U.PORTO

FACULDADE DE FARMÁCIA
UNIVERSIDADE DO PORTO

2nd Cycle Studies - Master Degree in Pharmaceutical Chemistry

***In vivo*, *in vitro* and *in silico* studies of new antifouling compounds obtained by synthesis**

Catarina Isabel Soares Gonçalves

July 2019

Advisor:

Professora Doutora Marta Ramos Pinto Correia da Silva Carvalho Guerra

Co-advisor:

Doutora Joana Reis de Almeida

(Author's signature/Assinatura do autor)

(Advisor: Professora Doutora Marta Correia da Silva Carvalho Guerra)

(Master Course Director: Professora Doutora Emília Sousa)

Support for this work was provided by the Strategic Funding UID/Multi/04423/2019 and under the project PTDC/AAG-TEC/0739/2014 (reference POCI-01-0145-FEDER-016793) supported through national funds provided by FCT – Foundation for Science and Technology and European Regional Development Fund (ERDF) through the COMPETE - Programa Operacional Factores de Competitividade (POFC) programme and the Project 9471 – Reforçar a Investigação, o Desenvolvimento Tecnológico e a Inovação (RIDTI, 9471) and under the Project POCI-01-0145-FEDER-028736, co-financed by COMPETE 2020, Portugal 2020 and the European Union through the ERDF and by FCT through national funds.

Some results presented in this dissertation are part of the following scientific communications:

- **Abstract in Conference proceedings**

Gonçalves, C.*; Neves, A.R.; Sousa, E.; Pinto, M.; Almeida, J.; Correia-da-Silva, M. "Discovery of a new polyphenol acetylcholinesterase inhibitor", Book of Abstracts of 4ª Edição da Escola de Inverno de Farmácia, Porto, Portugal, 18-27 Março, 2019, pp 43.

Gonçalves,C*.; Neves, A.R.; Sousa, E.; Pinto, M.; Vasconcelos, V.; Almeida, J.; Correia-da-Silva, M. "*In silico* and *in vivo* evaluation of new synthetic antifouling compounds." Book of Abstracts of 12th Meeting of Young Researchers of University of Porto (IJUP2019), 2019, pp 15383.

- **Poster communications**

Gonçalves, C.*; Neves, A.R.; Sousa, E.; Pinto, M.; Almeida, J.; Correia-da-Silva, M. "Discovery of a new polyphenol acetylcholinesterase inhibitor", 4ª Edição da Escola de Inverno de Farmácia, Porto, Portugal, 18-27 Março, 2019, pp 43.

Gonçalves,C*.; Neves, A.R.; Sousa, E.; Pinto, M.; Vasconcelos, V.; Almeida, J.; Correia-da-Silva, M. "*In silico* and *in vivo* evaluation of new synthetic antifouling compounds.", 12th Meeting of Young Researchers of University of Porto (IJUP2019), 2019, pp 15383.

* Presenting author

Agradecimentos

Este trabalho é o resultado de um longo percurso no qual tive o prazer de ser ajudada por pessoas fantásticas às quais preciso e quero muito agradecer.

Em primeiro lugar, à minha orientadora Professora Doutora Marta Correia da Silva pelo entusiamo com que me recebeu no seu grupo de investigação do “fouling”. Obrigada pela ajuda, dedicação e por todo o conhecimento transmitido. É para mim um grande exemplo de que com trabalho e persistência tudo se consegue.

À minha coorientadora Doutora Joana Reis de Almeida que sempre me acompanhou e que foi fundamental para o sucesso de todo o trabalho laboratorial desenvolvido ao longo deste ano. Obrigada por ter estado sempre disponível para me ajudar e ensinar.

Ao Jorge Antunes e à Sandra Pereira por me terem ajudado na realização dos bioensaios e por me transmitirem o vosso conhecimento.

A toda a equipa do CIIMAR, em especial à equipa do laboratório BBE por me ter acolhido tão bem e me ter feito sentir “em casa”. A vossa ajuda foi uma peça fundamental para alcançar o sucesso.

Aos meus amigos, por me transmitirem sempre a confiança que tudo iria correr bem, mesmo nos dias mais difíceis. Obrigada por serem a minha fonte de alegria.

Ao Hildebrando, agradecer por todo o carinho, amor e compreensão. Obrigada por animares os meus dias e estares incondicionalmente do meu lado.

À minha família, mas em especial aos meus pais, um gigante obrigada. Sem o vosso amor, apoio e esforço nada disto seria possível. Obrigada por me incentivarem a seguir os meus sonhos e por serem sempre os primeiros a apoiar-me, independentemente das dificuldades.

Obrigada.

Abstract

Biofouling is defined as the undesirable colonization of submerged man-made surfaces by fouling organisms (microfoulers and macrofoulers) and represents a major economic and environmental concern worldwide. The most commonly used strategy to combat this problem is the use of antifouling paints. Nowadays, booster biocides and significant levels of copper are being used as antifouling agents, however these compounds are toxic to marine organisms and to environment. Thus, the development of greener antifouling agents for practical use has been a priority.

In this work, 46 nature-inspired compounds synthesized in the Laboratory of Organic and Pharmaceutical Chemistry (LQOF) were tested as potential ecofriendly antifouling compounds. These compounds are divided into three main chemical classes: 15 flavonoids compounds (10 chalcones and 5 flavones), 19 phenolic compounds and 12 acetophenones.

All compounds were tested using an *in vivo* anti-settlement assay with mussel (*Mytilus galloprovincialis*) plantigrade larvae. Thirteen compounds (**3**, **4**, **4a**, **8**, **14**, **17**, **21**, **30**, **33**, **36**, **40**, **41** and **42**) were active against settlement of mussel larvae (EC_{50} < 25µg mL^{-1}). In order to assess the possible mechanism of action of these compounds, the inhibition of the acetylcholinesterase enzyme (AChE) was evaluated and only compound **30** was active at 100µM. The ability to inhibit the growth of biofilm forming bacteria was evaluated for compounds **21** and **30**. Both compounds were able to inhibit the growth of some marine bacterial strains at 15µM.

The ecotoxicity test on a non-target species, *Artemia salina,* revealed that only compounds **40**, **41** and **42** presented toxicity to this species. *In silico* calculation of Log KOW values showed that compounds **3**, **8**, **40** and **41** have some potential for bioaccumulation.

The structure-activity relationship studies have shown that almost all compounds that were active contained hydroxyl groups.

Keywords: biofouling; antifouling; ecofriendly; nature-inspired; synthesis; structure-activity relationship; mechanism of action; bioaccumulation.

Resumo

A bioincrustação marinha é definida como a colonização de superfícies submersas por organismos com propriedades adesivas (micro-organismos e macro-organismos) e representa uma importante preocupação económica e ambiental em todo o mundo. A estratégia mais utilizada para combater este problema é o uso de tintas anti-incrustantes. Atualmente, estão a ser utilizados biocidas e altos níveis de cobre para impedir a adesão destes organismos incrustantes, no entanto, estes compostos são tóxicos para os organismos marinhos e para o ambiente. Assim sendo, desenvolver compostos anti-incrustantes "verdes" para uso prático tem sido uma prioridade.

Neste trabalho, foram testados 46 compostos sintetizados no Laboratório de Química Orgânica e Farmacêutica (LQOF) como potenciais novos compostos anti-incrustantes ecológicos. Estes compostos pertencem a três classes químicas principais: 15 compostos flavonoides (10 calconas e 5 flavonas), 19 compostos fenólicos e 12 acetofenonas.

Todos os compostos foram testados usando um ensaio *in vivo* de avaliação da adesão de larvas de mexilhão (*Mytilus galloprovincialis*). Treze compostos (**3**, **4**, **4a**, **8**, **14**, **17**, **21**, **30**, **33**, **36**, **40**, **41** e **42**) foram ativos contra a adesão das larvas de mexilhão (EC_{50} ≤ 25µg mL^{-1}). A fim de identificar o possível mecanismo de ação destes compostos, avaliou-se a capacidade para inibirem a enzima acetilcolinesterase (AChE) e apenas o composto **30** foi ativo a 100µM. A capacidade de inibir o crescimento de bactérias formadoras de biofilme foi avaliada para os compostos **21** e **30**. Ambos mostraram alguma capacidade para inibir o crescimento de algumas estirpes de bactérias marinhas a 15µM.

Os testes de ecotoxicidade para a espécie de organismos não-alvo *Artemia salina* revelaram que apenas os compostos **40**, **41** e **42** são tóxicos. O cálculo *in silico* dos valores de Log KOW mostraram que os compostos **3**, **8**, **40** e **41** têm algum potencial de bioacumulação.

Os estudos de relação estrutura-atividade realizados mostraram que quase todos os compostos que foram ativos contêm grupos hidroxilo.

Palavras-chave: bioincrustação; anti-incrustantes; ecológicos; inspirados na natureza; síntese; relação estrutura-atividade; mecanismo de ação; bioacumulação.

General Index

Agradecimentos v
Abstract vi
Resumo vii
Figure Index x
Table Index xii
List of Abbreviations xiii
1. Introduction 1
1.1. Biofouling and antifouling 1
1.2. Nature-inspired synthetic compounds 5
1.2.1. Chalcones and flavones 6
1.2.2. Phenolic compounds 14
2. Aims and strategy of this work 16
3. Material and Methods 20
3.1. Antifouling bioassays 20
3.1.1. Mussel larvae anti-settlement bioassays 20
3.1.2. Anti-bacterial bioassays 20
3.2. *Mode of action assessment: in vitro* determination of acetylcholinesterase activity 21
3.3. Environmental fate parameters 21
3.3.1. *Artemia salina* ecotoxicity bioassay 21
3.3.2. *In silico* evaluation of bioaccumulation potential 22
3.4. Statistical analysis 22
4. Results and Discussion 23
4.1. *In vivo* bioassay against the settlement of mussel larvae 23
4.1.1. Chalcones and flavones 23
4.1.2. Phenolic compounds 28
4.1.3. Acetophenones 31
4.2. *In vitro* inhibition of Acetylcholinesterase (AChE) activity 34
4.2.1. Chalcones and flavones 34
4.2.2. Phenolic compounds 35
4.2.3. Acetophenones 36
4.3. Inhibition of biofilm-forming marine bacteria growth 37
4.4. Environmental fate parameters 38
4.4.1. *Artemia salina* ecotoxicity bioassay 38
4.4.2. *In silico* studies 41

5. Conclusion 43
6. References 45
7. Appendix 49
7.1. Appendix I 49
7.2. Appendix II 54
7.3. Appendix III 56

Figure Index

Figure 1. Simplified process of marine biofouling (adapted from [3]). 1
Figure 2. Structure of Tributyltin (TBT). 2
Figure 3. Structure of a chalcone. 6
Figure 4. Structure of 2-methoxy-2',4'-dichloro chalcone. 8
Figure 5. Structures of furylchalcones. 9
Figure 6. Structures of flavonoids. 10
Figure 7. Natural phenolic compounds active against *Bugula neritina* larvae settlement. 14
Figure 8. Structure of p-(sulfoxy) cinnamic acid (ZA). 14
Figure 9. Structures of ZA analogues. 15
Figure 10. Structure of gallic acid persulfate - AGS. 15
Figure 11. Structures of chalcones (**A**) and flavones (**B**). 17
Figure 12. Structures of phenolic compounds similar to zosteric acid (**A**) and similar to gallic acid persulfate (**B**). 18
Figure 13. Structures of acetophenones. 19
Figure 14. Percentage of settlement of Mytilus galloprovincialis larvae in the presence of chalcones and flavones at 50μM. B: DMSO control (0.01%); C: 5μM $CuSO_4$ as positive control. 23
Figure 15. The importance of 4'-substitutions in compounds **3** and **4**. 25
Figure 16. The influence of an ethoxyl chain in compounds **9**, **9a** and **12a**. 26
Figure 17. The influence of triazole in compound **7a**. 26
Figure 18. The importance of three methoxy groups in compounds **4** and **8**. 27
Figure 19. Percentage of settlement of Mytilus galloprovincialis larvae in the presence of phenolic compounds at 50μM. B: DMSO control (0.01%); C: 5μM $CuSO_4$ as positive control. 28
Figure 20. The influence of para-substitutions in compound **14**. 29
Figure 21. The influence of para-substitutions in compound **22**. 29
Figure 22. The influence of a para-substitution in compound **20**. 30
Figure 23. The influence of carboxy modifications in compound **14**. 30
Figure 24. The influence of double conjugation on the antifouling activity. 30
Figure 25. Percentage of settlement of Mytilus galloprovincialis larvae in presence of a series of acetophenones at 50μM. B: DMSO control (0.01%); C: 5μM $CuSO_4$ as positive control. 31
Figure 26. The influence of para-substitutions in compound **33**. 32
Figure 27. The influence of substitutions in compound **38**. 32
Figure 28. The influence of modifications in compound **39**. 33
Figure 29. Acetylcholinesterase (AChE) activity of the most promising chalcones (**3**-**5**,**8** and **9**) and flavones (**4a** and **12a**) compounds. B: 1% DMSO. C: Eserine (200μM). 34
Figure 30. Acetylcholinesterase (AChE) activity of the most promising phenolic compounds. B: 1% DMSO. C: Eserine (200μM). 35
Figure 31. Acetylcholinesterase (AChE) activity of acetophenones compounds. B: 1% DMSO. C: Eserine (200μM). 36
Figure 32. Antibacterial activity of compounds **21** and **30** at 15μM towards five biofilm-forming marine bacteria Cobetia marina, Vibrio harveyi, Pseudoalteromonas atlanti20, Halomonas aquamarina and Roseobacter litoralis. B: 0.1% DMSO. C: 1:100 penicilin-streptomycin-neomycin stabilized solution. 37
Figure 33. Mortality rate of Artemia salina nauplii after 48h of exposure to compounds **3**, **4**, **4a** and **8**. B: 1% DMSO in filtered seawater. C: $K_2C_{r2}O_7$ at 13.6μM. ECONEA® was used for comparative purposes. 38

Figure 34. Mortality rate of Artemia salina nauplii after 48h of exposure to compounds **21** and **30**. B: 1% DMSO in filtered seawater. C: $K_2C_{r2}O_7$ at 13.6μM. ECONEA® was used for comparative purposes. 39
Figure 35. Mortality rate of Artemia salina nauplii after 48h of exposure to the compounds **33**, **36**, **40**, **41** and **42**. B: 1% DMSO in filtered seawater. C: $K_2C_{r2}O_7$ at 13.6μM. ECONEA® was used for comparative purposes. 40
Figure 36. Compounds with the best results in this work. 44
Figure 37. Dose-response activity regarding anti-settlement of compound **3**. B: DMSO (0.01%) as negative control; C: 5μM $CuSO_4$ as positive control. 49
Figure 38. Dose-response activity regarding anti-settlement of compound **4**. B: DMSO (0.01%) as negative control; C: 5μM $CuSO_4$ as positive control. 49
Figure 39. Dose-response activity regarding anti-settlement of compound **4a**. B: DMSO (0.01%) as negative control; C: 5μM $CuSO_4$ as positive control. 50
Figure 40. Dose-response activity regarding anti-settlement of compound **5**. B: DMSO (0.01%) as negative control; C: 5μM $CuSO_4$ as positive control. 50
Figure 41. Dose-response activity regarding anti-settlement of compound **6**. B: DMSO (0.01%) as negative control; C: 5μM $CuSO_4$ as positive control. 51
Figure 42. Dose-response activity regarding anti-settlement of compound **8**. B: DMSO (0.01%) as negative control; C: 5μM $CuSO_4$ as positive control. 51
Figure 43. Dose-response activity regarding anti-settlement of compound **9**. B: DMSO (0.01%) as negative control; C: 5μM $CuSO_4$ as positive control. 52
Figure 44. Dose-response activity regarding anti-settlement of compound **9a**. B: DMSO (0.01%) as negative control; C: 5μM $CuSO_4$ as positive control. 52
Figure 45. Dose-response activity regarding anti-settlement of compound **12a**. B: DMSO (0.01%) as negative control; C: 5μM $CuSO_4$ as positive control. 53
Figure 46. Dose-response activity regarding anti-settlement of compound **14**. B: DMSO (0.01%) as negative control; C: $CuSO_4$ (5μM) as positive control. 54
Figure 47. Dose-response activity regarding anti-settlement of compound **17**. B: DMSO (0.01%) as negative control; C: $CuSO_4$ (5μM) as positive control. 54
Figure 48. Dose-response activity regarding anti-settlement of compound **21**. B: DMSO (0.01%) as negative control; C: $CuSO_4$ (5μM) as positive control. 55
Figure 49. Dose-response activity regarding anti-settlement of compound **30**. B: DMSO (0.01%) as negative control; C: $CuSO_4$ (5μM) as positive control. 55
Figure 50. Dose-response activity regarding anti-settlement of compound **33**. B: DMSO (0.01%) as negative control; C: $CuSO_4$ (5μM) as positive control. 56
Figure 51. Dose-response activity regarding anti-settlement of compound **36**. B: DMSO (0.01%) as negative control; C: $CuSO_4$ (5μM) as positive control. 56
Figure 52. Dose-response activity regarding anti-settlement of compound **40**. B: DMSO (0.01%) as negative control; C: CuSO4 (5μM) as positive control. 57
Figure 53. Dose-response activity regarding anti-settlement of compound **41**. B: DMSO (0.01%) as negative control; C: CuSO4 (5μM) as positive control. 57
Figure 54. Dose-response activity regarding anti-settlement of compound **42**. B: DMSO (0.01%) as negative control; C: CuSO4 (5μM) as positive control. 58

Table Index

Table 1. Booster biocides: mode of action and environmental fate parameters. 2
Table 2. Synthetic chalcones derivatives with anti-microfouling activity. 6
Table 3. Chalcones derivatives synthesized in LQOF with antifouling activities against both micro and macrofouling species. 8
Table 4. Natural flavonoids with antifouling activity. 11
Table 5. Results of the screening at 50µM of structurally related chalcones (**3**-**9**) and flavones (**3a**-**9a**) against the settlement of Mytilus galloprovincialis. 24
Table 6. Antifouling effectiveness of chalcones **3**-**6**,**8**,**9** and flavones **4a**, **9a** and **12a** towards mussel plantigrades larvae. 27
Table 7. Antifouling effectiveness of compounds **30**, **21**, **17** and **14** towards mussel plantigrades larvae. 31
Table 8. Antifouling effectiveness of compounds **33**, **36**, **40**, **41** and **42** towards mussel plantigrades 33
Table 9. Log KOW values obtained for chalcones (**3**, **4** and **8**) and flavones (**4a**). 41
Table 10. Log KOW values obtained for phenolic compounds. 42
Table 11. Log KOW values obtained for acetophenones compounds. 42

List of Abbreviations

AChE – Acetylcholinesterase

AF – Antifouling

AGS – Sulfated gallic acid

$CuSO_4$ – Copper sulphate

DMSO – Dimethylsulfoxide

EC_{50} – Half maximal effective concentration

EPA – Environmental Protection Agency

IMO – International Maritime Organization

$K_2Cr_2O_7$ – Potassium dichromate

Log KOW – Octanol – water partition coefficient

LQOF – Laboratório de Química Orgânica e Farmacêutica

MIC – Minimum inhibitory concentration

TBT – Tributylin

ZA – Zosteric acid

1. Introduction

1.1. Biofouling and antifouling

Biofouling is defined as the attachment and accumulation of micro and macro organisms such as bacteria, algae, protozoans, seaweeds, tubeworms, bryozoans, ascidians, barnacles and mussels on submerged surfaces.[1] This undesirable colonization of artificial structures causes serious problems and have a large economic impact to maritime industry worldwide because it increases fuel consumption as a result of water resistance and increase of weight, as well as due to promotion of corrosion.[1] Biofouling also creates a series of environmental problems such as the introduction of invasive species in new ecossystems which contributes to biodiversity reduction.[2]

This phenomenon usually involves a sequence of phases (Figure 1):[1]

1) adsorption of organic compounds (proteins, polysaccharides and lipids) present in seawater;
2) biofilm formation (bacteria);
3) microalgae and protozoa colonization;
4) settlement of macroalgal and invertebrate larvae.

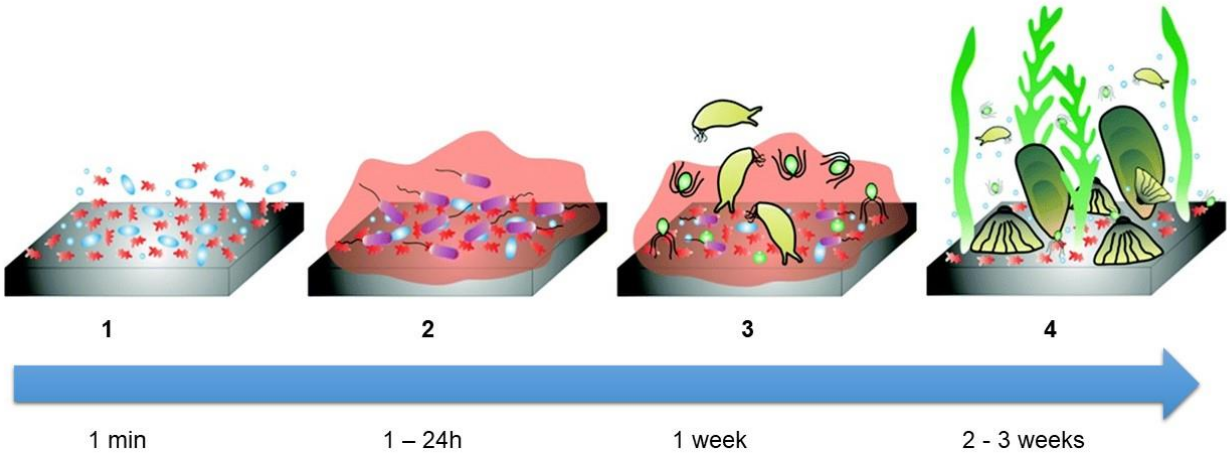


Figure 1. Simplified process of marine biofouling (adapted from [3]).

There are more than 4000 fouling species in the sea.[3] However, the dominant fouling organisms constituting very distinct biofouling communities may vary with the temperature, pH, and salinity of the marine environment.[3]

The use of antifouling paints has been the most widely used method to control biofouling. Until recently, organotins such as tributyltin (TBT) (Figure 2) were employed to prevent the attachment of fouling organisms.[1] However, several toxic effects of these compounds have been identified on both target and non-target organisms. For example, several oyster farms in France reported a reduction in oyster spatfall, anomalies in larval development and shell malformation, affecting 80–100% of individual oysters.[4] Another negative effect associated with TBT contamination was the *Imposex* phenomenon, which is characterized by the development of male characteristics on female gastropods.[4] For these reasons, TBT was banned worldwide by the International Maritime Organization (IMO).[1, 5]

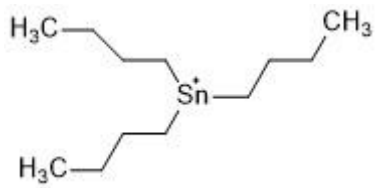
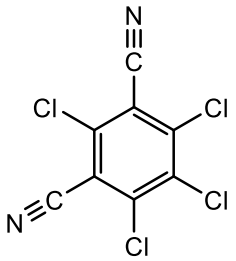
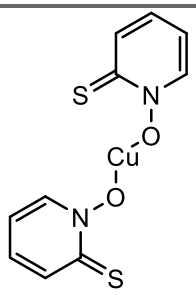
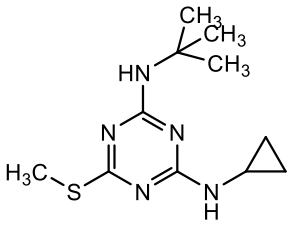
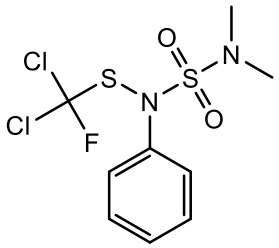
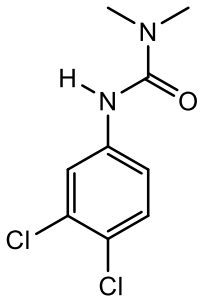


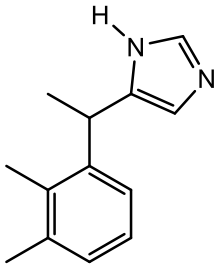
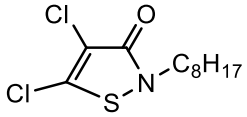
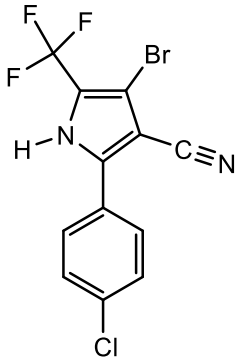
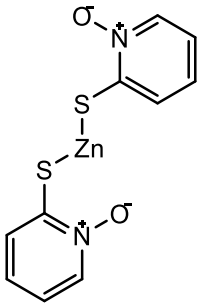
Figure 2. Structure of Tributyltin (TBT).

