evaluate the potential of these organisms. Additionally, the study pinpoints underexplored target species with greater ecological and socioeconomic importance.

In parallel, this thesis underscores the critical role of AMPs from aquatic invertebrates, including cnidarians, in addressing the growing antimicrobial resistance crisis. It catalogs approximately 350 AMPs from 65 protein families across 10 invertebrate phyla, detailing their diverse structures and bioactivities. These peptides present promising sustainable alternatives to antibiotics in aquaculture, with potential applications as feed additives, therapeutic agents, or through genetic engineering to enhance farmed species. However, challenges such as the scarcity of *in vivo* studies and the need for AMP optimization must be addressed to advance with their practical application.

Furthermore, this thesis fills some knowledge gaps in cnidarian omics by investigating the proteomes of two unexplored cnidarian species, each representing a major cnidarian clade: the sea anemone *A. fragacea* (Anthozoa), and the white-spotted jellyfish *P. punctata* (Medusozoa).

The proteomic profiling of *A. fragacea* employed two distinct protocols: one for venom nematocyst extraction and another for tissue and mucus secretion analyses. This approach identified 4,011 different proteins (13,276 distinct peptides) grouped into 3,383 protein clusters. Among these, 83 putative toxins, including actinoporins and neurotoxins, were detected. Additionally, 1,406 peptides were predicted as potential AMPs, including a putative Aurelin-like peptide. Numerous bioactive molecules involved in immune defense, such as antimicrobial enzymes, PRPs, and neuropeptides, were identified, highlighting the species' molecular complexity and biotechnological potential.

Similarly, the proteomic analysis of *P. punctata* integrated proteomic and publicly available transcriptomic data, identifying 2,764 proteins and 25,045 peptides. This included venom components such as jellyfish toxins (JFTs) and phospholipase A2 (PLA2). In addition, advanced deep learning and machine learning approaches identified 274 promising AMP candidates, including structurally characterized α-helical and β-like peptides, further emphasizing *P. punctata'*s untapped biotechnological potential.

While this thesis provides valuable insights into cnidarian ecology, diversity, molecular complexity, and biotechnological applications, certain limitations must be acknowledged. A primary constraint is the scarcity of genomic and transcriptomic data from cnidarians, which limits the depth of proteomic studies, including those on *A. fragacea* and *P. punctata*. Future research should prioritize generating genomic and transcriptomic datasets to complement proteomic analyses, thereby improving protein identification and functional annotation.

Additionally, while numerous potential AMPs have been identified, they remain unvalidated. Future studies should focus on *in vitro* microbial assays (e.g., MIC, MBC, time-kill kinetics, anti-biofilm assays), to evaluate their antimicrobial efficacy. Cytotoxicity assays (e.g., MTT, LDH release, hemolysis assays) are also essential to determine their safety, while mechanistic studies such as liposome leakage, membrane permeabilization, and fluorescence microscopy can elucidate their mechanisms of action. Stability testing under different conditions (e.g., pH, temperature, salinity), drug interaction studies, and pharmacokinetic evaluations (e.g. gastrointestinal permeability tests, plasma protein binding assays), are crucial to determine peptide absorption, distribution, and bioavailability in aquaculture species. Furthermore, investigating potential additional bioactivities of these peptides, such as immunomodulatory, antioxidant, anti-inflammatory, and growth-promoting properties, could yield further valuable insights. Ultimately, the most promising AMPs should undergo *in vivo* testing to assess their sustainable therapeutic efficacy in aquaculture, including challenge tests, toxicity assessments, evaluations of growth and health performance, and environmental impact analysis.

Overall, this thesis advances our understanding of cnidarians, their ecology, molecular composition, and immense biotechnological potential. Future research efforts should aim at closing omics data gaps, validating AMPs, and exploring novel applications, ultimately supporting sustainable marine biotechnology and aquaculture management.

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# Attachments

# Attachment 1 – Chapter 2

Additional data and supplementary materials related to Chapter 2 are available online:

- **Regional Studies in Marine Science (Elsevier):** https://doi.org/10.1016/j.rsma.2024.103462
- **Global Biodiversity Information Facility (GBIF):** https://doi.org/10.15468/j7349s

**Table S 1** Public reports of Medusozoa identified at least at the genus level in the Iberian region.

**Table S 2** Checklist of the medusozoans recorded in the Iberian region.

**Table S 3** Highest reported hydrozoans and scyphozoans in the Iberian region.

**Table S 4** Cnidaria genomic (WGA, WGS) and transcriptomic (RNA-Seq) dataset from NCBI SRA database (until November 2023).

**Table S 5** Cnidaria proteomic dataset from ProteomeXchange, PRIDE, MassIVE, iProX, and UniProt Proteome repositories, along with revisited literature (until November 2023).

**Fig. S 1** Distribution heatmap of the medusozoans in the Iberian region. Darker red areas correspond to areas with more reports of Medusozoa. The heatmap represented correspond to all the reports in the Table S 1.