Currently, booster biocides and significant levels of copper are being used as antifouling agents (Table 1).

Table 1. Booster biocides: mode of action and environmental fate parameters.

Biocide (Commercial name)	**Structure of booster biocide**	**Mode of action**	**Environmental fate parameters**	**Ref.**
Chlorothalonil		Inhibits photosynthesis and carbon incorporation	Log KOW = 2.6-4.4 Ecotoxicity: Highly toxic to aquatic species; *Artemia salina* - mortality at 24h.	[4, 6-7]
Copper pyrithione		Disrupt the integrity of cell membrane	Log KOW = 2.84 Ecotoxicity: *Artemia salina* - mortality at 24h.	[6]

Biocide (Commercial name)	Structure of booster biocide	Mode of action	Environmental fate parameters	Ref.
				
Cybutryne (Irgarol 1051)		Oxidative stress inducer	Log KOW = 2.8-4.1 Ecotoxicity: Highly toxic to periphyton and macrophytes *Artemia salina* - mortality at 24h.	7-9
Dichlofluanid		Inhibits photosynthesis and carbon incorporation	Log KOW = 2.8-3.7 Ecotoxicity: Toxic for most of biodiversity.	7
3-(3,4-dichlorophenyl)-1,1-dimethylurea (Diuron®)		Inhibits photosynthesis and carbon incorporation	Log KOW = 2.82 Ecotoxicity: Slightly toxic to mammals and birds, moderately toxic to fishes and highly toxic to aquatic invertebrates; *Artemia salina* - mortality at 24h.	6-7, 10
Medetomidine (Selektope®)		Stimulates the octopamine receptor	Log KOW = 2.6 Ecotoxicity: Very toxic to aquatic life.	11

Biocide (Commercial name)	Structure of booster biocide	Mode of action	Environmental fate parameters	Ref.
				
4,5-dichloro-2-n-octyl-4-isothiazolin-3-one (Sea-Nine 211®)		Inhibitor of acetylcholine esterase	Log KOW = 2.85 Ecotoxicity: Broad-spectrum antifoulant.	[7]
Tralopyril (Econea®)		Inhibitor of mitochondrial electron transport	Log KOW = 3.47 Ecotoxicity: Very toxic to aquatic life and potentially harmful to non-target aquatic organisms; *Artemia salina* - mortality at 24h.	[7]
Zinc pyrithione		Oxidative stress inducer	Log KOW = 0.9 Ecotoxicity: Highly toxic to aquatic plants and animals; *Artemia salina* - mortality at 24h.	[6]

It is known that some booster biocides, such as Irgarol 1051 and Diuron are relatively persistent in seawater.[12] Moreover, the degradation products of Irgarol 1051 are more toxic than the original compound.[5] Dichlofluanid have also been reported to occur in sediments.[12] Beside that, it seems that marine aquatic species are sensitive to all booster biocides.[1] For this reason, the development of new non-toxic and environmental friendly antifouling compounds has been a priority.

Through millions of years of evolution, several marine organisms have developed strategies to combat the settlement of other fouling organisms, including the production of secondary metabolites with antifouling properties.[13] Therefore, these organisms have become a natural source of "green" antifouling compounds, since they are less toxic and biodegradable.[14] Most of these natural compounds are identified as terpenoids, steroids, carotenoids, phenolics, furanones, alkaloids, peptides and lactones.[15]

Nevertheless, the use of natural marine sources to extract bioactive compounds on a large scale is unsustainable, since the concentrations obtained are very low and the demand exceeds the supply. In addition, most of these natural compounds are structurally complex, making commercial production almost impossible.[3] Therefore, synthesis of promising antifouling candidates in the laboratory has been used as an alternative. Furthermore, through the synthesis, it is possible to optimize their antifouling activity.

According to the Biocidal Product Regulation (EU) 528/2012, a clear description of environmental fate for new AF compounds is now required to their introduction into the market. Ideally, a new effective and environmentally compatible antifouling compound must have: broad spectrum of action through the biologically diverse biofouling community; low toxicity to target and non-target species; a balance between low solubility in water and low bioaccumulation potential and low environmental persistence.[10]

Recent structure-activity relationship studies show that through natural products or their optimized derivatives, it is indeed possible to obtain antifouling compounds with the necessary balance between antifouling potency and low or no toxicity to the environment.

1.2. Nature-inspired synthetic compounds

The synthesis of new compounds inspired in natural products has gained increasingly interest in the discovery of new antifouling compounds. Therefore, many derivatives of natural products have been studied as promising anti-fouling compounds, namely, stilbenes, 2,5-diketopiperazines, bromotyrosines, hemibastadins, glucosamine-based isocyanides and polygodial derivatives.[16-21] This section will focus on the flavonoids, chalcones and other phenolic compounds.

1.2.1. Chalcones and flavones

Chalcones, also known as α-β-unsaturated ketones (Figure 3), are natural products precursors of flavonoids that can also be obtained synthetically using a relatively simple synthesis procedure.[2, 22]

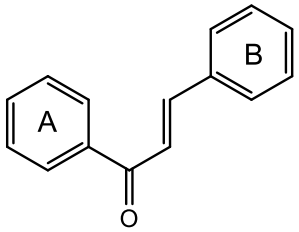
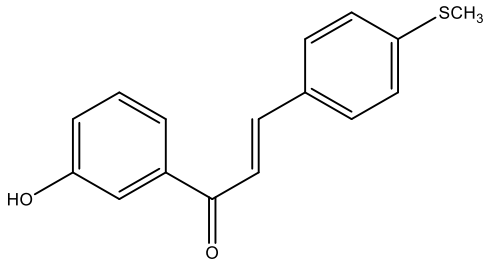


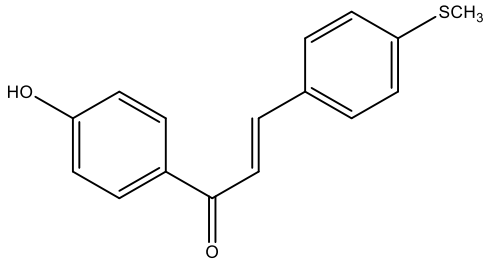
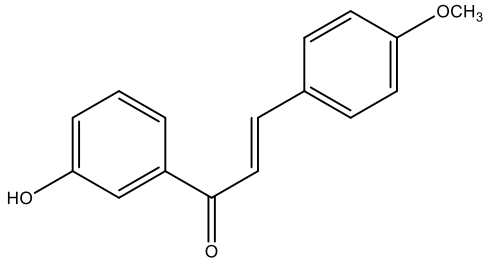
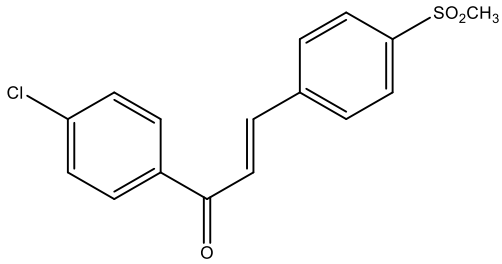
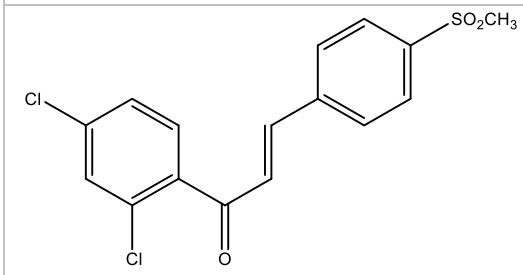
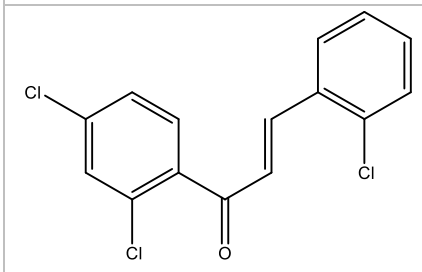
Figure 3. Structure of a chalcone.

They exhibit various useful biological activities, namely, antioxidant, anticancer, anti-tubercular, anti-inflammatory, antimalarial, antifungal and antiviral activities [23] and it is believed that the presence of double bond in conjugation with carbonyl functionality is responsible for these activities.[22] A series of clorochalcones and furylchalcones are also used as insecticides.[24-25] Some derivatives were described with antibacterial, slimicidal and anticorrosive properties. [26] All these characteristics make chalcones ideal candidates for use in antifouling coatings.

From a study where 47 chalcone derivatives were synthesized and evaluated for their ability to inhibit bacterial growth of three species of marine bacteria: *Bacillus flexus*, *Pseudomonas fluorescens* and *Vibrio natriegens*, results showed that six compounds were active and in the majority of the cases these compounds had hydroxyl substitutions (Table 2).[26]

Table 2. Synthetic chalcones derivatives with anti-microfouling activity.

Chalcone	**Anti-microfouling activity**			**Ref.**
	B.flexus	*P.fluorescens*	*V.natriegens*	
	MIC = 0.014µM	MIC = 0.029µM	MIC = 0.058µM	[26]

Chalcone	Anti-microfouling activity			Ref.
	B.flexus	*P.fluorescens*	*V.natriegens*	
	MIC= 0.058µM	MIC = 0.014µM	MIC = 0.116µM	
	MIC = 0.031µM	MIC = 0.031µM	MIC = 0.061µM	
	MIC = 0.195µM	MIC = 0.006µM	MIC = 0.024µM	
	MIC = 0.088µM	MIC = 0.044µM	MIC = 0.088µM	
	MIC = 0.002µM	MIC = 0.014µM	MIC = 0.060µM	

MIC = minimum inhibitory concentration

In another study, the 2-methoxy-2',4'-dichloro chalcone (Figure 4) showed to reduce the biofilm formation of the marine bacteria *Vibrio natriegens*.[27]

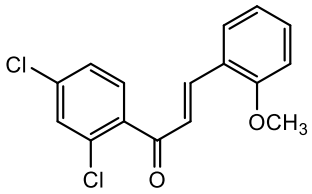
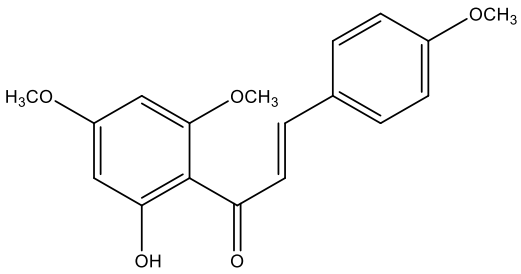
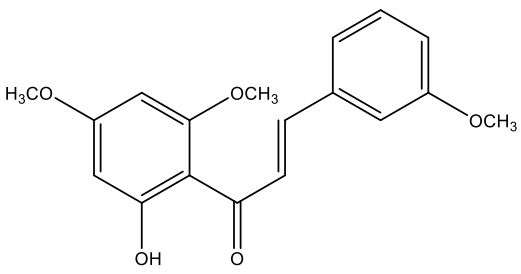
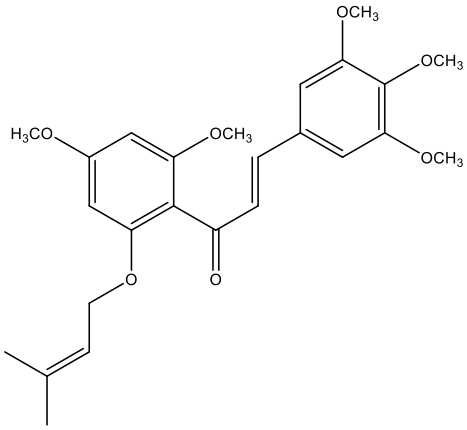


Figure 4. Structure of 2-methoxy-2',4'-dichloro chalcone.

From a recent series of chalcones synthesized in Laboratório de Química Orgânica e Farmacêutica (LQOF), three chalcones showed promising antifouling profile through the inhibition of the settlement of mussel larvae and inhibition of biofilm forming bacteria and diatoms growth (Table 3). The importance of the presence of a polymethoxylated B ring associated with a lipophilic side chain in A ring of the chalcones seems to influence the AF activity.[2]

Table 3. Chalcones derivatives synthesized in LQOF with antifouling activities against both micro and macrofouling species.

Chalcone	**Antifouling activity**		**Ref.**
	Anti-macrofouling	**Anti-microfouling**	
	**Mussel** *Mytilus galloprovincialis:* EC_{50} = 34.63µM	n.a.	[2]
	**Mussel** *Mytilus galloprovincialis:* EC_{50} = 7.24µM	**Bacteria** *Halomonas aquamarina*: EC_{50} = 18.67µM *Roseobacter litoralis*: EC_{50} = 4.09µM	

Chalcone	Antifouling activity		Ref.
	Anti-macrofouling	**Anti-microfouling**	
	**Mussel** *Mytilus galloprovincialis:* EC_{50} = 16.48µM	**Bacteria** *Halomonas aquamarina*: EC_{50} = 18.67µM *Roseobacter litoralis*: EC_{50} = 4.09µM **Diatom** *Cylindrotheca sp.*: EC_{50} = 7.04µM *Halamphora sp.*: EC_{50}= 14.65µM *Nitzschia sp.*: EC_{50} = 20.31µM *Navicula sp.*: EC_{50} = 6.75µM	

EC_{50} = concentration that inhibits 50% of attachment of selected test organism; n.a = not active.

These chalcones were also found to be non-toxic to the brine shrimp (*Artemia salina*) nauplii at 50µM (the maximum concentration tested), which make them more suitable for AF agents.[2]

More recently, six furylchalcones were synthesized and incorporated into antifouling paints (Figure 5).[28]

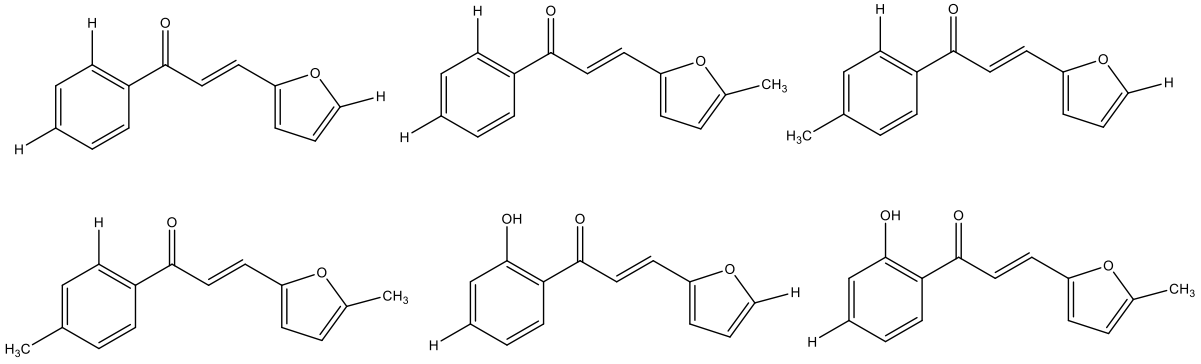


Figure 5. Structures of furylchalcones.

After 45 days exposure in the sea, this antifouling paints with furylchalcones showed great anti-protozoal, anti-diatom, and anti-macrofouling activities.[28] Furthermore, this study revealed that the replacement of the B ring (phenyl) by a furan ring was associated with an increased anti-macrofouling activity.[28]

Flavonoids (Figure 6) are also natural products which have diverse recognized biological activities, that includes: antioxidative properties, antimicrobial, antioxidant, antifeedant and antitumor activities. [29-32]

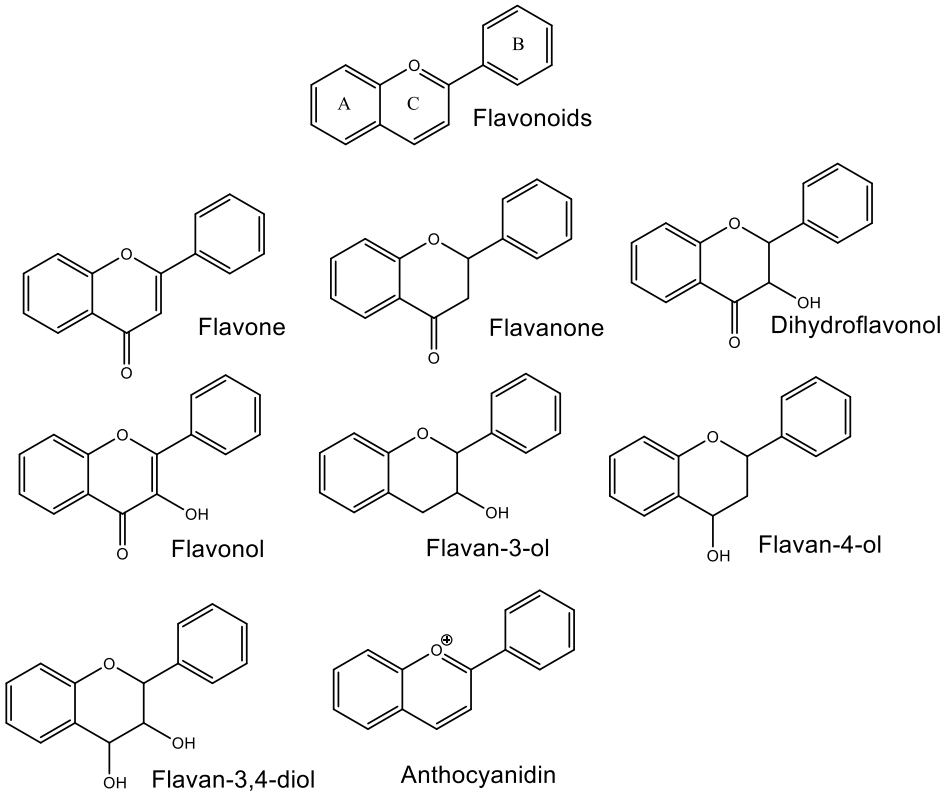
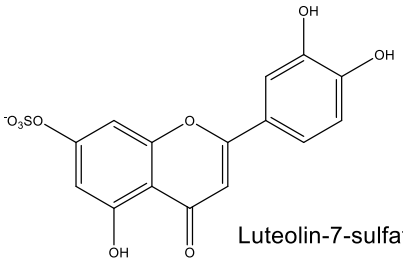
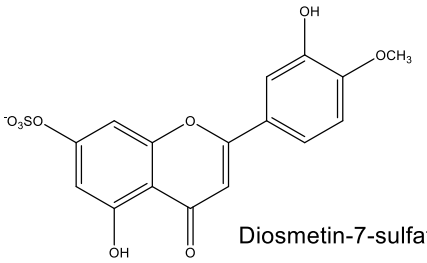
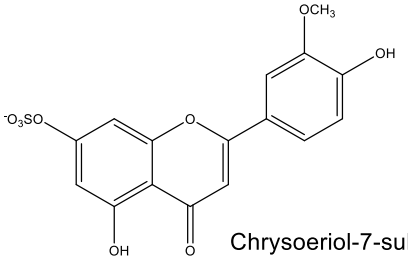
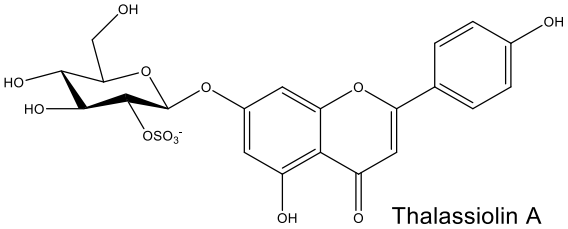
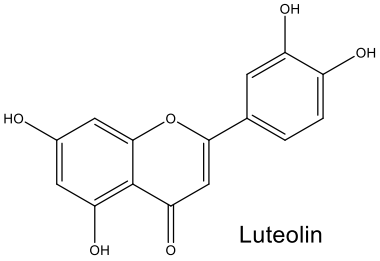
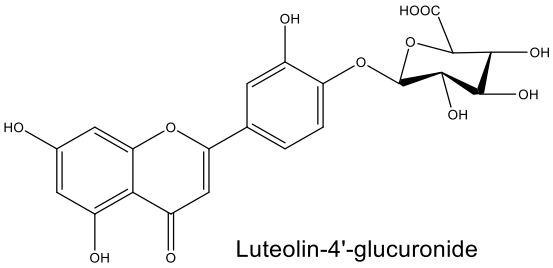
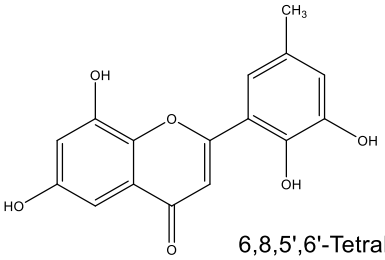
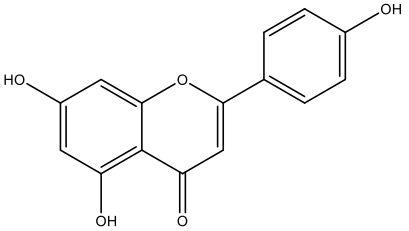
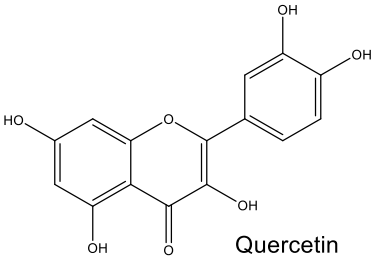
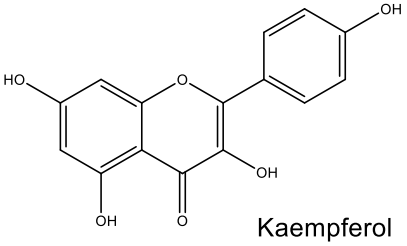
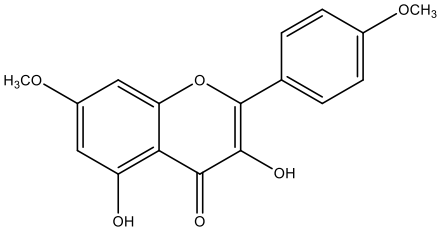


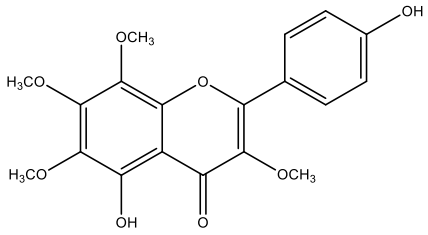
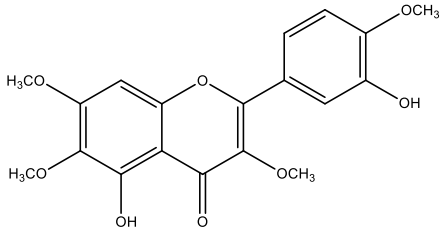
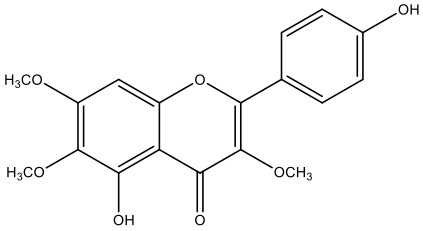
Figure 6. Structures of flavonoids.

Several studies have shown that some natural flavonoids also exhibit antifouling activity (Table 4).[32-36]

Table 4. Natural flavonoids with antifouling activity.

Natural Flavonoids	Antifouling Activity	Ref.
Flavones		
 Luteolin-7-sulfate	Prevention of the settlement of *Vibrio cyclitrophicus* and *Marivita litorea*	33
 Diosmetin-7-sulfate		
 Chrysoeriol-7-sulfate		
 Thalassiolin A	Reduction of the attachment of *Schizochytrium aggregatum* motile zoospores	34
 Luteolin	Inhibition the growth of *Loktanella hongkongensis* and the settlement of *Balanus amphitrite* larvae	32, 36

Natural Flavonoids	Antifouling Activity	Ref.
Flavones		
 Luteolin-4'-glucuronide	Inhibition the growth of the bacteria *Loktanella hongkongensis* and *Vibrio halioticoli* and the larval settlement of *Bugula neritina*	32
 6,8,5',6'-Tetrahydroxy-3'-methylflavone	Inhibited the settlement of *Balanus amphitrite* larvae	35
 Apigenin	Inhibition the settlement of *Balanus amphitrite* larvae	36
Flavonol		
 Quercetin	Inhibition of the growth of the bacteria *Bacillus thuringiensis*, *Pseudoalteromonas elyakovii* and *Pseudomonas aeruginosa*	37
 Kaempferol	Inhibition the settlement of *Balanus amphitrite* larvae	36
 3,5-Dihydroxy-7,4'-dimethoxyflavone		

Natural Flavonoids	Antifouling Activity	Ref.
Flavonols derivatives		
 Calycopterin	Inhibition the settlement of *Balanus amphitrite* larvae	36
 Casticin		
 5,40-Dihydroxy-3,6,7-trimethoxyflavone		

At the best of our knowledge, no studies concerning synthetic flavonoids with antifouling activity have been conducted.

1.2.2. Phenolic compounds

Phenolic compounds are characterized by the presence of a hydroxyl group, attached to a benzene ring or other complex aromatic ring structures.[38] This natural compounds are present in plant essential oils and show high antifungal, antibacterial and insecticidal efficacy.[38] Thymol, eugenol and guaiacol are natural phenolic compounds used in coatings with medical application and also in food industry, due to its anti-adhesion, anti-biofilm and antibacterial properties.[38] Moreover, there are several natural phenolic compounds described as antifouling agents.[39-40] Figure 7 shows some of these compounds that were active against *Bugula neritina* larvae settlement.[39-40]

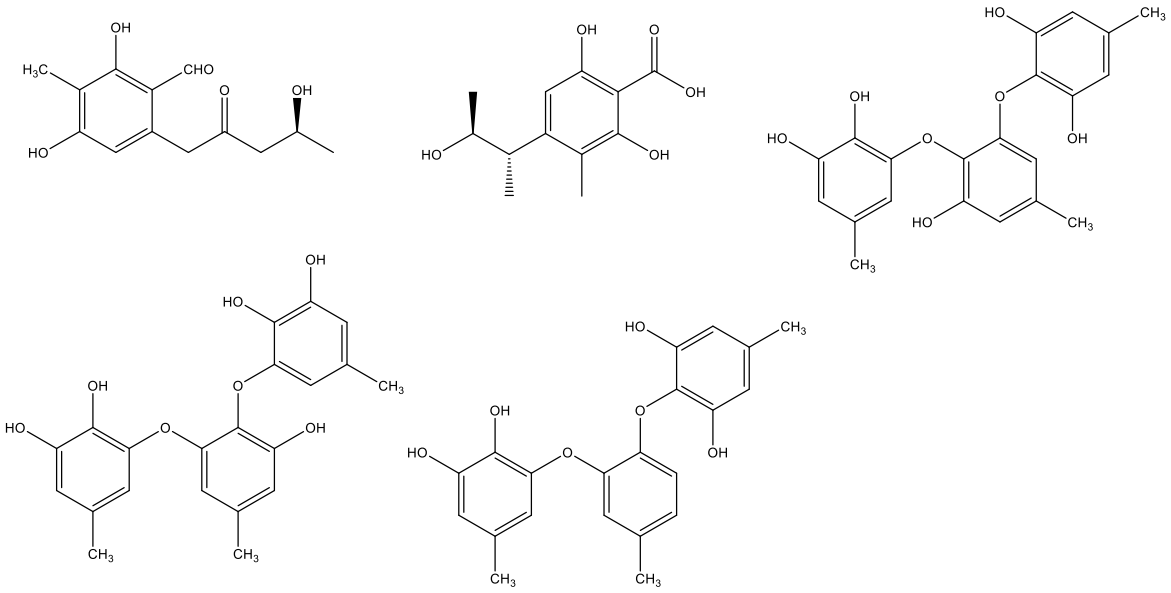


Figure 7. Natural phenolic compounds active against *Bugula neritina* larvae settlement.

One of the best known examples of a natural phenolic compound with antifouling properties is the secondary metabolite identified as Zosteric acid or p-(sulfoxy) cinnamic acid (ZA) produced by the seagrass *Zostera marina* (Figure 8), that is capable of preventing the attachment of bacteria, [41-42] fungus[43-44] and higher-order organisms[45] at nontoxic concentrations.[46] However, ZA is highly water soluble what makes its release from conventional antifouling paints difficult to control.[47-48]

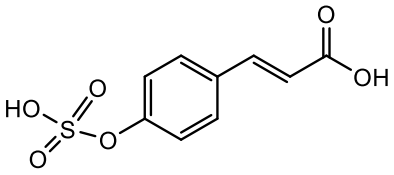
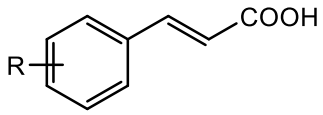


Figure 8. Structure of p-(sulfoxy) cinnamic acid (ZA).

A series of ZA analogues were synthesized in order to identify important structural determinants to their anti-biofilm activity (Figure 9).[41]



R = 4-OH; 4-CHO; 4-COOH; 4-CH_3; 4-Cl; 4-NH_2; 4-CF_3; 2-Cl;

Figure 9. Structures of ZA analogues.

This study revealed that the activity of ZA analogues depends of the presence of a carboxylate anion and a conjugated aromatic system.[41] In addition, it has been concluded that the sulfoxy group is not necessary for the anti-biofilm activity.[41]

Recently, our research group synthesized a series of synthetic sulfated compounds, including phenolic compounds, and the most promising compound was structurally-related to ZA, the gallic acid persulfate - AGS (Figure 10).[46]

This compound proved to be effective against settlement of *Mytilus galloprovincialis* larvae (EC_{50} = 17.65µM) and non-toxic to non-target organism *Artemia salina* even at 250µM .[46] However, its Log KOW value is extremely low (- 7.02), indicating that this compound is also very water soluble. [48] Moreover, this compound can be obtained using a relatively simple synthesis procedure.[46]

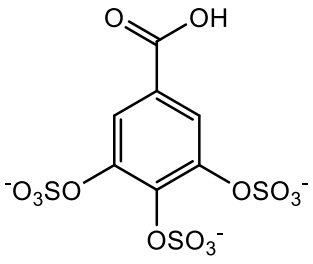


Figure 10. Structure of gallic acid persulfate - AGS.

2. Aims and strategy of this work

The main objective of this work was to evaluate the antifouling activity of nature-inspired compounds obtained by synthesis in LQOF and assess their potential as new ecofriendly agents.

As previously shown, there are several studies that associate chalcones and flavonoids with antifouling activity, therefore, they were selected as one of the chemical classes to be tested in this work (Figure 11). Furthermore, it was intended to compare the results obtained with other flavonoids derivatives previously synthesized in LQOF (Figure 11, blue). Phenolic compounds similar to the natural compound ZA and to the synthetic compound AGS (Figure 12) were also one of the classes evaluated in this work given the good results described for both scaffolds. A widely used strategy in the discovery of new drugs is the random screening of synthetic intermediates as possible new drugs. In this direction, we tested a series of acetophenones (Figure 13), which were synthesized as synthetic intermediates of chalcones.

All compounds were screened using an *in vivo* anti-settlement bioassay with mussel *Mytilus galloprovincialis* larvae at 50µM. The most promising compounds were selected for dose-response studies and mode of action assessment for their ability to inhibit the activity of acetylcholinesterase enzyme (AChE). In addition, environmental parameters such as ecotoxicity to non-target organisms (*Artemia salina*) and the potential for bioaccumulation were assessed.

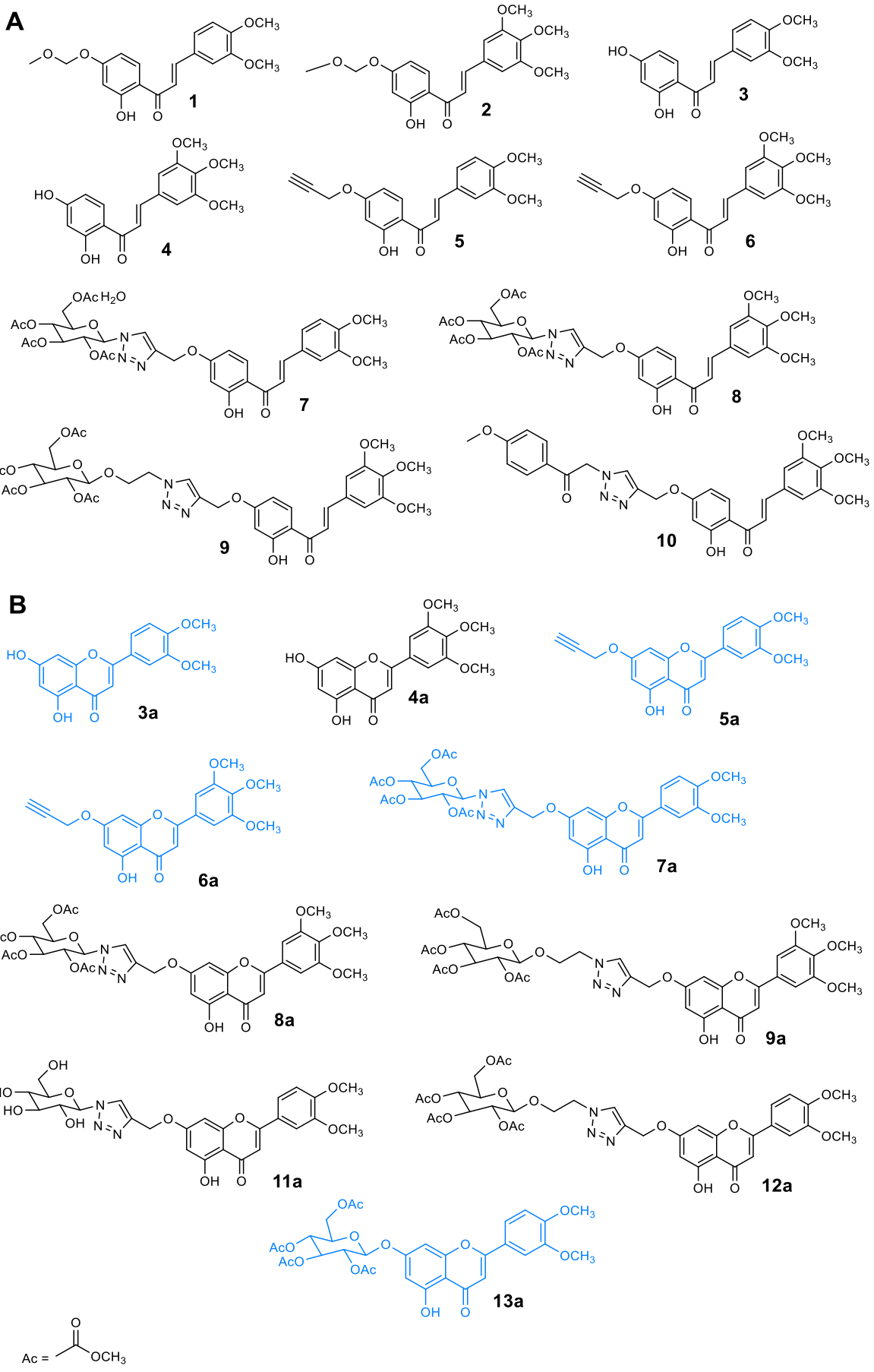


Figure 11. Structures of chalcones (**A**) and flavones (**B**).

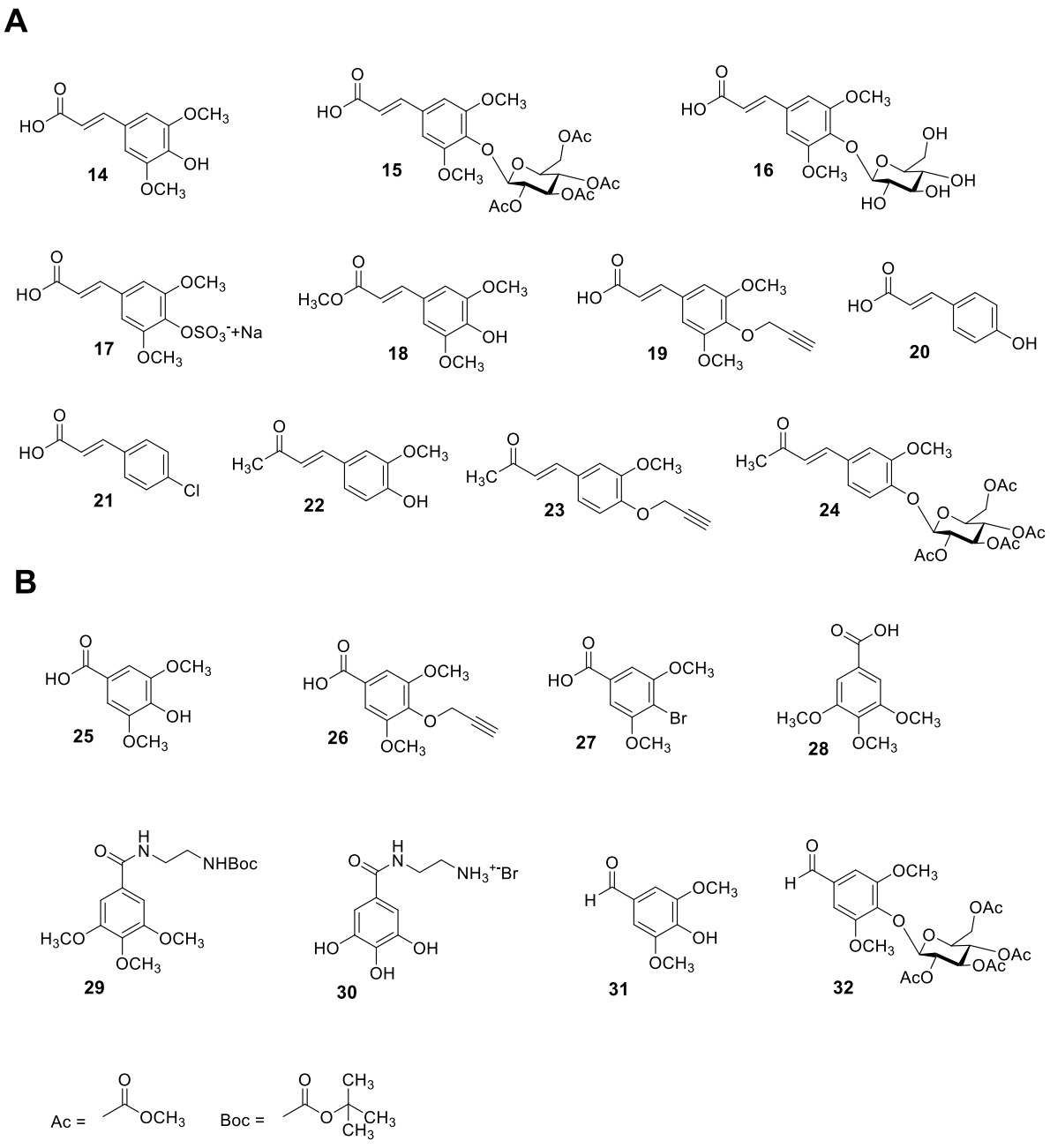


Figure 12. Structures of phenolic compounds similar to zosteric acid (**A**) and similar to gallic acid persulfate (**B**).

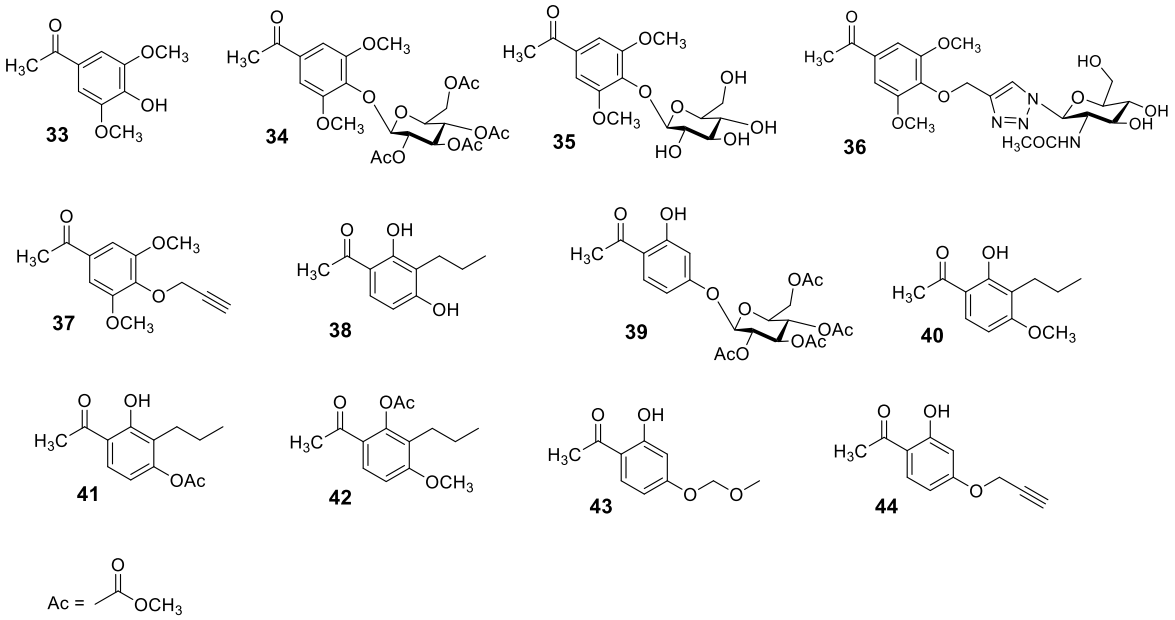


Figure 13. Structures of acetophenones.

3. Material and Methods

All compounds tested in this study were synthesized in LQOF.

3.1. Antifouling bioassays

3.1.1. Mussel larvae anti-settlement bioassays

Mussel (*Mytilus galloprovincialis*) juvenile (0.5cm shell length) aggregates were collected during low neap tides at Memória beach, Matosinhos, Portugal. In laboratory, healthy mussel plantigrade larvae (0.5 - 2mm with functional foot) were selected from the mussel aggregates in a binocular magnifier (Olympus SZX2-ILLT) and isolated in a petri dish with filtered seawater.

After selection of the *Mytilus galloprovincialis* mussel larvae that showed exploring behavior, they were exposed to the test compounds at concentration for antifouling screening purposes at 50µM in 24-well microplates with 4 well replicates per condition and 5 larvae per well for 15h, at 18 ± 1°C, in the darkness. Test solutions were obtained by dilution of the compounds stock solutions (50mM) in DMSO and prepared with filtered seawater. All bioassays included a negative control with DMSO and a positive control with $CuSO_4$, a potent antifouling agent. After the exposure period, the anti-settlement activity was determined by the presence / absence of attached byssal threads produced by each individual larvae.

All compounds that caused more than 60% of settlement inhibition (≤ 40% of settlement) at 50µM in the screening bioassay were considered active and selected for the determination of the semi-maximum response concentration that inhibited 50% of larval settlement (EC_{50}), which is performed in the same way as the screening bioassay but with increasing concentrations (3.12, 6.25, 12.5, 25, 50, 100, 200µM) of the test compound.

3.1.2. Anti-bacterial bioassays

For anti-bacterial screening five strains of marine biofilm-forming bacteria from the Spanish Type Culture Collection (CECT): *Cobetia marina* CECT 4278, *Vibrio harveyi* CECT 525*, Halomonas aquamarina* CECT 5000, *Pseudoalteromonas atlantica* CECT 570, and *Roseobacter litoralis* CECT 5395 were used. Bacteria were inoculated and incubated for 24h at 26°C in marine broth (Difco) at an initial density of 0.1 (OD_{600}) in 96 well flat-bottom

microtiter plates and exposed to the test compounds at 15µM. Test solutions were obtained by dilution of the compounds stock solutions (50mM) in DMSO. The negative control used were a solution of marine broth with DMSO and the positive control were a solution of marine broth with penicillin-streptomycin-neomycin. Bacterial growth inhibition was determined in quadruplicate at 600nm using a microplate reader (Biotek Synergy HT, Vermont, USA).

3.2. *Mode of action assessment: in vitro* determination of acetylcholinesterase activity

The ability of test compounds to inhibit acetylcholinesterase (AChE) was tested to assess the potential mode of action (neurotransmission disruption) of the promising antifouling compounds. AChE activity was evaluated using Electrophorus electric acetylcholinesterase Type V-S (SIGMA C2888, E.C. 3.1.1.7), according to Ellman et al. (1961)[49] with some modifications.[50-51] Reaction solution containing phosphate buffer 1M pH 7.2, dithiobisnitrobenzoate (DTNB) 10mM (acid dithiobisnitrobenzoate and sodium hydrogen carbonate in phosphate buffer) and acetylcholine iodide 0.075M was added to pure acetylcholinesterase enzyme (0.25U/mL) and each test compound (final concentration of 25, 50 and 100µM) in quadruplicate. All test included a positive control with eserine and a negative control with DMSO. The optical density was measured at 412nm in a microplate reader (Biotek Synergy HT, Vermont, USA) during 5min at 25°C.

3.3. Environmental fate parameters

3.3.1. *Artemia salina* ecotoxicity bioassay

The brine shrimp (*Artemia salina*) nauplii lethality test was used to determine the toxicity of promising antifouling compounds to non-target organisms.[52] *Artemia salina* eggs were allowed to hatch in nutrient-enriched seawater for 48h at 25ºC. Bioassays were performed in 96-wells microplates with 15-20 nauplii per well and 200µL of the test solution. Test solutions were prepared in filtered seawater at concentrations of 50µM and 25µM. All tests included $K_2Cr_2O_7$ as positive control and DMSO as negative control. Thereafter, the bioassays run in the dark at 25°C, and the percentage of mortality was determined after 48h of exposure.

3.3.2. *In silico* evaluation of bioaccumulation potential

Bioaccumulation potential of each promising antifouling candidate was assessed by *in silico* calculation of log Kow (octanol-water partition coefficient) was obtained through the program KOWWIN™ v1.68 (log octanol-water partition coefficient calculation program) developed by Syracuse Research Cooperation jointly with the Environmental Protection Agency (EPA).

3.4. Statistical analysis

EC_{50} values for each compound were calculated using Probit regression analysis. The software IBM SPSS Statistics 25 and Excel 2017 were used for statistical analysis.