**Fig. S 2** Distribution heatmap of the medusozoans in the Iberian region. Darker red areas correspond to areas with more reports of Medusozoa. The heatmap represented correspond to all the reports in the Table S 1.

**Fig. S 3** Distribution map of the sessile (red dots) and free-swimming (blue dots) marine medusozoans in the Iberian region. The data points represented correspond to the marine reports in the Table S 1.

**Fig. S 4** Seasonality of Medusozoa reports by EEZ. IR: Iberian region, PT: Portugal, SP: Spain, AZ: Azores, MAD: Madeira, CAN: Canary. The data represented corresponds to the reports available in Table S 1 with information on the month of the report.

**Fig. S 5** Distribution map of the holoplanktonic (yellow dots), meroplanktonic (purple dots), and benthic (cyan dots) marine medusozoan species in the Iberian region. The data points represented correspond to the marine reports in the Table S 1.

**Fig. S 6** Cumulative number of cnidarian omics entries through years. Entries correspond to NCBI SRA genomic (WGA, and WGS) and transcriptomic (RNA-Seq) entries, and, proteomic (ProteomeXchange, PRIDE, MassIVE, iProX, UniProt Proteome, and literature) retrieved until November 2023. For detailed information see Table S 1.

## Attachment 2 – Chapter 3

Additional data and supplementary materials related to Chapter 3 are available online:

- **Microorganisms (MDPI):** https://www.mdpi.com/article/10.3390/microorganisms13010156/s1.

**Table S 6** Pathogenicity profiles of the microorganisms targeted by AMPs derived from aquatic invertebrates.
**Table S 7** Database of the AMPs obtained from aquatic invertebrates.
**Table S 8** Antimicrobial activities of AMPs from aquatic invertebrate annelids against pathogens relevant to aquaculture and human health.
**Table S 9** Antimicrobial activities of AMPs from aquatic invertebrate arthropods against pathogens relevant to aquaculture and human health.
**Table S 10** Antimicrobial activities of AMPs from aquatic invertebrate chordates against pathogens relevant to aquaculture and human health.
**Table S 11** Antimicrobial activities of AMPs from cnidaria against pathogens relevant to aquaculture and human health.
**Table S 12** Antimicrobial activities of AMPs from echinoderms against pathogens relevant to aquaculture and human health.
**Table S 13** Antimicrobial activity of AMPs from mollusks against pathogens relevant to aquaculture and human health.
**Table S 14** Antimicrobial activity of AMPs from other invertebrates against microorganisms including pathogens relevant to aquaculture and human health.
**Table S 15** Activity of “non classical AMPs” derived from aquatic invertebrates against pathogens relevant to aquaculture and human health.

## Attachment 3 – Chapter 4

Additional data and supplementary materials related to Chapter 4 are available online:

- **Marine Drugs (MDPI):**
  https://www.mdpi.com/1660-3397/23/2/79

**Table S 16** Comprehensive protein report derived from *A. fragacea* samples.

**Table S 17** InterProScan results report from general and uncharacterized proteins identified in *A. fragacea* proteome.

**Table S 18** GO Annotation results for proteins identified in *A. fragacea* proteome.

**Table S 19** List of putative toxins identified in *A. fragacea* proteome.

**Table S 20** List of putative immune-related proteins identified in *A. fragacea* proteome.

**Table S 21** Predicted AMPs in *A. fragacea* proteome based on CAMPR4 and Antimicrobial Peptide Scanner vr.2 analyses.

**Fig. S 7** Distribution of GO levels across biological process, molecular function, and cellular component domains in *A. fragacea* samples.

**Fig. S 8** Enzyme Commission (EC) classes of enzymes identified through GO annotation in the *A. fragacea* proteome.

**Fig. S 9** Enzyme Commission (EC) classes of hydrolases identified through GO annotation in the *A. fragacea* proteome.

# Attachment 4 – Chapter 5

Additional data and supplementary materials related to Chapter 4 are available online:

- **Biomolecules (MDPI):** https://www.mdpi.com/2218-273X/15/8/1121#B37-biomolecules-15-01121

**Table S 22** Comprehensive protein list identified from *Phyllorhiza punctata* tissues.

**Table S 23** InterProScan annotation of identified proteins.

**Table S 24** Gene Ontology (GO) enrichment analysis of identified proteins.

**Table S 25** KEGG pathway enrichment analysis of identified proteins.

**Table S 26** Putative toxins identified in the *P. punctata* proteome.

**Table S 27** Predicted antimicrobial peptides (AMPs) in the *P. punctata* proteome.

**File S 1** De novo transcriptome assembly of *P. punctata*.

**File S 2** Multiple sequence alignment of jellyfish toxin family (JFT) proteins.

**File S 3** Structural predictions of selected putative AMPs.