4. Results and Discussion

4.1. *In vivo* bioassay against the settlement of mussel larvae

Mussels are one of the main macrofouling organisms present on ships and all submerged maritime structures, thus they are a target species used in settlement inhibition assays.[53-54] Due to the presence of a muscular sensory foot, mussel larvae are highly specialized in adhesion to the submerged surfaces and the fixation is made through the production of byssal threads.[55] Therefore, the first screening assay for the evaluation of antifouling activity of the synthetic compounds was the anti-settlement of *Mytilus galloprovincialis* larvae at 50µM.

4.1.1. Chalcones and flavones

Figure 14 shows the results obtained in the initial screening at 50µM. In this bioassay 10 chalcones (**1 - 10**) and 5 flavones (**4a**, **8a**, **9a**, **11a** and **12a**) were tested.

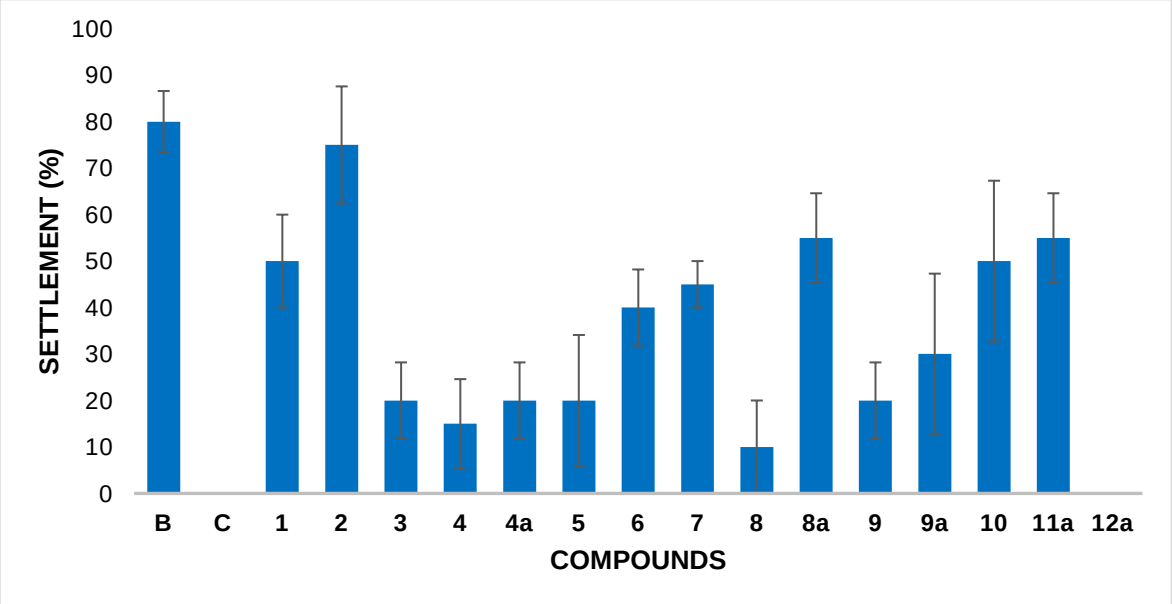
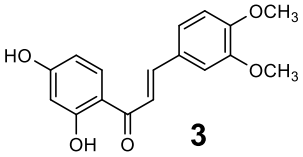
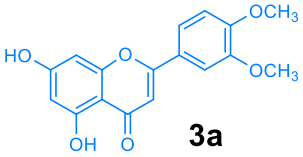
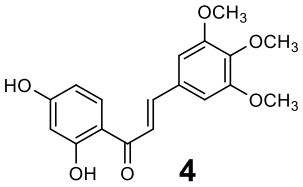
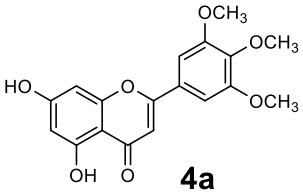
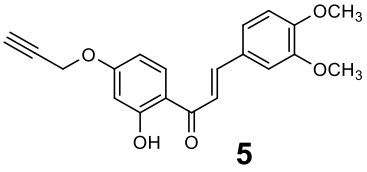
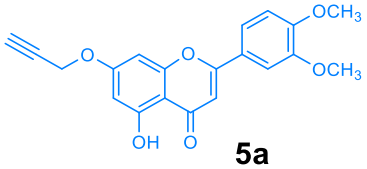
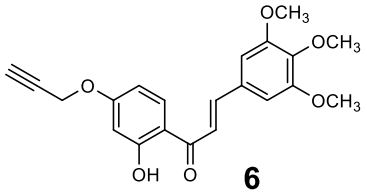
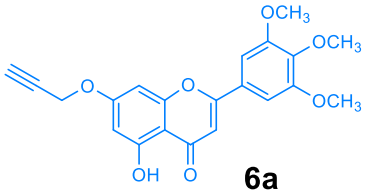
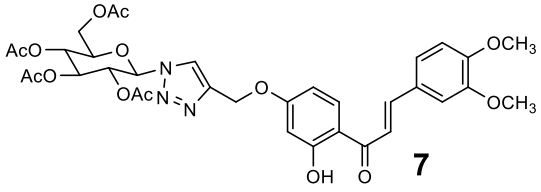
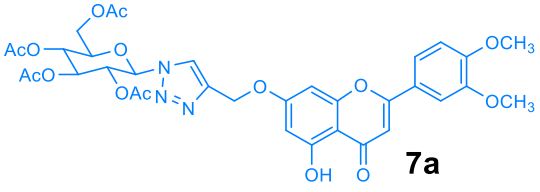


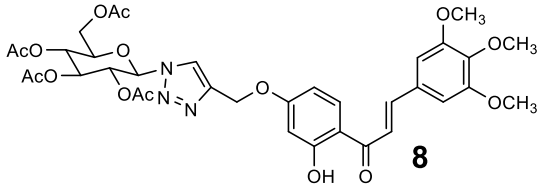
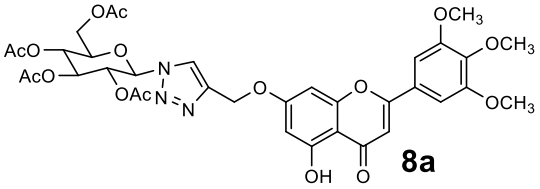
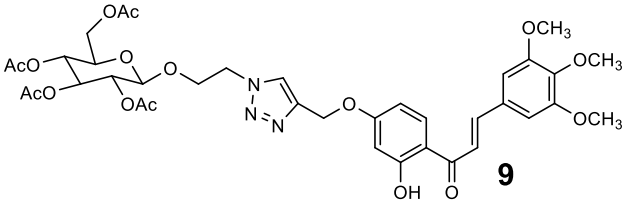
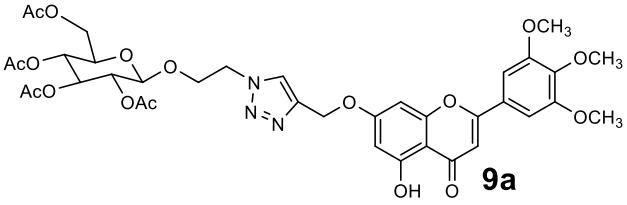
Figure 14. Percentage of settlement of *Mytilus galloprovincialis* larvae in the presence of chalcones and flavones at 50µM. B: DMSO control (0.01%); C: 5µM $CuSO_4$ as positive control.

According to this first screening, 6 chalcones (compounds **3, 4, 5, 6, 8** and **9** - Figure 11) and 3 flavones (compounds **4a**, **9a**, and **12a** - Figure 11) showed a percentage of settlement ≤ 40%, which makes them promising antifouling compounds.

The results obtained in this bioassay were compared with those previously obtained with structurally related flavones (marked at blue in Table 5) and it appears that chalcones have a greater capacity to inhibit the adhesion of mussel larvae than flavones (Table 5).

Table 5. Results of the screening at 50µM of structurally related chalcones (**3**-**9**) and flavones (**3a**-**9a**) against the settlement of *Mytilus galloprovincialis*.

Chalcones **(% settlement at 50µM)**	**Flavones** **(% settlement at 50µM)**
 **3** (20%)	 **3a** (55%)
 **4** (15%)	 **4a** (20%)
 **5** (20%)	 **5a** (70%)
 **6** (40%)	 **6a** (70%)
 **7** (45%)	 **7a** (90%)

Chalcones (% settlement at 50μM)	Flavones (% settlement at 50μM)
 (10%)	 (55%)
 (20%)	 (30%)

It is also important to highlight some structure-activity relationship. For example, when comparing compound **1** with compound **3** and compound **2** with compound **4** (Figure 15), it seems that the introduction of methoxymethyl decreased the inhibition of settlement.

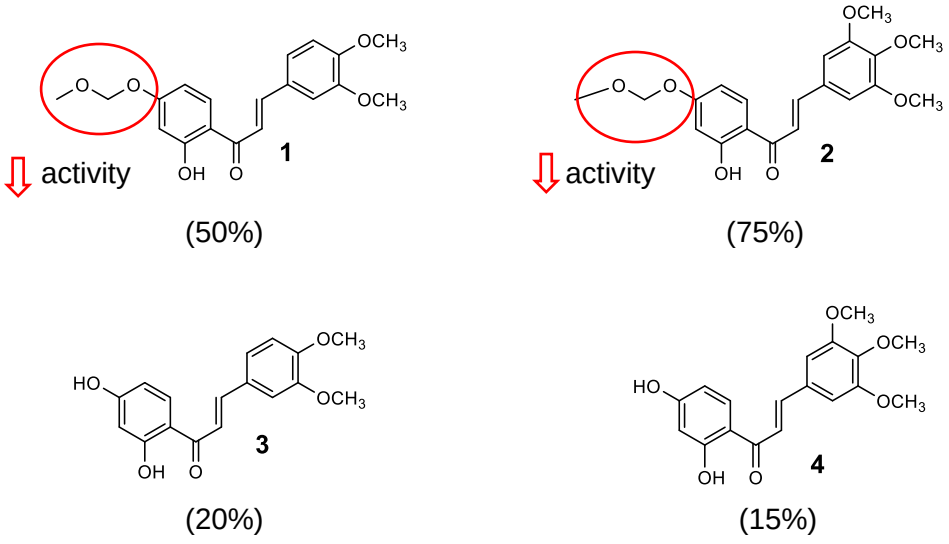


Figure 15. The importance of 4'-substitutions in compounds **3** and **4**.

On the other hand, when comparing compound **7a** with compound **12a,** compound **8a** with compound **9a**, and compound **8** with compound **9** (Figure 16), it seems that the presence of an ethoxyl chain between the triazole linkages is related to an increased in the inhibition of the settlement in flavones and a decreased in chalcones.

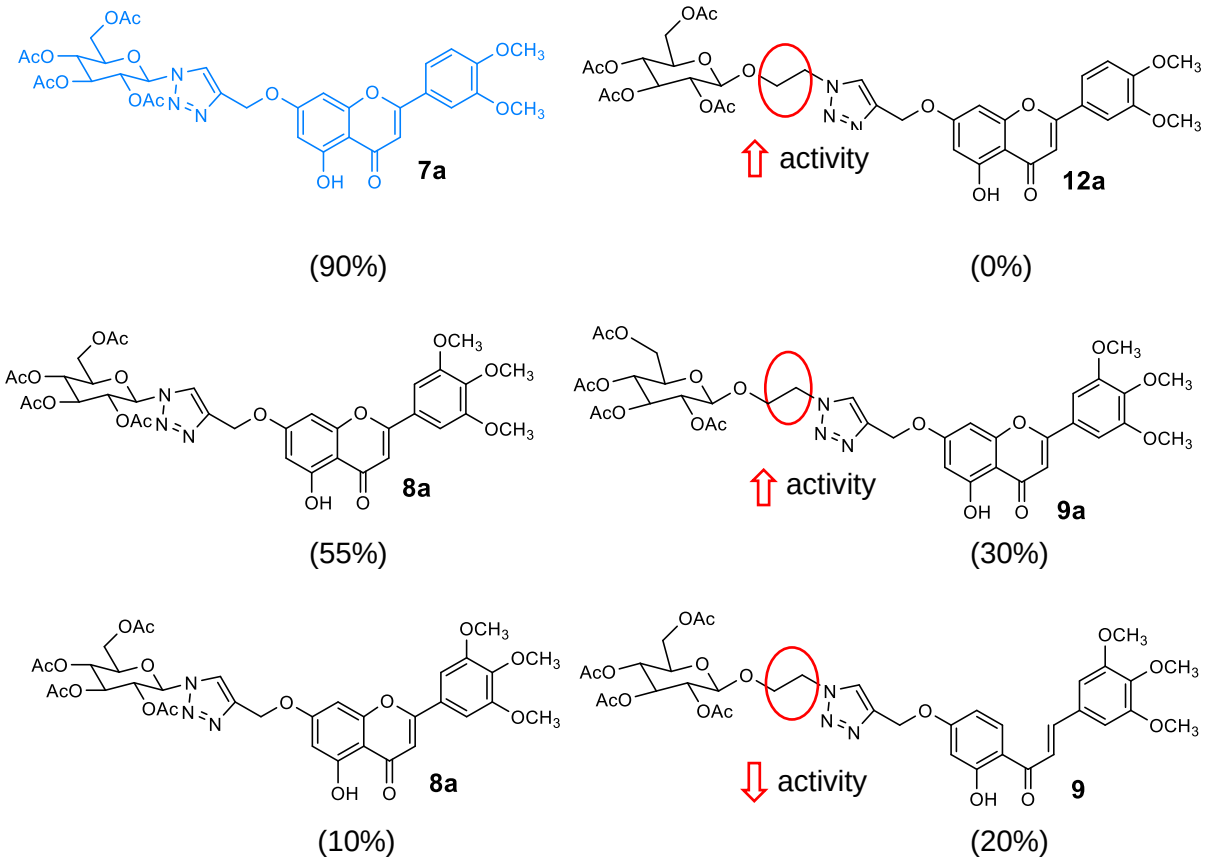


Figure 16. The influence of an ethoxyl chain in compounds **9**, **9a** and **12a**.

The introduction of a triazole linkage in compound **7a** did not improve the antifouling activity when compared to compound **13a** (Figure 17).

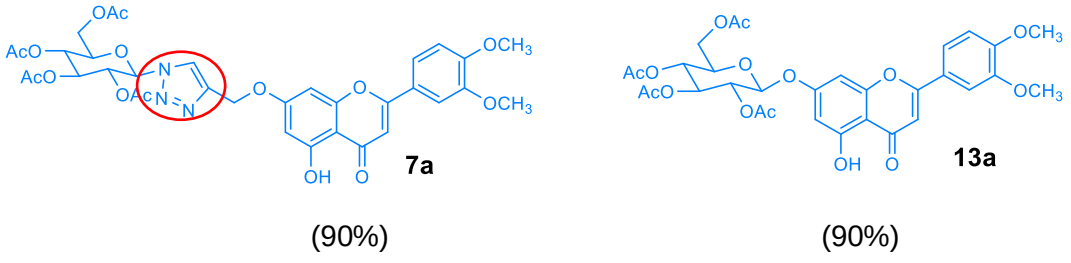


Figure 17. The influence of triazole in compound **7a**.

Concerning to B ring, the presence of three methoxy groups is associated with an increase in antifouling activity as can be seen by comparing the antifouling activity of compound **3** with compound **4**, and compound **7** with compound **8** (Figure 18).

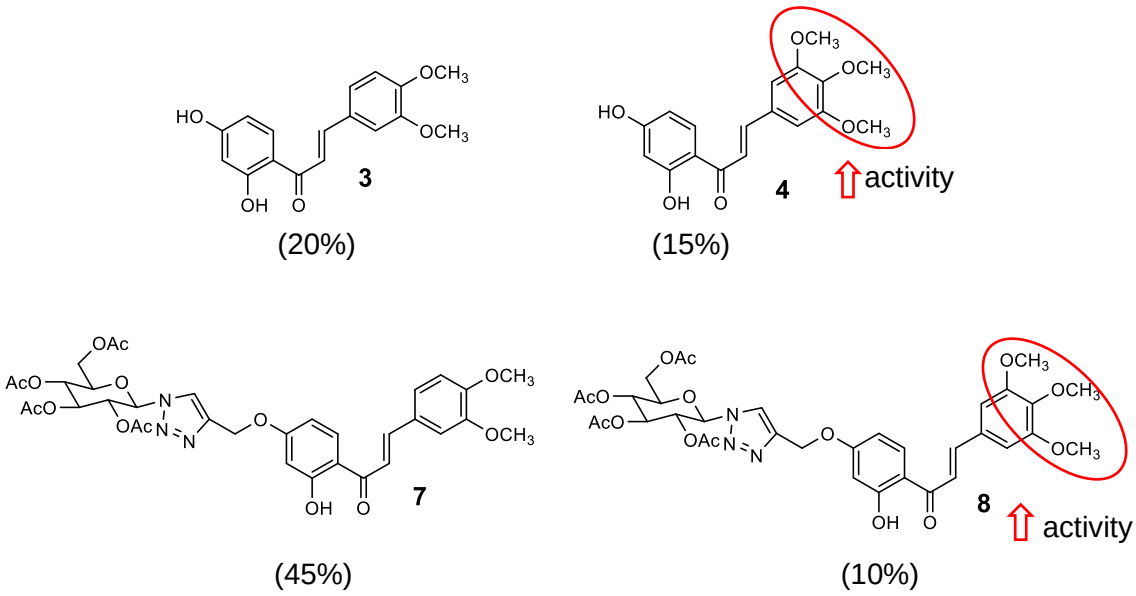


Figure 18. The importance of three methoxy groups in compounds **4** and **8**.

From this series of flavonoids derivatives tested, six chalcones (compounds **3**, **4**, **5**, **6**, **8**, **9**) and three flavones (compounds **4a**,**9a**, and **12a**) were selected for dose-response studies (Appendix I) to determine the EC_{50} values.

Compound **8** showed to be the most effective one, followed by compound **4a**, compound **4** and compound **3** (Table 6). Compounds **5, 6, 9, 9a**, and **12a** presented higher values than those recommended by the US Navy guidelines (Table 6).

Table 6. Antifouling effectiveness of chalcones **3**-**6**,**8**,**9** and flavones **4a**, **9a** and **12a** towards mussel plantigrades larvae.

Compound	**EC_{50} (μM)**	**EC_{50} (μg mL^{-1})**
8	3.28 (95% CI: 1.97 - 4.74)	2.43
4a	8.34 (95% CI: 4.22 – 13.36)	2.87
4	9.64 (95% CI: 3.85 – 17.22)	3.18
3	18.10 (95% CI: 13.95 – 23.44)	5.44
9a	48.22 (95% CI: 30.57 – 85.40)	38.56

Compound	EC_{50} (μM)	EC_{50} (μg mL^{-1})
9	53.90 (95% CI: 29.98 – 126.88)	42.35
12a	84.48 (95% CI: 51.28 – 184.54)	65.02
5	84.52 (95% CI: 45.07 – 267.02)	28.60
6	85.56 (95% CI: 44.84 – 291.41)	31.52

EC_{50}: minimum concentration that inhibited 50% of larval settlement; CI: confidence interval. EC_{50} are recommend to be less than 25μg mL^{-1}.[56]

Even thought, it should be noted that flavone **4a** showed better results than the structurally related chalcone **4**, it is possible to observe that the most potent compounds (EC_{50} values ≤ 25μg mL^{-1}), were mainly chalcones.

4.1.2. Phenolic compounds

Figure 19 shows the results of the initial screening of 19 phenolic derivatives (Figure 12) at 50μM.

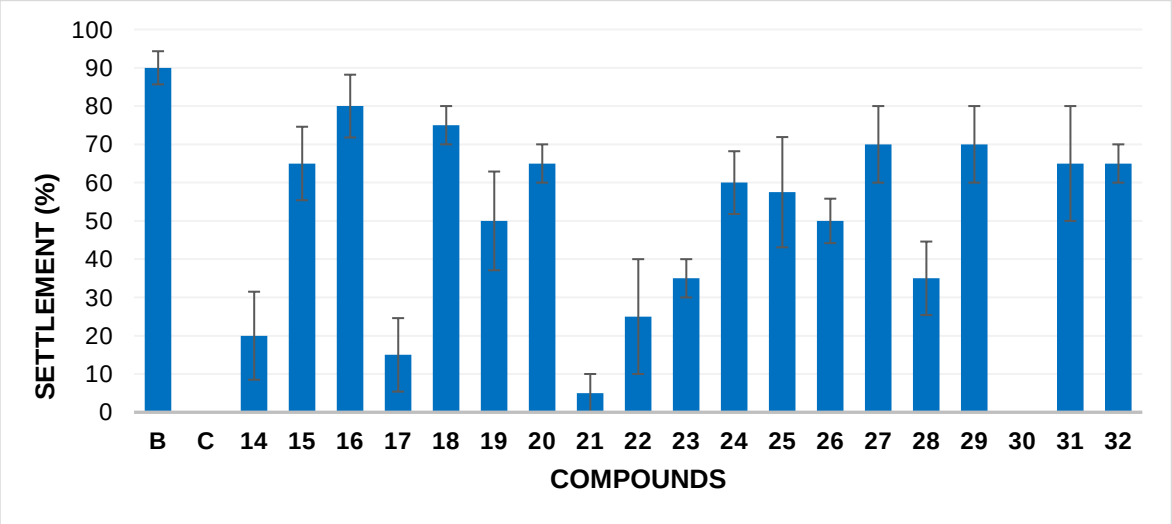


Figure 19. Percentage of settlement of *Mytilus galloprovincialis* larvae in the presence of phenolic compounds at 50μM. B: DMSO control (0.01%); C: 5μM $CuSO_4$ as positive control.

According to these results, seven phenolic derivatives (compounds **14**, **17**, **21**, **22**, **23**, **28** and **30** – Figure 12) presented very significant anti-settlement responses at 50µM (percentage of settlement ≤ 40%) against mussel *M. galloprovincialis* larvae when compared to the negative control (Figure 19).

Substitutions made on compound **14** showed that the introduction of a propargyl group (compound **19**) and both acetylated and deacetylated O-glycosyl portions (compounds **15** and **16**) decreased the ability to inhibit the settlement of larvae. In contrast, the introduction of sulfate group (compound **17**) seems to increase this inhibition (Figure 20).

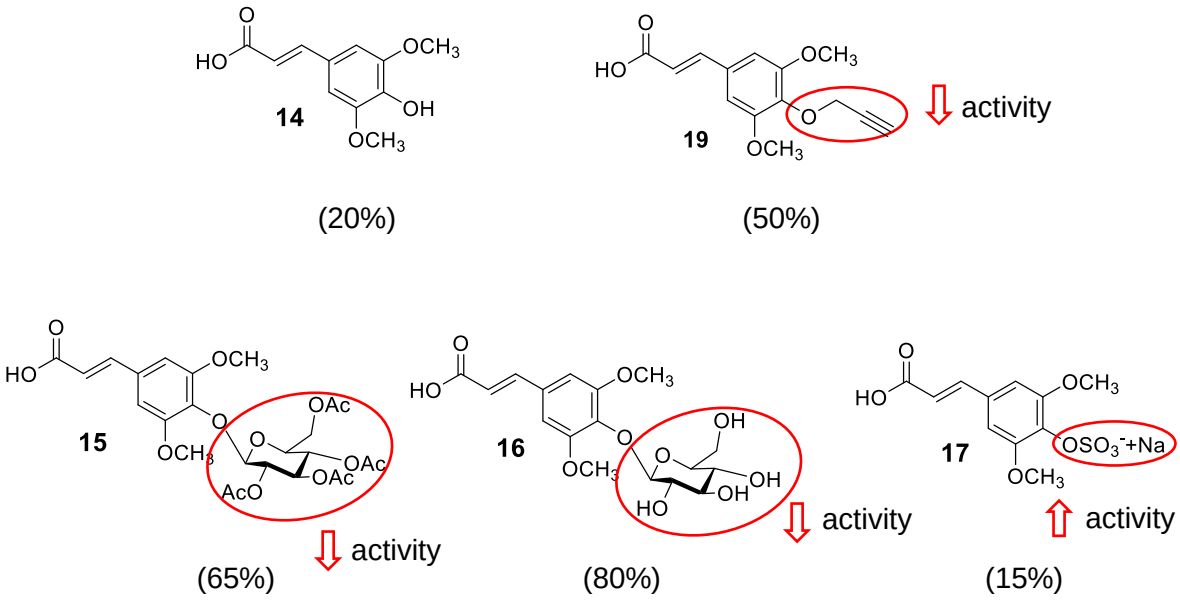


Figure 20. The influence of *para*-substitutions in compound **14**.

The same substitutions made on the compound **22** were also not favorable for the antifouling activity, when compared to compounds **23** and **24** (Figure 21).

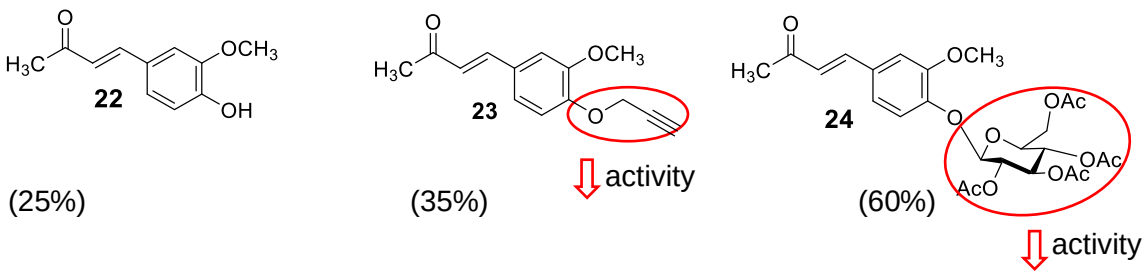


Figure 21. The influence of *para*-substitutions in compound **22**.

The introduction of the chlorine in compound **21** seems to be associated with a more favorable anti-settlement activity, when compared to compound **20** (Figure 22).

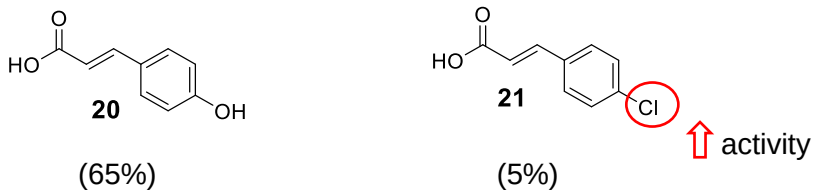


Figure 22. The influence of a *para*-substitution in compound **20**.

When carboxylic acid was replaced by an ester (compound **18**), there was also a decreased in inhibition of settlement of mussels larvae (Figure 23).

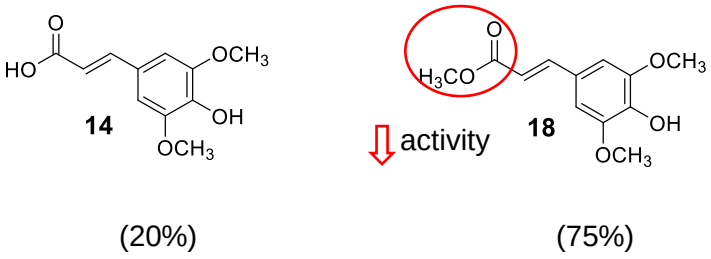


Figure 23. The influence of carboxy modifications in compound **14**.

When comparing compound **14** with compound **25**, the presence of double conjugation seems to positively influence the activity. The presence of an aldehyde (compound **31**) instead a carboxylic acid (compound **25**) was not favorable (Figure 24).

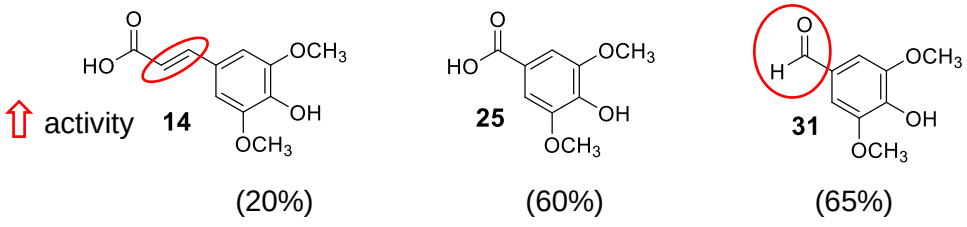


Figure 24. The influence of double conjugation on the antifouling activity.

From this series of phenolic derivatives, four compounds (**14**, **17**, **21** and **30**) were selected for dose-response studies (Appendix II) in order to determine the minimum concentration that inhibited 50% of larval settlement (EC_{50}) values (Table 7).

Compound **30** proved to be the most active one followed by compounds **21**, **17** and **14** (Table 7) (Appendix II).

Table 7. Antifouling effectiveness of compounds **30**, **21**, **17** and **14** towards mussel plantigrades larvae.

Compound	EC_{50} (μM)	EC_{50} (μg mL^{-1})
30	2.74 (95% CI: 2.04 - 3.60)	0.80
21	9.36 (95% CI: 6.29 - 12.87)	1.71
17	29.34 (95% CI: 20.27 – 43.08)	9.57
14	60.24 (95% CI: 30.81-191.15)	13.51

EC_{50}: minimum concentration that inhibited 50% of larval settlement; CI: confidence interval. EC_{50} are recommend to be less than 25μg mL^{-1}.[56]

According to these results, all compounds have EC_{50} values below 25μg mL^{-1}. Furthermore, these results confirm that compounds structurally related to ZA (Figure 8) and to AGS (Figure 10) are promising hit compounds for the development of novel antifouling agents.

4.1.3. Acetophenones

Figure 25 shows the results obtained in the initial screening of acetophenones at 50μM.

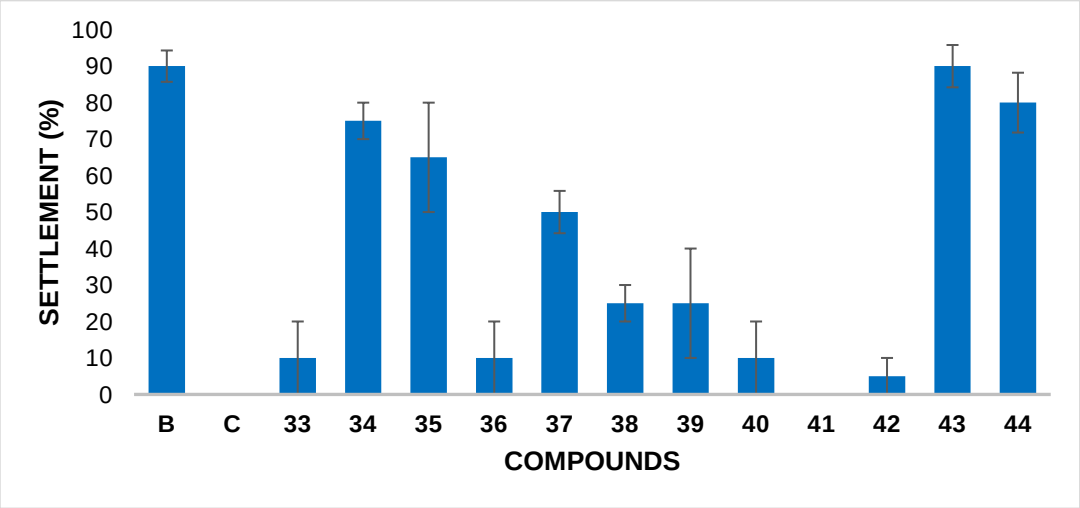


Figure 25. Percentage of settlement of *Mytilus galloprovincialis* larvae in presence of a series of acetophenones at 50μM. B: DMSO control (0.01%); C: 5μM $CuSO_4$ as positive control.

Seven compounds (**33**, **36**, **38**, **39**, **40**, **41** and **42**) presented significant anti-settlement responses (percentage of settlement ≤ 40%) against mussel *M. galloprovincialis* larvae when compared to the negative control (Figure 25).

Substitutions made on compound **33** with the introduction of a propargyl group (compound **37**) and both acetylated and deacetylated O-glycosyl portions (compounds **34** and **35**) decreased the ability to inhibit the settlement of larvae (Figure 26). However, when an acetoglucosamine was linked by a triazole (compound **36**), the activity did not decrease.

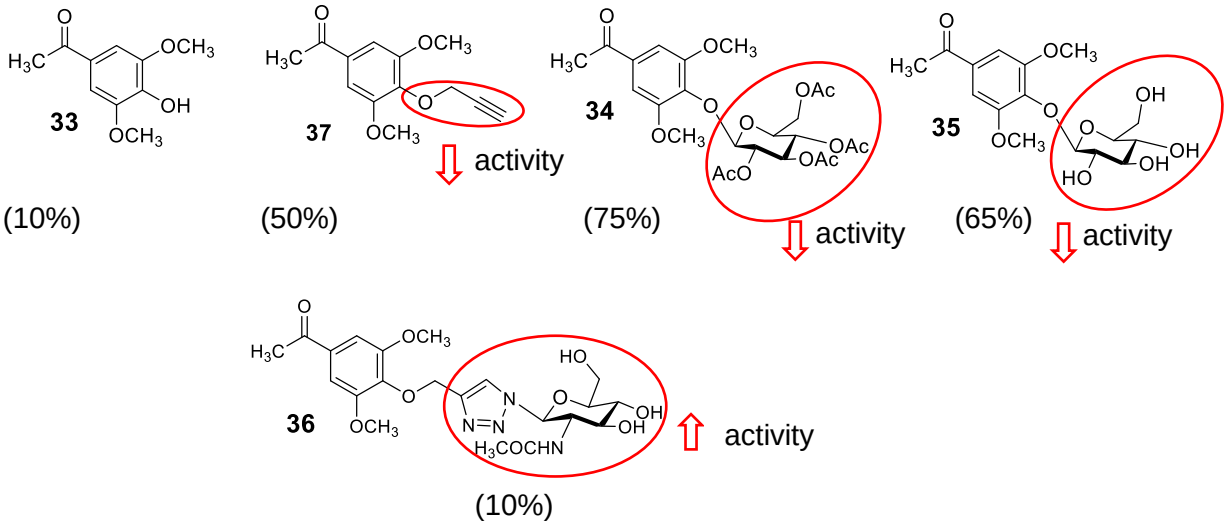


Figure 26. The influence of *para*-substitutions in compound **33**.

The substitution of the hydroxyl group in compound **38** by a methoxy group (compound **40)** and an acethyl group (compound **41**) seems to be favorable for the antifouling activity (Figure 27).

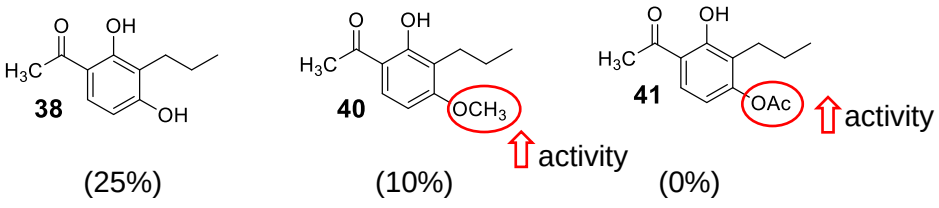


Figure 27. The influence of substitutions in compound **38**.

When comparing compound **39** with compound **43** and compound **44**, it seems that the introduction of a methoxymethyl and a propargyl group decreased the inhibition of settlement (Figure 28).

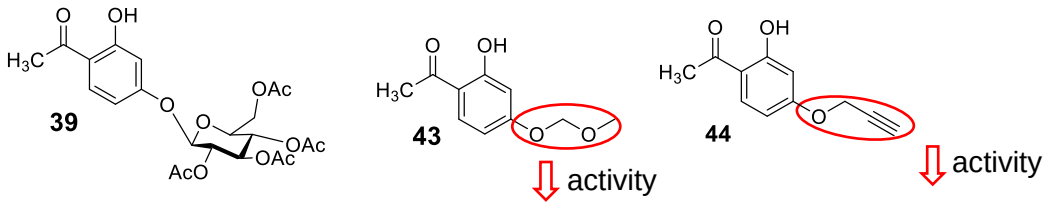


Figure 28. The influence of modifications in compound **39**.

Five compounds (**33**, **36**, **40**, **41** and **42**) were selected for dose-response studies (Appendix III) in order to determine the minimum concentration that inhibited 50% of larval settlement (EC_{50}) values (Table 8).

Compound **40** proved to be the most effective, followed by compounds **33**, **42**, **41** and **36** (Table 8) (Appendix III).

Table 8. Antifouling effectiveness of compounds **33**, **36**, **40**, **41** and **42** towards mussel plantigrades.

Compound	**EC_{50} (µM)**	**EC_{50} (µg ml^{-1})**
40	5.03 (95% CI: 2.86 – 7.19)	1.05
33	13.94 (95% CI: 7.59 – 22.82)	2.74
42	16.37 (95% CI: 10.15 – 26.04)	4.10
41	19.43 (95% CI: 9.97 – 35.97)	4.59
36	20.68 (95% CI: 9.70 – 40.75)	9.94

EC_{50}: minimum concentration that inhibited 50% of larval settlement; CI: confidence interval. EC_{50} are recommend to be less than 25µg mL^{-1}.[56]

All compounds show EC_{50} values below 25µg mL^{-1}. These results highlight that the scaffold of acetophenones can also be considered a good hit compound in the development of new effective antifouling compounds.

4.2. *In vitro* inhibition of Acetylcholinesterase (AChE) activity

The identification of the mechanism of action associated with antifouling activity remains a challenge for the scientific community. According to Qian et al. (2013) some antifoulants appear to affect settlement through distinct patterns, which can be classified roughly into several categories such as inhibitors of ion channel function, inhibitors of quorum sensing, blockers of neurotransmission or inhibitors of adhesive production or release.[57] Moreover, some specific target molecules in fouling organisms have been determined, such as acetylcholinesterase (AChE) which seems to be involved in cholinergic neural signaling during the settlement.[58] It is known that the commercial booster biocide Sea-Nine 211 acts by this mechanism[59-60], as well as two natural compounds isolated from marine organisms: Territrem and Pulmonarin.[61-62]

For this reason, the ability to inhibit the acetylcholinesterase enzyme (AChE) was evaluated for compounds with a percentage of settlement ≤ 20%. Besides that, acetylcholinesterase inhibitors are also widely used in Alzheimer's disease and myasthenia gravis, which can be explored in the future.

4.2.1. Chalcones and flavones

Inhibition of the acetylcholinesterase activity was not detected for any of these compounds, indicating that the antifouling activity of these compounds could not be related to this target (Figure 29).

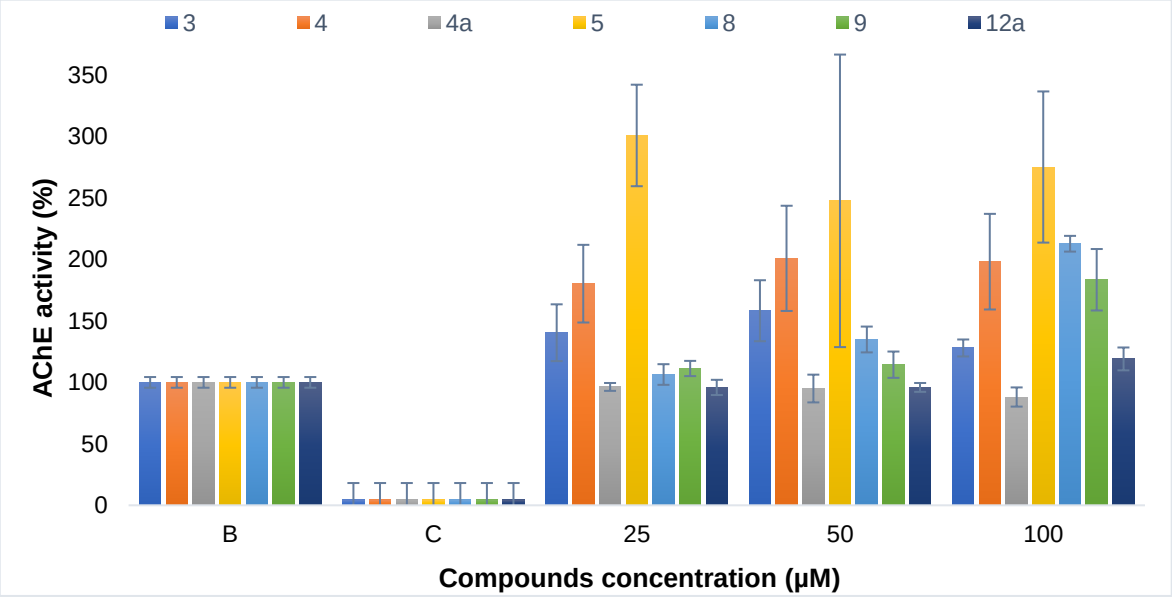


Figure 29. Acetylcholinesterase (AChE) activity of the most promising chalcones (**3**-**5**,**8** and **9**) and flavones (**4a** and **12a**) compounds. B: 1% DMSO. C: Eserine (200µM).

4.2.2. Phenolic compounds

Results showed that acetylcholinesterase activity was inhibited by compound **30** reaching 35% of inhibition at 100µM (Figure 30). However, the EC_{50} value of this compound is much less than 100µM, so, we cannot say that this is the mechanism of action that regulates the anti-settlement activity of the compound **30**.

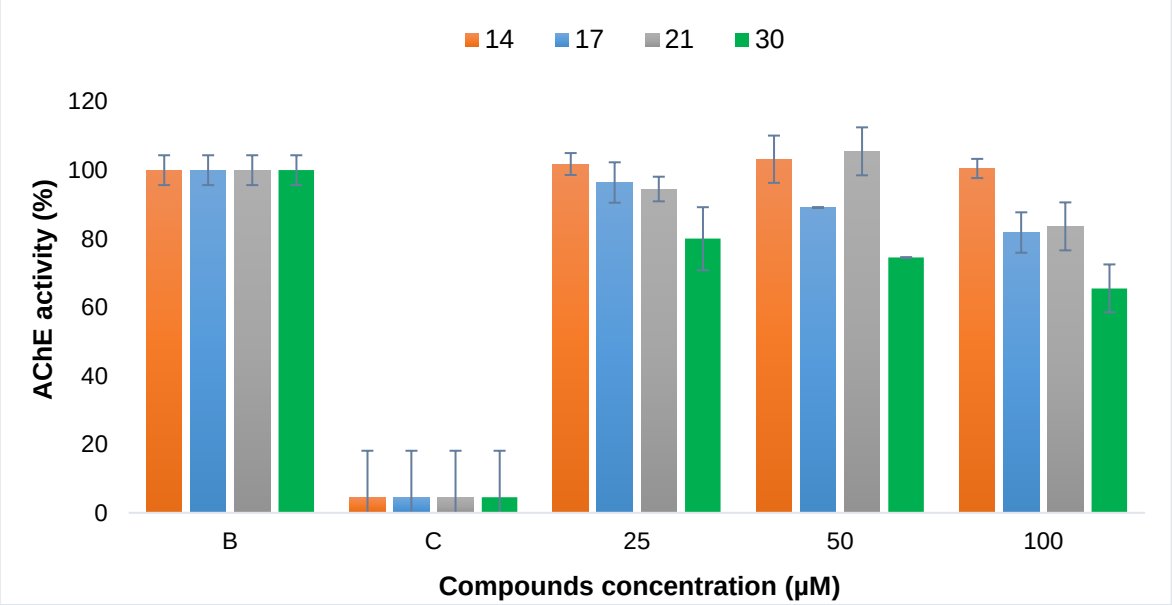


Figure 30. Acetylcholinesterase (AChE) activity of the most promising phenolic compounds. B: 1% DMSO. C: Eserine (200µM).

4.2.3. Acetophenones

Inhibition of the acetylcholinesterase activity was not detected for any of these compounds, indicating that the antifouling activity detected in these compounds is not related to this mechanism of action (Figure 31).

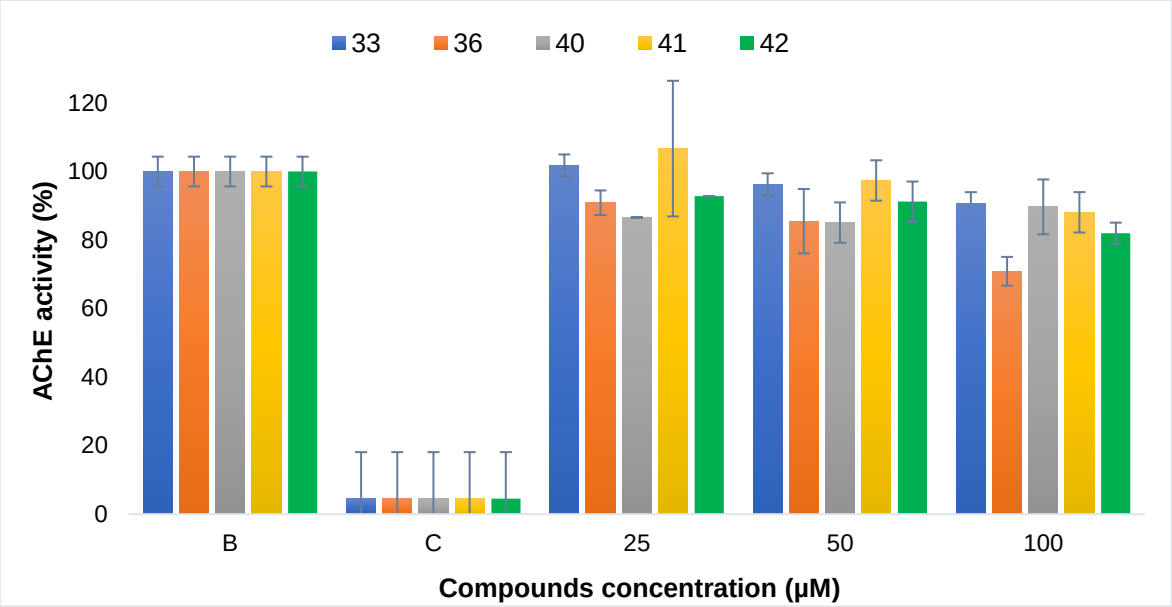


Figure 31. Acetylcholinesterase (AChE) activity of acetophenones compounds. B: 1% DMSO. C: Eserine (200µM).

4.3. Inhibition of biofilm-forming marine bacteria growth

Marine biofilms consist of aggregates of bacteria and diatoms inserted into a matrix of extracellular polymers, that secrete chemical cues to attract the successive fouling species, namely invertebrate larvae and macro-algal spores.[63-64] Consequently, for controlling biofouling it is very important to act also against the growth of marine bacteria. Therefore, the ability of the two most potent compounds ($EC_{50} \leq 2\mu g\ mL^{-1}$) to inhibit the growth of five species of marine bacteria were evaluated.

The screening at 15μM showed that compound **30** inhibited 43% of the bacterial growth of *Cobetia marina,* and compound **21** inhibited 48% of *Cobetia marina*, 52% of the bacterial growth of *Vibrio harveyi*, and 28% of *Roseobacter litoralis* (Figure 32). The compound **21** seemed to be more effective towards bacterial biofilms given the broad-spectrum effect against different bacterial strains.

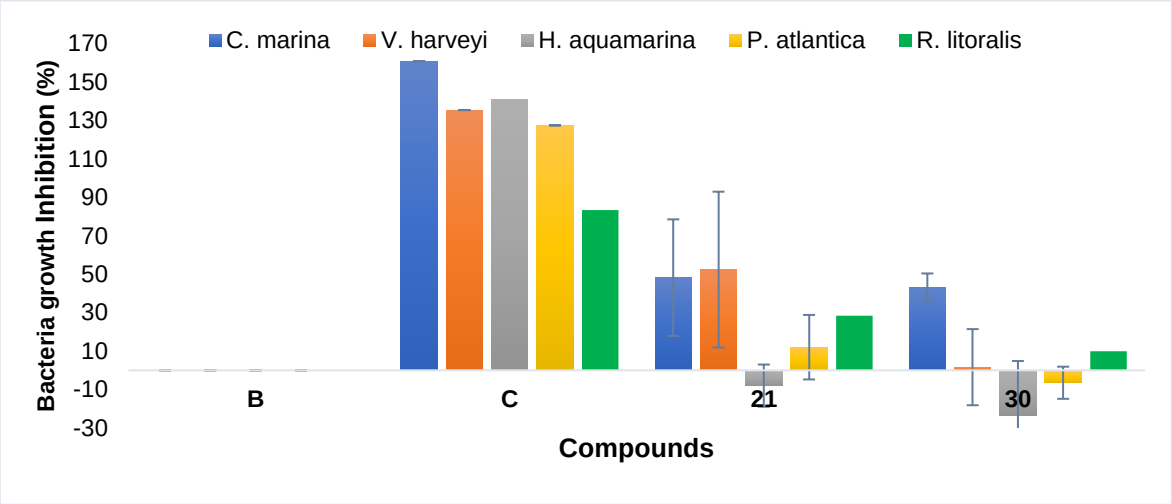


Figure 32. Antibacterial activity of compounds **21** and **30** at 15μM towards five biofilm-forming marine bacteria *Cobetia marina*, *Vibrio harveyi*, *Pseudoalteromonas atlanti20*, *Halomonas aquamarina* and *Roseobacter litoralis*. B: 0.1% DMSO. C: 1:100 penicilin-streptomycin-neomycin stabilized solution.

4.4. Environmental fate parameters

4.4.1. *Artemia salina* ecotoxicity bioassay

Ecotoxicity assays carried out on non-target organisms aim to understand how tested compounds can alter the health status of non-target organisms or even cause their death.[65] *Artemia salina* are small crustaceans that live in salty marine environments and are used as test organisms because of their easy culture, short generation time, cosmopolitan distribution and commercial availability of their eggs in latent form.[6] In this bioassay all compounds with $EC_{50} \leq 25\mu g\ mL^{-1}$ were tested.

4.4.1.1. Chalcones and flavones

Results showed that compounds **3**, **4**, **4a** and **8** are non-toxic to *Artemia salina* at both concentrations tested (25 and 50µM). The commercial AF agent ECONEA® showed 100% lethality at the same concentrations (Figure 33).

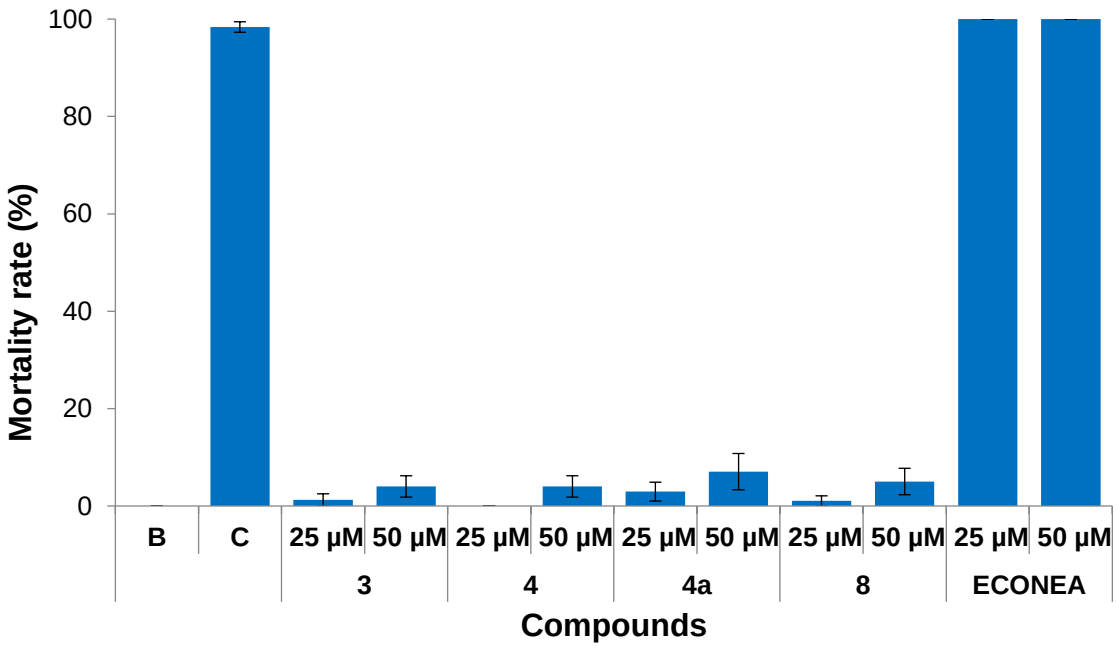


Figure 33. Mortality rate of *Artemia salina* nauplii after 48h of exposure to compounds **3**, **4**, **4a** and **8**. B: 1% DMSO in filtered seawater. C: $K_2Cr_2O_7$ at 13.6µM. ECONEA® was used for comparative purposes.

4.4.1.2. Phenolic compounds

In contrast to the commercial AF agent ECONEA® which showed 100% lethality at both concentrations tested, compounds **21** and **30** were not toxic to *Artemia salina* even at 50µM (Figure 34).

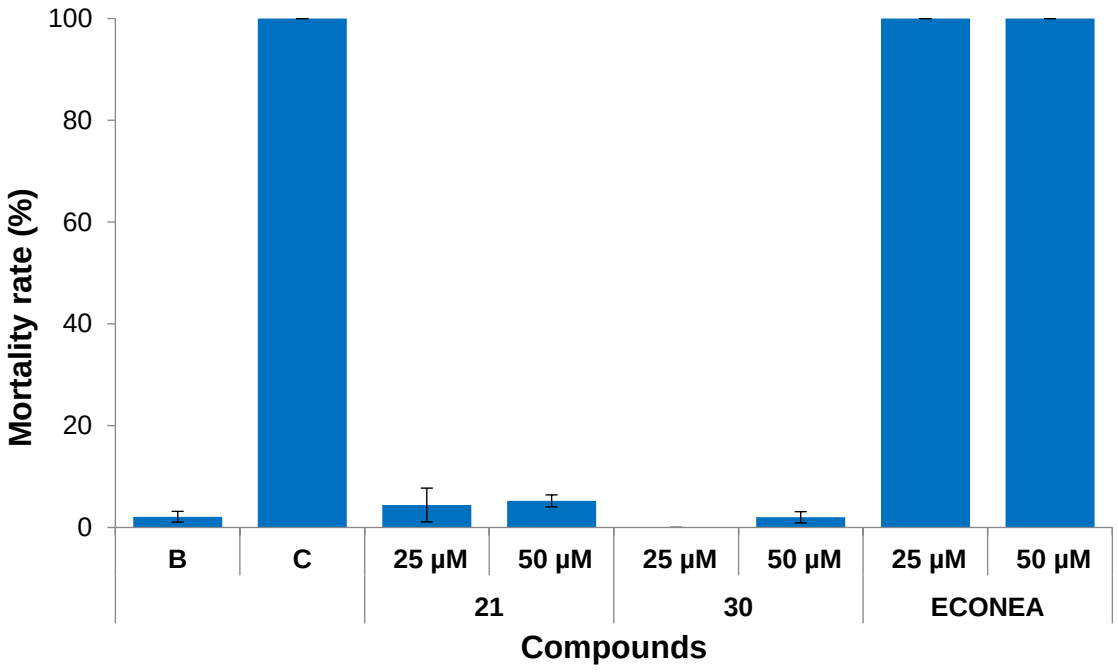


Figure 34. Mortality rate of *Artemia salina* nauplii after 48h of exposure to compounds **21** and **30**. B: 1% DMSO in filtered seawater. C: $K_2Cr_2O_7$ at 13.6µM. ECONEA® was used for comparative purposes.

4.4.1.3. Acetophenones

Compounds **33** and **36** were also not toxic to *Artemia salina* at 25 and 50µM (Figure 35). However, compounds **40**, **41** and **42** showed considerable levels of toxicity even at 25µM.

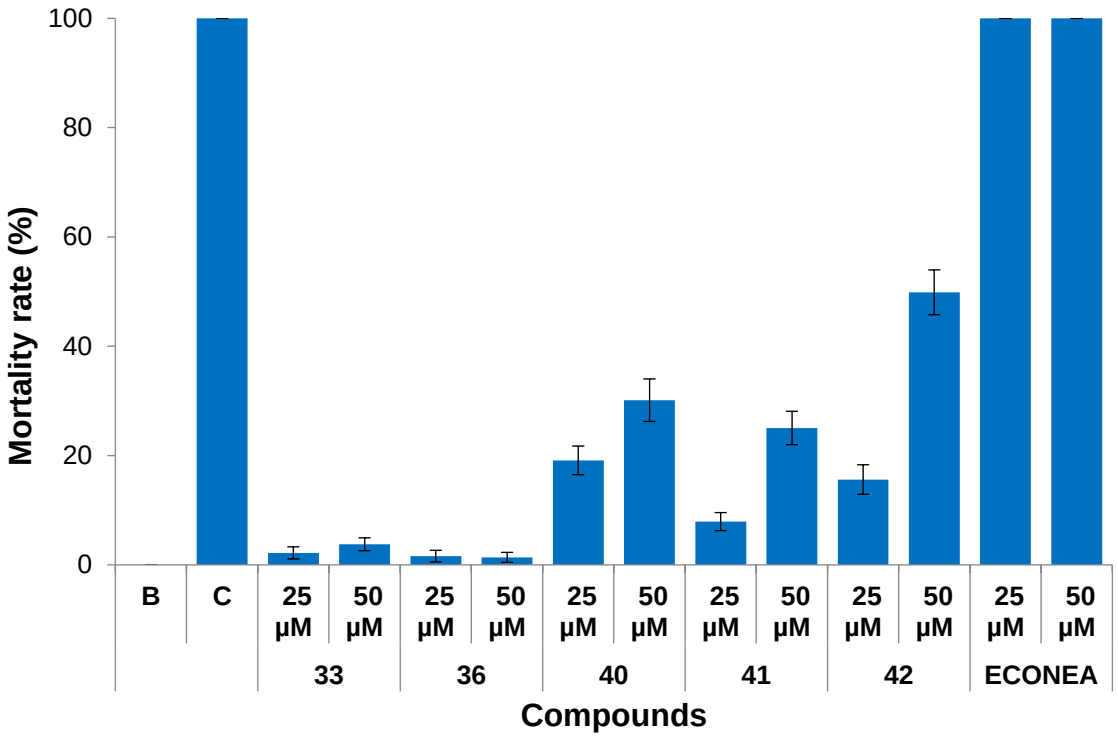


Figure 35. Mortality rate of *Artemia salina* nauplii after 48h of exposure to the compounds **33**, **36**, **40**, **41** and **42**. B: 1% DMSO in filtered seawater. C: $K_2Cr_2O_7$ at 13.6µM. ECONEA® was used for comparative purposes.

4.4.2. *In silico* studies

An important aspect to take into consideration in the development of new antifouling compounds is the determination of the bioaccumulation potential.

By using the octanol-water partition coefficient (P), which expresses the relationship between the solubility of a compound in octanol (non-polar solvent) and the solubility in water (polar solvent), we can predict the probability of the compound being bioaccumulated.[66] If a compound is lipophilic it tends to be stored in the adipose tissue of the fish and consequently be biomagnified to higher trophic levels, including its human consumers.[47] It is considered that a compound is potentially bioaccumulative if it has a Log KOW value greater than 3.[46]

In this work, the determinations of Log KOW values were made using the KOWWIN™ (estimates the log octanol-water partition coefficient) in the EPI Suit ™, a software developed by Syracuse Research Cooperation (SRC) jointly with Environmental Protection Agency (EPA) which contains a based suite of physical/chemical property and environmental fate estimation programs.[67] All compounds with $EC_{50} \leq 25\mu g\ mL^{-1}$ were evaluated.

4.4.2.1. Chalcones and flavones

Calculated values showed that compounds **4** and **4a** can be selected as promising non-bioaccumulate AF agents (Table 9).

Table 9. Log KOW values obtained for chalcones (**3**, **4** and **8**) and flavones (**4a**).

Compound	Log KOW
3	3.12
4	2.99
4a	2.84
8	3.55

4.4.2.2. Phenolic compounds

All phenolic compounds tested have low potential for bioaccumulation since Log KOW values were less than 3 (Table 10).

Table 10. Log KOW values obtained for phenolic compounds.

Compound	Log KOW
14	1.24
17	-1.83
21	2.72
30	-0.88

4.4.2.3. Acetophenones

Calculated values showed that acetophenones **33**, **36** and **42** had Log KOW less than 3, indicating their low bioaccumulation potential (Table 11).

Table 11. Log KOW values obtained for acetophenones compounds.

Compound	Log KOW
33	0.84
36	0.57
40	3.58
41	3.10
42	2.88

5. Conclusion

Chemical synthesis has been an increasingly used alternative to mimic products obtained from natural sources or even to produce new compounds. Through this process we can perform structure-activity relationship studies and thus optimize the activity.

All compounds tested in this work were obtained using a relatively simple synthesis procedure, having potential to be produced at large scale.

With this work it can be concluded that:

- ✓ three chalcones (**3**, **4** and **8**) one flavone (**4a**), four phenolic compounds (**14**, **17**, **21** and **30**) and five acetophenones (**33**, **36**, **40**, **41** and **42**) showed high anti-settlement potency (EC_{50} < 25µg mL^{-1}) against larvae of *Mytilus galloprovincialis*; only compound **30** inhibited the activity of the acetylcholinesterase enzyme at 100µM;
- ✓ two phenolic compounds (**21** and **30**) were also active against biofilm forming bacteria showing inhibitory activity in growth at 15µM;
- ✓ the ecotoxicity to non-target species *Artemia salina* was evaluated for compounds **3**, **4**, **4a**, **8**, **21**, **30**, **33**, **36**, **40**, **41** and **42** and only three compounds (**40**, **41** and **42**) presented toxicity to this species (>10 % lethality at 25µM);
- ✓ only compounds **3**, **8**, **40** and **41** presented log KOW values upper than 3, showing potential for bioaccumulation.

Overall, six compounds can be selected as the most promising ones (Figure 36): 1 chalcone, 1 flavone, 2 phenolic derivatives and 2 acetophenones. It is possible to observe that all contain polar groups (hydroxyl groups/carboxy groups) and lipophilic portions (methoxyl, chloride, aliphatic chains, triazole).

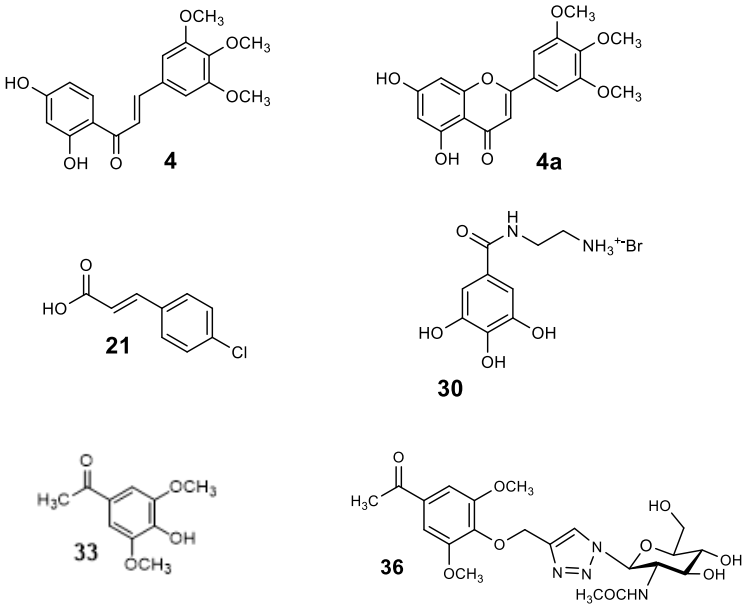


Figure 36. Compounds with the best results in this work.

In the future, it is important to explore the mode of action of the most active compounds as well as to perform incorporation studies in paints.

6. References

1. Amara, I.; Miled, W.; Slama, R. B.; Ladhari, N., Antifouling processes and toxicity effects of antifouling paints on marine environment. A review. *Environmental toxicology and pharmacology* **2018,** *57*, 115-130.
2. Almeida, J. R.; Moreira, J.; Pereira, D.; Pereira, S.; Antunes, J.; Palmeira, A.; Vasconcelos, V.; Pinto, M.; Correia-da-Silva, M.; Cidade, H., Potential of synthetic chalcone derivatives to prevent marine biofouling. *The Science of the total environment* **2018,** *643*, 98-106.
3. Xie, Q.; Pan, J.; Ma, C.; Zhang, G., Dynamic surface antifouling: mechanism and systems. *Soft Matter* **2019,** *15* (6), 1087-1107.
4. Dafforn, K. A.; Lewis, J. A.; Johnston, E. L., Antifouling strategies: History and regulation, ecological impacts and mitigation. *Marine pollution bulletin* **2011,** *62* (3), 453-465.
5. Cui, Y. T.; Teo, S. L.; Leong, W.; Chai, C. L., Searching for "environmentally-benign" antifouling biocides. *International journal of molecular sciences* **2014,** *15* (6), 9255-84.
6. Koutsaftis, A.; Aoyama, I., Toxicity of four antifouling biocides and their mixtures on the brine shrimp Artemia salina. *The Science of the total environment* **2007,** *387* (1-3), 166-74.
7. Guardiola, F. A.; Cuesta, A.; Meseguer, J.; Esteban, M. A., Risks of Using Antifouling Biocides in Aquaculture. *International journal of molecular sciences* **2012,** *13* (2), 1541.
8. Arrhenius, A.; Backhaus, T.; Hilvarsson, A.; Wendt, I.; Zgrundo, A.; Blanck, H., A novel bioassay for evaluating the efficacy of biocides to inhibit settling and early establishment of marine biofilms. *Marine pollution bulletin* **2014,** *87* (1-2), 292-299.
9. Okamura, H.; Aoyama, I.; Liu, D.; Maguire, R. J.; Pacepavicius, G. J.; Lau, Y. L., Fate and ecotoxicity of the new antifouling compound Irgarol 1051 in the aquatic environment. *Water Research* **2000,** *34* (14), 3523-3530.
10. Castro, Í. B., TINTAS ANTI-INCRUSTANTES DE TERCEIRA GERAÇÃO: NOVOS BIOCIDAS NO AMBIENTE AQUÁTICO. *Quimica Nova* **2011,** *34* (6), 1021-1031.
11. Executive, H. a. S. *CLH report for medetomidine: proposal for harmonised classification and labelling.*; Chemicals Regulation Directorate: UK, 2014; p 64.
12. Thomas, K. V.; Brooks, S., The environmental fate and effects of antifouling paint biocides. *Biofouling* **2010,** *26* (1), 73-88.
13. Escobar, A.; Pérez, M.; Sathicq, Á.; García, M.; Paola, A.; Romanelli, G.; Blustein, G., Alkyl 2-furoates obtained by green chemistry procedures as suitable new antifoulants for marine protective coatings. *Journal of Coatings Technology and Research* **2019,** *16* (1), 159-166.
14. Raveendran, T. V.; Mol, V. P. L., Natural product antifoulants. *Current Science* **2009,** *97* (4), 508-520.
15. Wang, K. L.; Wu, Z. H.; Wang, Y.; Wang, C. Y.; Xu, Y., Mini-Review: Antifouling Natural Products from Marine Microorganisms and Their Synthetic Analogs. *Marine drugs* **2017,** *15* (9).
16. Moodie, L. W. K.; Trepos, R.; Cervin, G.; Brathen, K. A.; Lindgard, B.; Reiersen, R.; Cahill, P.; Pavia, H.; Hellio, C.; Svenson, J., Prevention of Marine Biofouling Using the Natural Allelopathic Compound Batatasin-III and Synthetic Analogues. *Journal of natural products* **2017,** *80* (7), 2001-2011.
17. Sjögren, M.; Johnson, A.-L.; Hedner, E.; Dahlström, M.; Göransson, U.; Shirani, H.; Bergman, J.; Jonsson, P. R.; Bohlin, L., Antifouling activity of synthesized peptide analogs of the sponge metabolite barettin. *Peptides* **2006,** *27* (9), 2058-2064.
18. Trepos, R.; Cervin, G.; Hellio, C.; Pavia, H.; Stensen, W.; Stensvag, K.; Svendsen, J. S.; Haug, T.; Svenson, J., Antifouling compounds from the sub-arctic ascidian

Synoicum pulmonaria: synoxazolidinones A and C, pulmonarins A and B, and synthetic analogues. *Journal of natural products* **2014,** *77* (9), 2105-13.
19. Niemann, H.; Hagenow, J.; Chung, M. Y.; Hellio, C.; Weber, H.; Proksch, P., SAR of Sponge-Inspired Hemibastadin Congeners Inhibiting Blue Mussel PhenolOxidase. *Marine drugs* **2015,** *13* (5), 3061-71.
20. Umezawa, T.; Hasegawa, Y.; Novita, I. S.; Suzuki, J.; Morozumi, T.; Nogata, Y.; Yoshimura, E.; Matsuda, F., Design, Synthesis, and Antifouling Activity of Glucosamine-Based Isocyanides. *Marine drugs* **2017,** *15* (7).
21. Moodie, L. W.; Trepos, R.; Cervin, G.; Larsen, L.; Larsen, D. S.; Pavia, H.; Hellio, C.; Cahill, P.; Svenson, J., Probing the Structure-Activity Relationship of the Natural Antifouling Agent Polygodial against both Micro- and Macrofoulers by Semisynthetic Modification. *Journal of natural products* **2017,** *80* (2), 515-525.
22. Singh, P.; Anand, A.; Kumar, V., Recent developments in biological activities of chalcones: a mini review. *European journal of medicinal chemistry* **2014,** *85*, 758-77.
23. Rani, A.; Anand, A.; Kumar, K.; Kumar, V., Recent developments in biological aspects of chalcones: the odyssey continues. *Expert opinion on drug discovery* **2019,** *14* (3), 249-288.
24. Thirunarayanan, G.; Surya, S.; Srinivasan, S.; Vanangamudi, G.; Sathiyendiran, V., Synthesis and insect antifeedant activities of some substituted styryl 3,4-dichlorophenyl ketones. *Spectrochimica acta. Part A, Molecular and biomolecular spectroscopy* **2010,** *75* (1), 152-6.
25. Subramanian, M.; Vanangamudi, G.; Thirunarayanan, G., Hydroxyapatite catalyzed aldol condensation: synthesis, spectral linearity, antimicrobial and insect antifeedant activities of some 2,5-dimethyl-3-furyl chalcones. *Spectrochimica acta. Part A, Molecular and biomolecular spectroscopy* **2013,** *110*, 116-23.
26. Sivakumar, P. M.; Prabhawathi, V.; Doble, M., Antibacterial activity and QSAR of chalcones against biofilm-producing bacteria isolated from marine waters. *SAR and QSAR in environmental research* **2010,** *21* (3-4), 247-63.
27. Sivakumar, P. M.; Prabhawathi, V.; Doble, M., 2-Methoxy-2',4'-dichloro chalcone as an antimicrofoulant against marine bacterial biofilm. *Colloids and surfaces. B, Biointerfaces* **2010,** *81* (2), 439-46.
28. Sathicq, Á.; Paola, A.; Pérez, M.; Dallesandro, O.; García, M.; Roldán, J. P.; Romanelli, G.; Blustein, G., Furylchalcones as new potential marine antifoulants. *International Biodeterioration & Biodegradation* **2019,** *143*, 104730.
29. Krylova, N. V.; Popov, A. M.; Leonova, G. N.; Artiukov, A. A.; Maistrovskaia, O. S., [Comparative study of antiviral activity of luteolin and 7,3'-disulfate luteolin]. *Antibiotiki i khimioterapiia = Antibiotics and chemoterapy [sic]* **2011,** *56* (11-12), 7-10.
30. Behery, F. A.; Naeem, Z. E.; Maatooq, G. T.; Amer, M. M.; Ahmed, A. F., A novel antioxidant phenanthrenoid dimer from Juncus acutus L. *Natural product research* **2013,** *27* (2), 155-63.
31. Hou, X. M.; Wang, C. Y.; Gu, Y. C.; Shao, C. L., Penimethavone A, a flavone from a gorgonian-derived fungus Penicillium chrysogenum. *Natural product research* **2016,** *30* (20), 2274-7.
32. Qi, S.-H.; Zhang, S.; Qian, P.-Y.; Wang, B.-G., Antifeedant, antibacterial, and antilarval compounds from the South China Sea seagrass Enhalus acoroides. In *Botanica Marina*, 2008; Vol. 51, p 441.
33. Guan, C.; Parrot, D.; Wiese, J.; Sonnichsen, F. D.; Saha, M.; Tasdemir, D.; Weinberger, F., Identification of rosmarinic acid and sulfated flavonoids as inhibitors of microfouling on the surface of eelgrass Zostera marina. *Biofouling* **2017,** *33* (10), 867-880.
34. Jensen, P. R.; Jenkins, K. M.; Porter, D.; Fenical, W., Evidence that a New Antibiotic Flavone Glycoside Chemically Defends the Sea Grass Thalassia testudinum against Zoosporic Fungi. *Applied and environmental microbiology* **1998,** *64* (4), 1490-6.
35. Bao, J.; Sun, Y. L.; Zhang, X. Y.; Han, Z.; Gao, H. C.; He, F.; Qian, P. Y.; Qi, S. H., Antifouling and antibacterial polyketides from marine gorgonian coral-associated fungus Penicillium sp. SCSGAF 0023. *The Journal of antibiotics* **2013,** *66* (4), 219-23.

36. Zhou, X.; Zhang, Z.; Xu, Y.; Jin, C.; He, H.; Hao, X.; Qian, P. Y., Flavone and isoflavone derivatives of terrestrial plants as larval settlement inhibitors of the barnacle Balanus amphitrite. *Biofouling* **2009,** *25* (1), 69-76.
37. Kong, N. N.; Fang, S. T.; Liu, Y.; Wang, J. H.; Yang, C. Y.; Xia, C. H., Flavonoids from the halophyte Apocynum venetum and their antifouling activities against marine biofilm-derived bacteria. *Natural product research* **2014,** *28* (12), 928-31.
38. Perez, M.; Garcia, M.; Blustein, G., Evaluation of low copper content antifouling paints containing natural phenolic compounds as bioactive additives. *Marine environmental research* **2015,** *109*, 177-84.
39. Huang, Z.; Nong, X.; Ren, Z.; Wang, J.; Zhang, X.; Qi, S., Anti-HSV-1, antioxidant and antifouling phenolic compounds from the deep-sea-derived fungus Aspergillus versicolor SCSIO 41502. *Bioorganic & medicinal chemistry letters* **2017,** *27* (4), 787-791.
40. Nong, X. H.; Zheng, Z. H.; Zhang, X. Y.; Lu, X. H.; Qi, S. H., Polyketides from a marine-derived fungus Xylariaceae sp. *Marine drugs* **2013,** *11* (5), 1718-27.
41. Catto, C.; Dell'Orto, S.; Villa, F.; Villa, S.; Gelain, A.; Vitali, A.; Marzano, V.; Baroni, S.; Forlani, F.; Cappitelli, F., Unravelling the Structural and Molecular Basis Responsible for the Anti-Biofilm Activity of Zosteric Acid. *PloS one* **2015,** *10* (7), e0131519.
42. Newby, B.-m. Z.; Cutright, T.; Barrios, C. A.; Xu, Q., Zosteric acid—An effective antifoulant for reducing fresh water bacterial attachment on coatings. *JCT Research* **2006,** *3* (1), 69-76.
43. Villa, F.; Albanese, D.; Giussani, B.; Stewart, P. S.; Daffonchio, D.; Cappitelli, F., Hindering biofilm formation with zosteric acid. *Biofouling* **2010,** *26* (6), 739-52.
44. Villa, F.; Pitts, B.; Stewart, P. S.; Giussani, B.; Roncoroni, S.; Albanese, D.; Giordano, C.; Tunesi, M.; Cappitelli, F., Efficacy of zosteric acid sodium salt on the yeast biofilm model Candida albicans. *Microbial ecology* **2011,** *62* (3), 584-98.
45. Ram, J. L.; Purohit, S.; Newby, B. M.; Cutright, T. J., Evaluation of the natural product antifoulant, zosteric acid, for preventing the attachment of quagga mussels--a preliminary study. *Natural product research* **2012,** *26* (6), 580-4.
46. Almeida, J. R.; Correia-da-Silva, M.; Sousa, E.; Antunes, J.; Pinto, M.; Vasconcelos, V.; Cunha, I., Antifouling potential of Nature-inspired sulfated compounds. *Scientific Reports* **2017,** *7*, 42424.
47. Polo, A.; Foladori, P.; Ponti, B.; Bettinetti, R.; Gambino, M.; Villa, F.; Cappitelli, F., Evaluation of Zosteric Acid for Mitigating Biofilm Formation of Pseudomonas putida Isolated from a Membrane Bioreactor System. *International journal of molecular sciences* **2014,** *15* (6), 9497.
48. Vilas-Boas, C.; Sousa, E.; Pinto, M.; Correia-da-Silva, M., An antifouling model from the sea: a review of 25 years of zosteric acid studies. *Biofouling* **2017,** *33* (10), 927-942.
49. Ellman, G. L.; Courtney, K. D.; Andres, V., Jr.; Feather-Stone, R. M., A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical pharmacology* **1961,** *7*, 88-95.
50. Almeida, J. R.; Freitas, M.; Cruz, S.; Leao, P. N.; Vasconcelos, V.; Cunha, I., Acetylcholinesterase in Biofouling Species: Characterization and Mode of Action of Cyanobacteria-Derived Antifouling Agents. *Toxins* **2015,** *7* (8), 2739-56.
51. Cunha, I.; Garcia, L. M.; Guilhermino, L., Sea-urchin (Paracentrotus lividus) glutathione S-transferases and cholinesterase activities as biomarkers of environmental contamination. *JOURNAL OF ENVIRONMENTAL MONITORING* **2005,** *7*, 288-294.
52. Meyer, B. N.; Ferrigni, N. R.; Putnam, J. E.; Jacobsen, L. B.; Nichols, D. E.; McLaughlin, J. L., Brine shrimp: a convenient general bioassay for active plant constituents. *Planta medica* **1982,** *45* (5), 31-4.
53. Kojima, R.; Kobayashi, S.; Satuito, C. G.; Katsuyama, I.; Ando, H.; Seki, Y.; Senda, T., A Method for Evaluating the Efficacy of Antifouling Paints Using Mytilus galloprovincialis in the Laboratory in a Flow-Through System. *PloS one* **2016,** *11* (12), e0168172.

54. Briand, J. F., Marine antifouling laboratory bioassays: an overview of their diversity. *Biofouling* **2009,** *25* (4), 297-311.
55. Aldred, N.; Ista, L. K.; Callow, M. E.; Callow, J. A.; Lopez, G. P.; Clare, A. S., Mussel (Mytilus edulis) byssus deposition in response to variations in surface wettability. *Journal of the Royal Society, Interface* **2006,** *3* (6), 37-43.
56. Qian, P. Y.; Xu, Y.; Fusetani, N., Natural products as antifouling compounds: recent progress and future perspectives. *Biofouling* **2010,** *26* (2), 223-34.
57. Qian, P. Y.; Chen, L.; Xu, Y., Mini-review: Molecular mechanisms of antifouling compounds. *Biofouling* **2013,** *29* (4), 381-400.
58. Chen, L.; Qian, P. Y., Review on Molecular Mechanisms of Antifouling Compounds: An Update since 2012. *Marine drugs* **2017,** *15* (9).
59. Chen, L.; Zhang, H.; Sun, J.; Wong, Y. H.; Han, Z.; Au, D. W.; Bajic, V. B.; Qian, P. Y., Proteomic changes in brain tissues of marine medaka (Oryzias melastigma) after chronic exposure to two antifouling compounds: butenolide and 4,5-dichloro-2-n-octyl-4-isothiazolin-3-one (DCOIT). *Aquatic toxicology (Amsterdam, Netherlands)* **2014,** *157*, 47-56.
60. Chen, L.; Au, D. W.; Hu, C.; Peterson, D. R.; Zhou, B.; Qian, P. Y., Identification of Molecular Targets for 4,5-Dichloro-2-n-octyl-4-isothiazolin-3-one (DCOIT) in Teleosts: New Insight into Mechanism of Toxicity. *Environmental science & technology* **2017,** *51* (3), 1840-1847.
61. Nong, X. H.; Wang, Y. F.; Zhang, X. Y.; Zhou, M. P.; Xu, X. Y.; Qi, S. H., Territrem and butyrolactone derivatives from a marine-derived fungus Aspergillus terreus. *Marine drugs* **2014,** *12* (12), 6113-24.
62. Tadesse, M.; Svenson, J.; Sepcic, K.; Trembleau, L.; Engqvist, M.; Andersen, J. H.; Jaspars, M.; Stensvag, K.; Haug, T., Isolation and synthesis of pulmonarins A and B, acetylcholinesterase inhibitors from the colonial ascidian Synoicum pulmonaria. *Journal of natural products* **2014,** *77* (2), 364-9.
63. Dobretsov, S., Inhibition and Induction of Marine Biofouling by Biofilms. In *Marine and Industrial Biofouling*, Flemming, H.-C.; Murthy, P. S.; Venkatesan, R.; Cooksey, K., Eds. Springer Berlin Heidelberg: Berlin, Heidelberg, 2009; pp 293-313.
64. Yang, C.; Yu, Y.; Sun, W.; Xia, C., Indole derivatives inhibited the formation of bacterial biofilm and modulated Ca2+ efflux in diatom. *Marine pollution bulletin* **2014,** *88* (1-2), 62-9.
65. Katranitsas, A.; Castritsi-Catharios, J.; Persoone, G., The effects of a copper-based antifouling paint on mortality and enzymatic activity of a non-target marine organism. *Marine pollution bulletin* **2003,** *46* (11), 1491-4.
66. Spargo, P. L., Green Engineering: Environmentally Conscious Design of Chemical Processes By David T. Allen and David R. Shonnard. Prentice Hall PTR: New Jersey. 2002. 552 pp. £51.99. ISBN 0-13-061908-6. *Organic Process Research & Development* **2004,** *8* (6), 1083-1083.
67. Agency, U. S. E. P. EPI Suite™-Estimation Program Interface. https://www.epa.gov/tsca-screening-tools/epi-suitetm-estimation-program-interface (accessed 19/07/2019).

7. Appendix

7.1. Appendix I

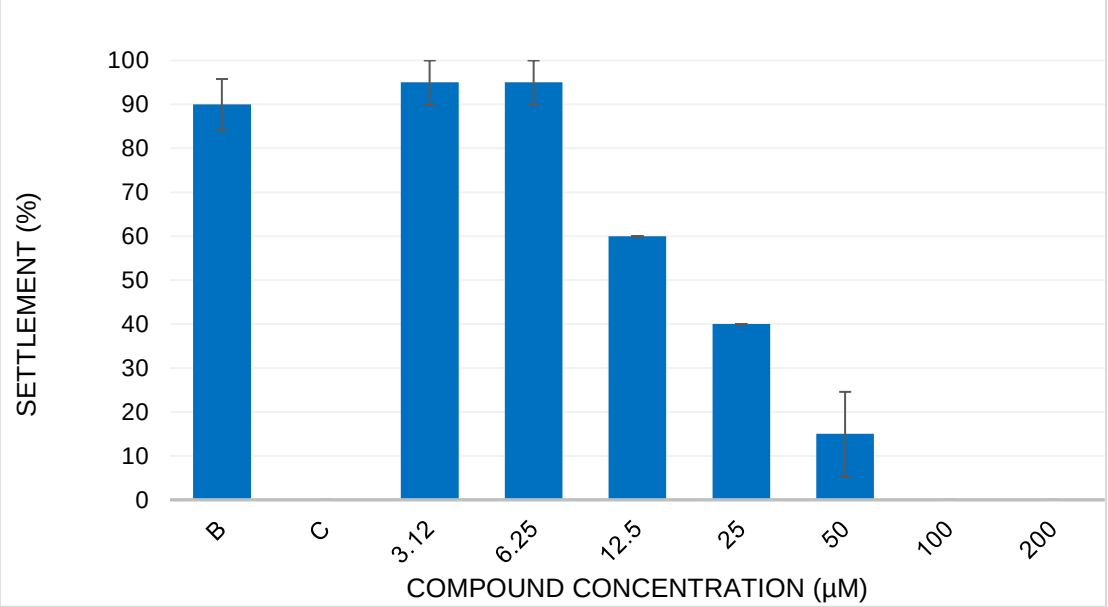


Figure 37. Dose-response activity regarding anti-settlement of compound **3**. B: DMSO (0.01%) as negative control; C: 5µM $CuSO_4$ as positive control.

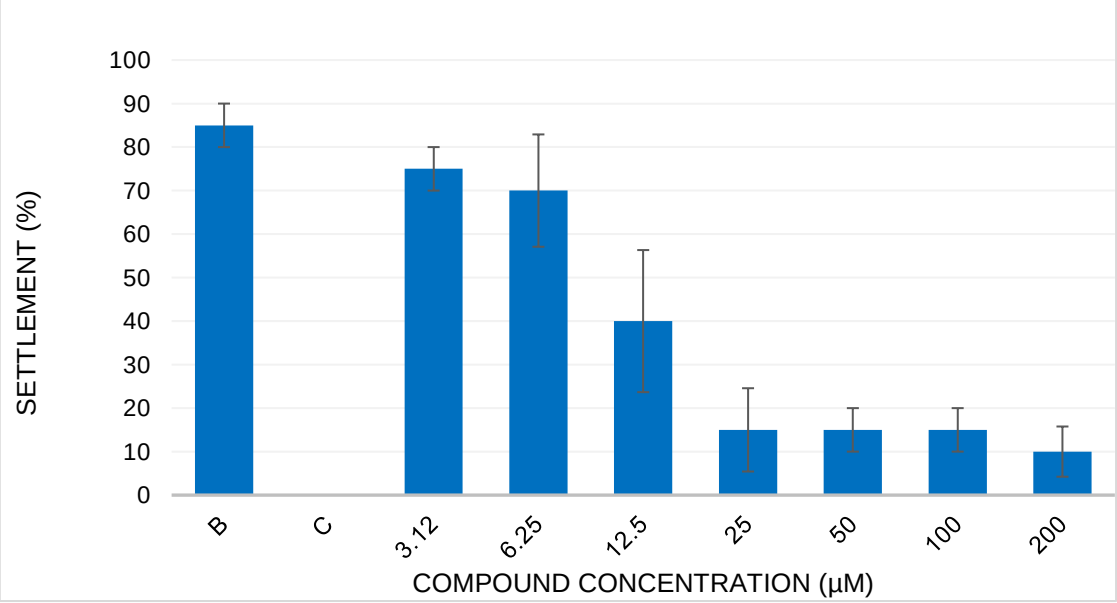


Figure 38. Dose-response activity regarding anti-settlement of compound **4**. B: DMSO (0.01%) as negative control; C: 5µM $CuSO_4$ as positive control.

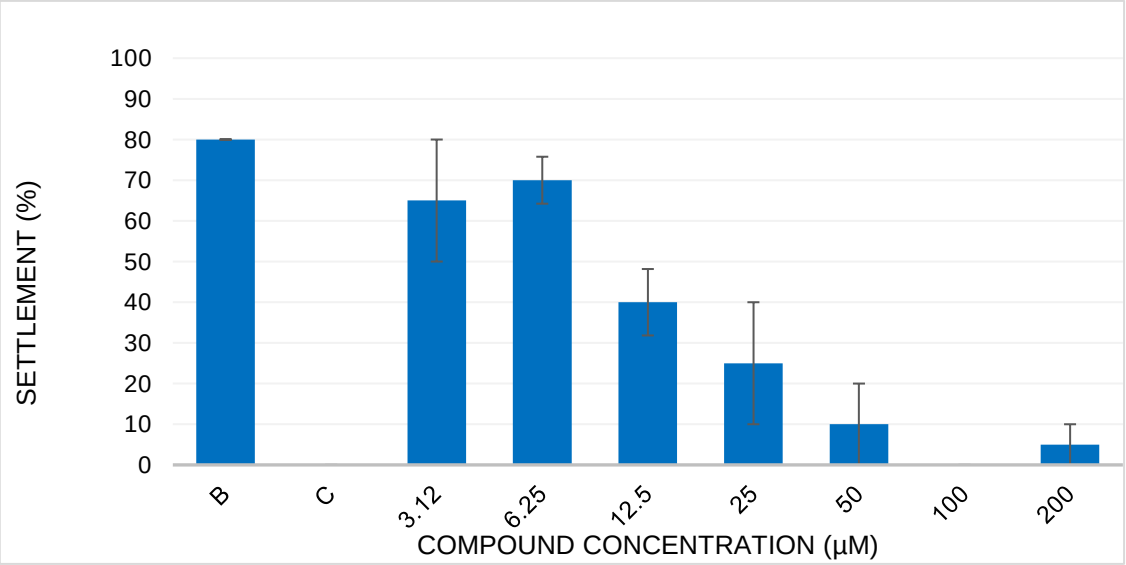


Figure 39. Dose-response activity regarding anti-settlement of compound **4a**. B: DMSO (0.01%) as negative control; C: 5µM $CuSO_4$ as positive control.

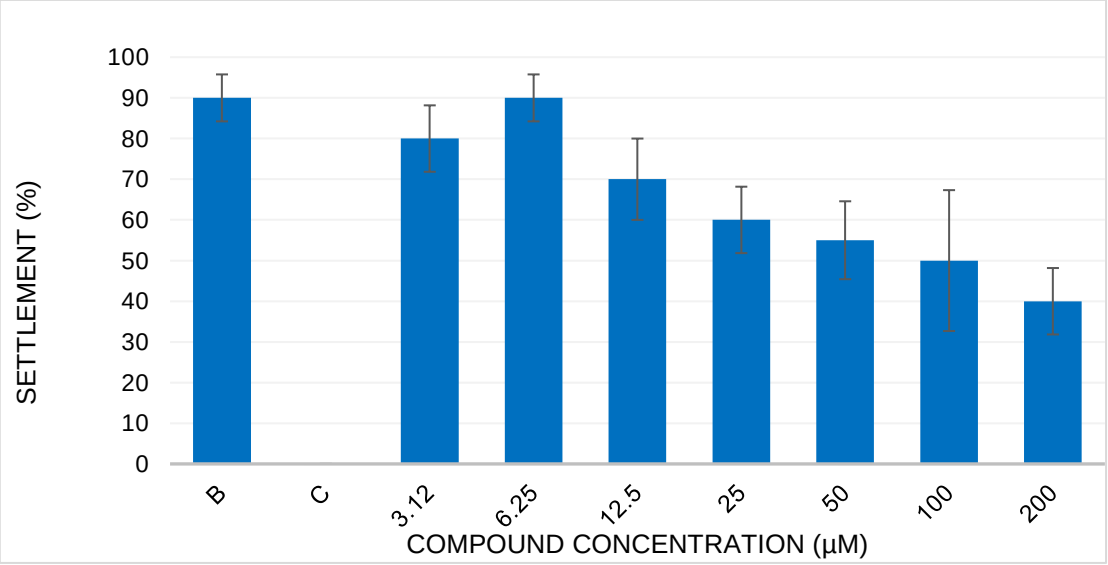


Figure 40. Dose-response activity regarding anti-settlement of compound **5**. B: DMSO (0.01%) as negative control; C: 5µM $CuSO_4$ as positive control.

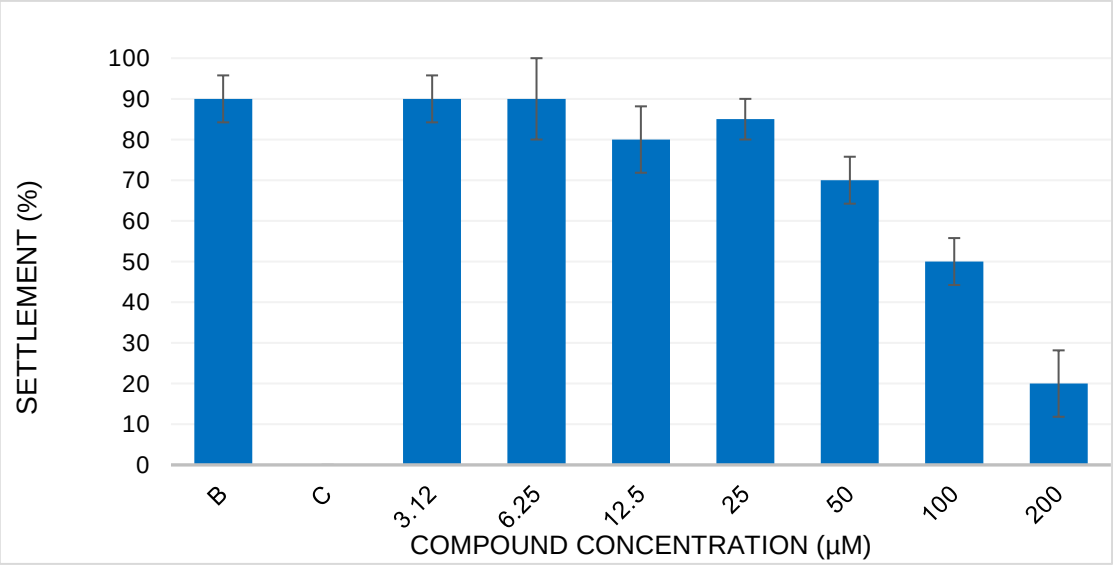


Figure 41. Dose-response activity regarding anti-settlement of compound **6**. B: DMSO (0.01%) as negative control; C: 5µM $CuSO_4$ as positive control.

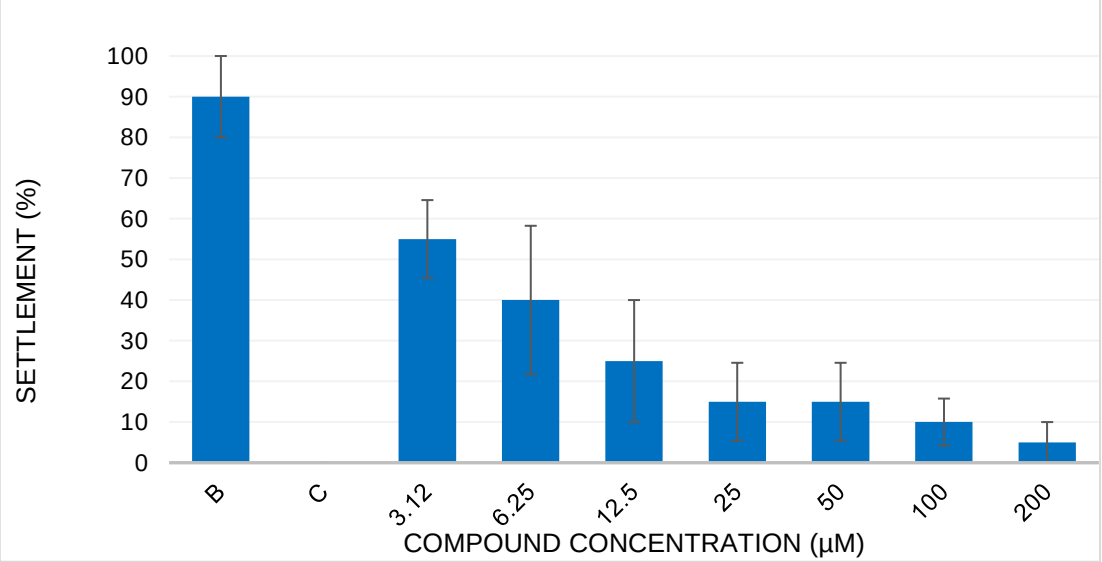


Figure 42. Dose-response activity regarding anti-settlement of compound **8**. B: DMSO (0.01%) as negative control; C: 5µM $CuSO_4$ as positive control.

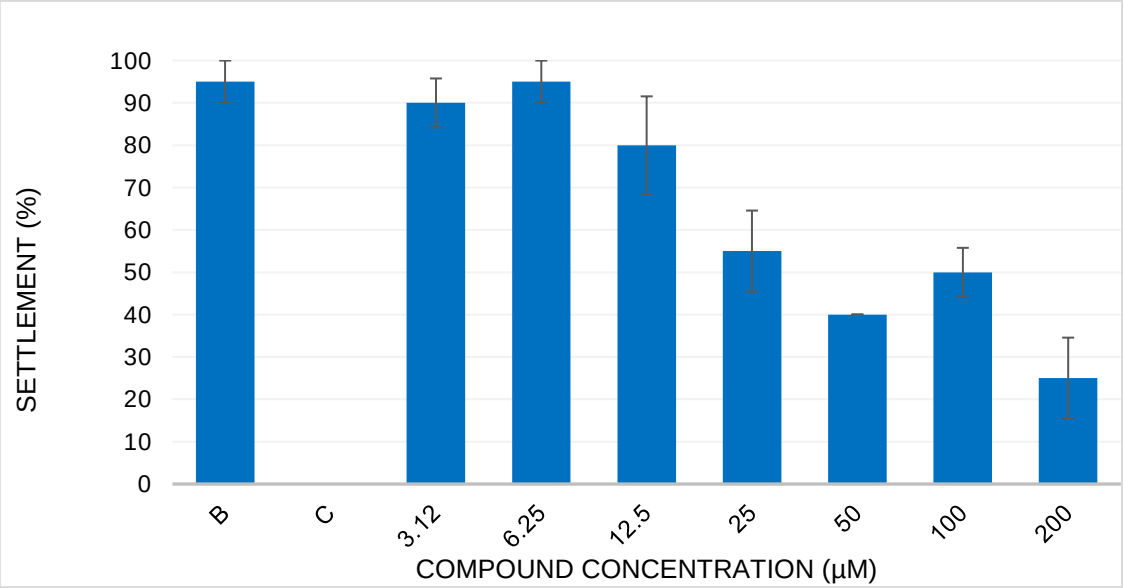


Figure 43. Dose-response activity regarding anti-settlement of compound **9**. B: DMSO (0.01%) as negative control; C: 5µM $CuSO_4$ as positive control.

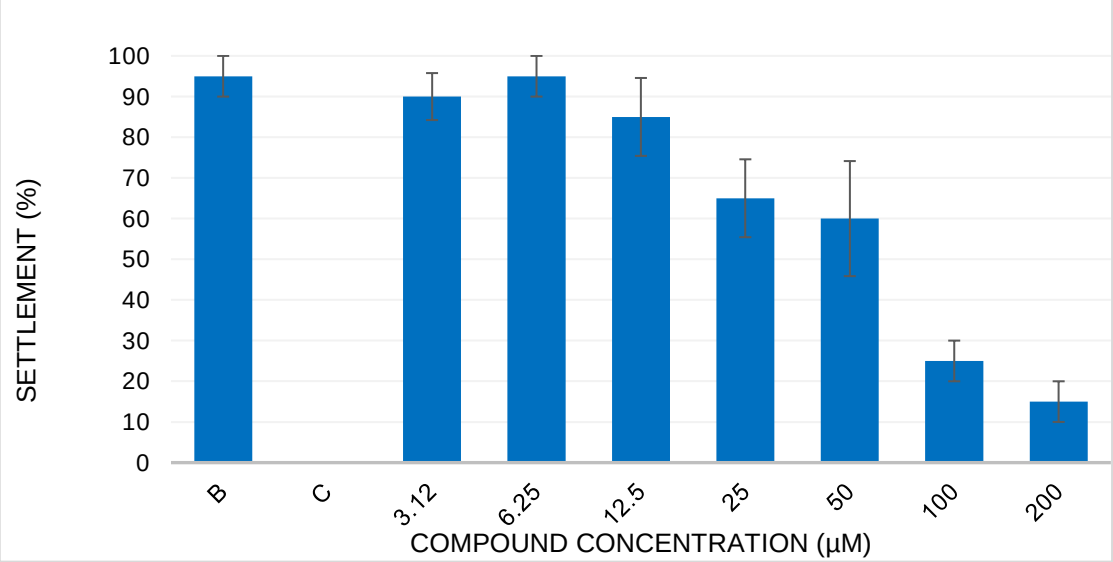


Figure 44. Dose-response activity regarding anti-settlement of compound **9a**. B: DMSO (0.01%) as negative control; C: 5µM $CuSO_4$ as positive control.

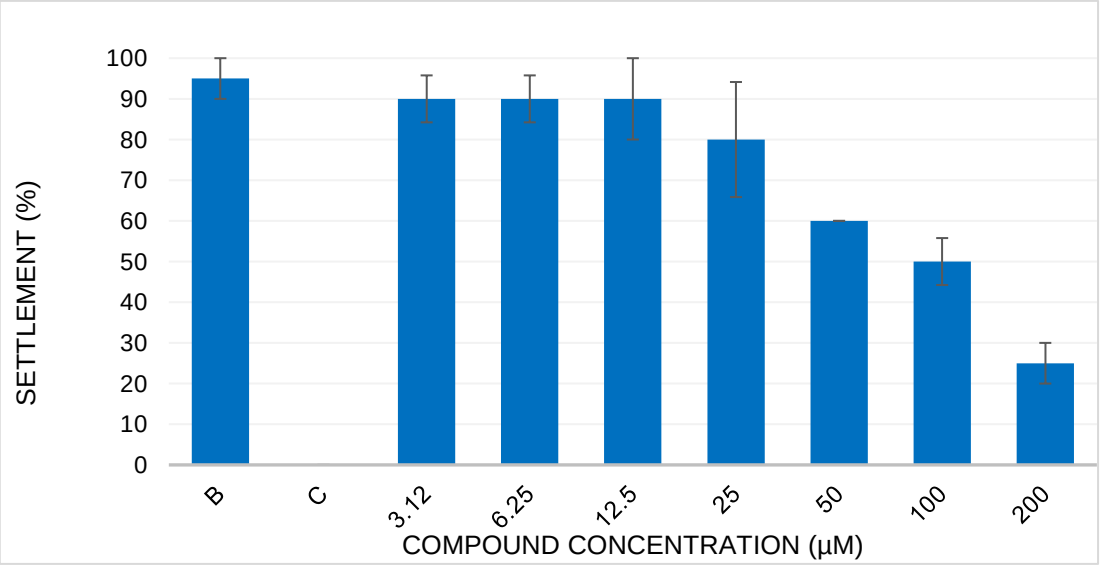


Figure 45. Dose-response activity regarding anti-settlement of compound **12a**. B: DMSO (0.01%) as negative control; C: 5µM $CuSO_4$ as positive control.

7.2. Appendix II

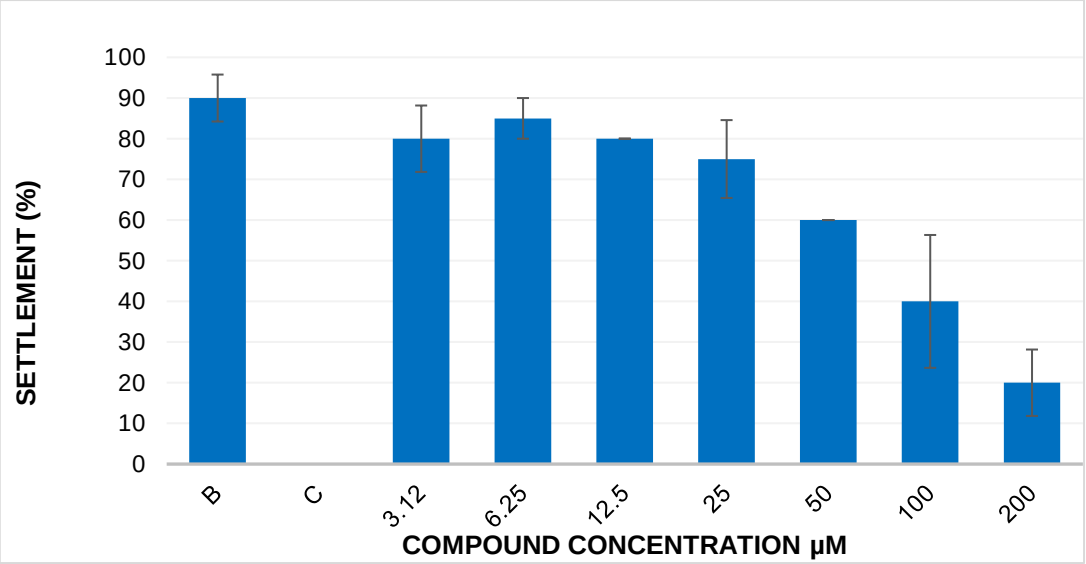


Figure 46. Dose-response activity regarding anti-settlement of compound **14**. B: DMSO (0.01%) as negative control; C: $CuSO_4$ (5µM) as positive control.

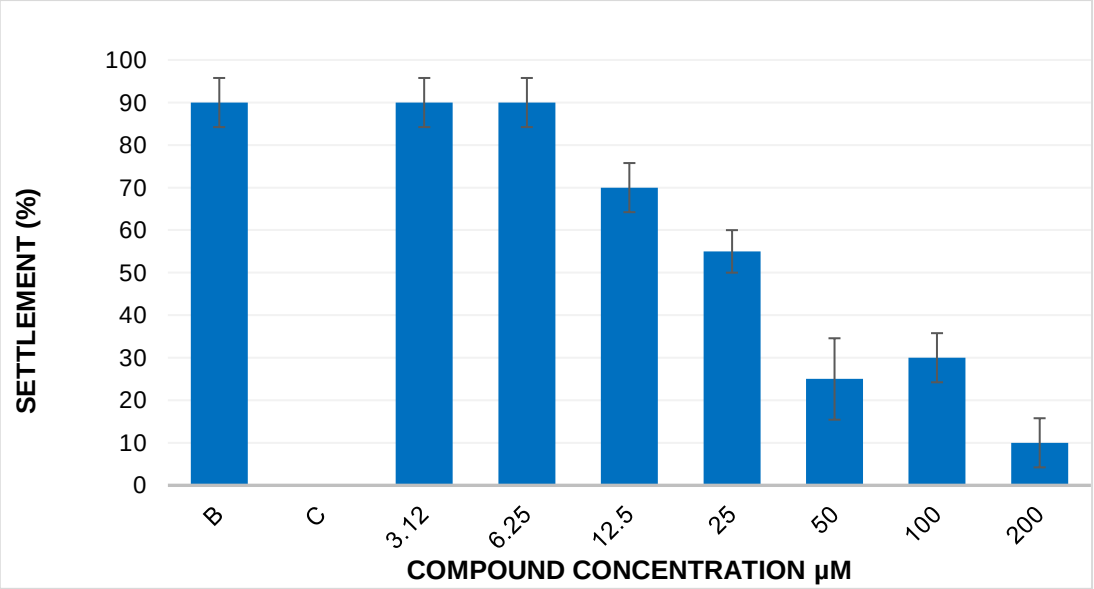


Figure 47. Dose-response activity regarding anti-settlement of compound **17**. B: DMSO (0.01%) as negative control; C: $CuSO_4$ (5µM) as positive control.

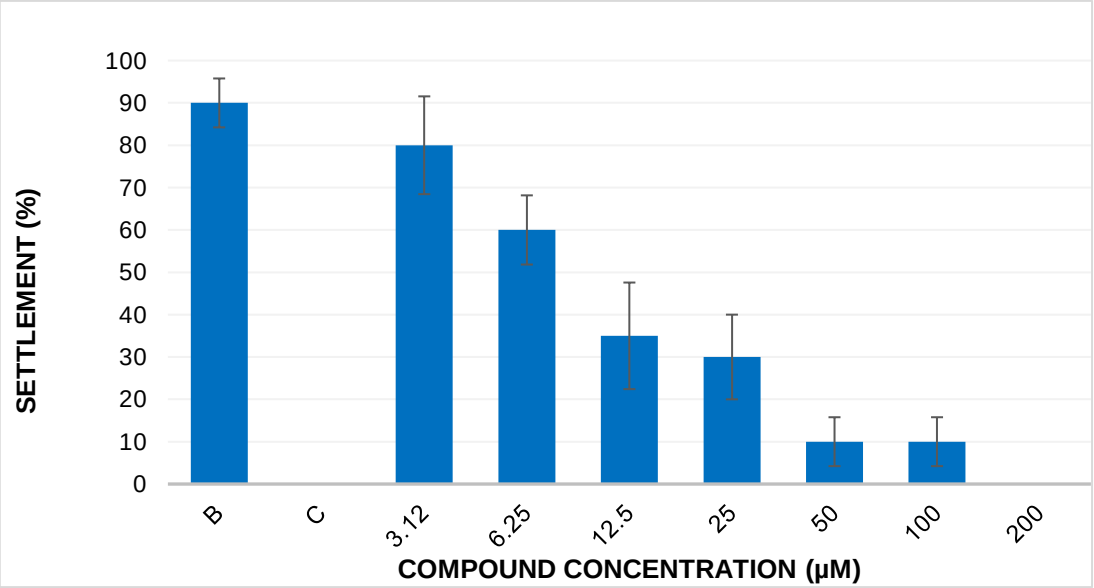


Figure 48. Dose-response activity regarding anti-settlement of compound **21**. B: DMSO (0.01%) as negative control; C: $CuSO_4$ (5µM) as positive control.

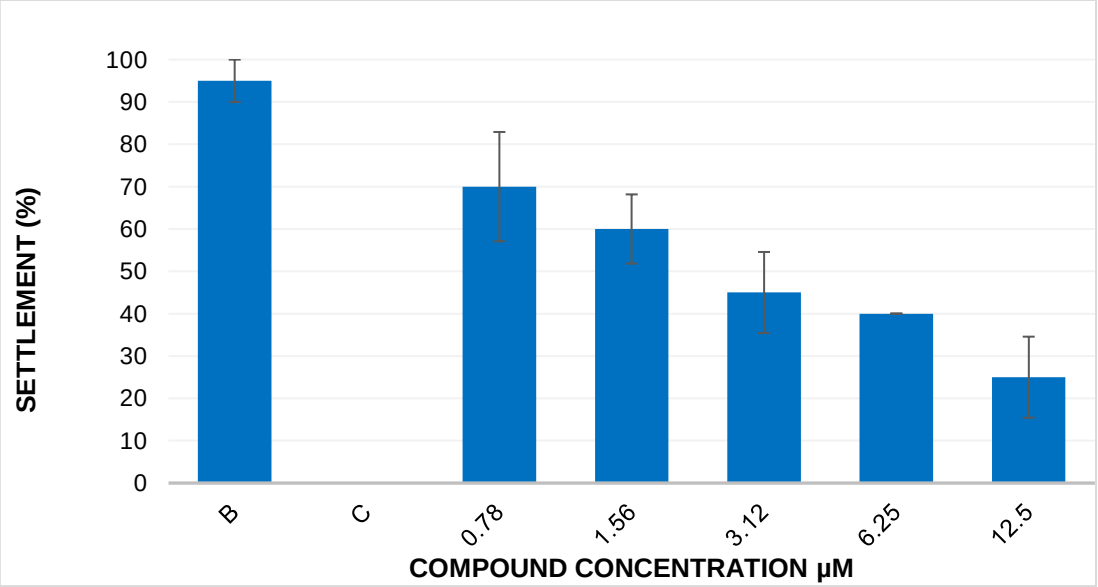


Figure 49. Dose-response activity regarding anti-settlement of compound **30**. B: DMSO (0.01%) as negative control; C: $CuSO_4$ (5µM) as positive control.

7.3. Appendix III

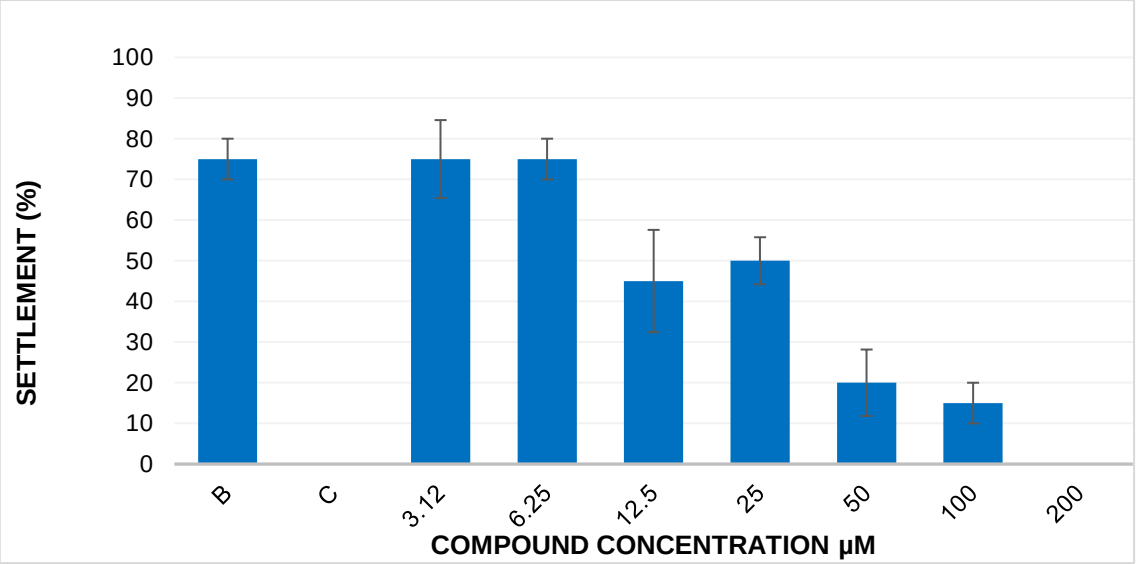


Figure 50. Dose-response activity regarding anti-settlement of compound **33**. B: DMSO (0.01%) as negative control; C: $CuSO_4$ (5µM) as positive control.

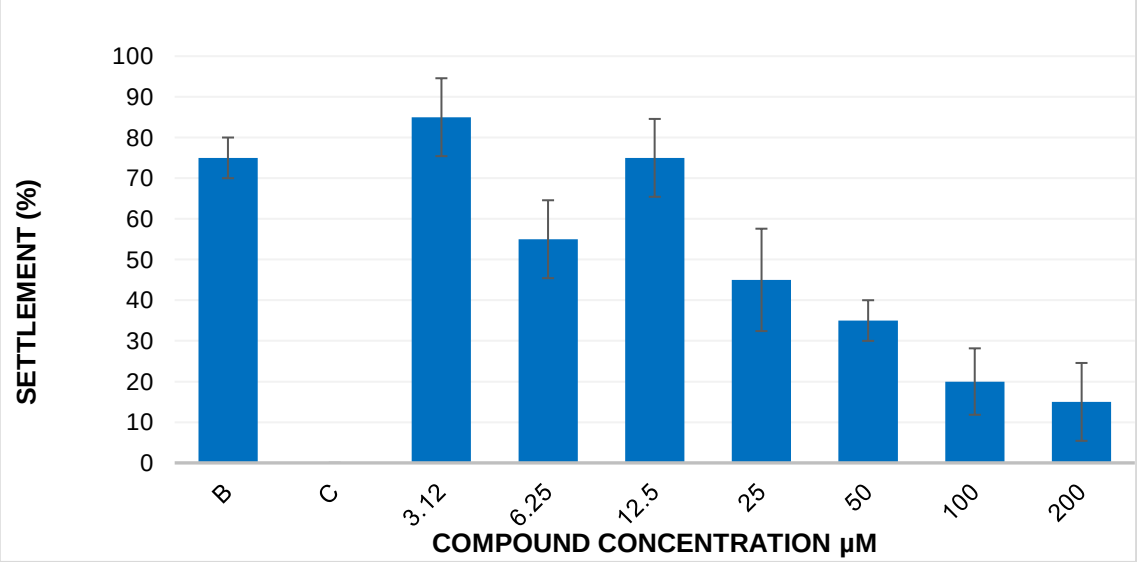


Figure 51. Dose-response activity regarding anti-settlement of compound **36**. B: DMSO (0.01%) as negative control; C: $CuSO_4$ (5µM) as positive control.

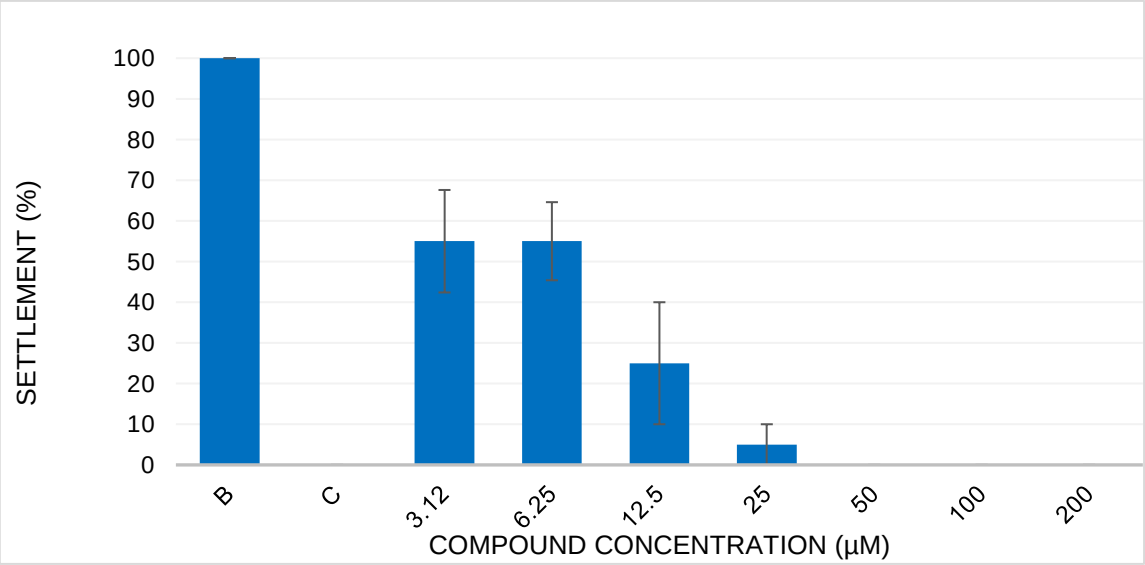


Figure 52. Dose-response activity regarding anti-settlement of compound **40**. B: DMSO (0.01%) as negative control; C: CuSO4 (5µM) as positive control.

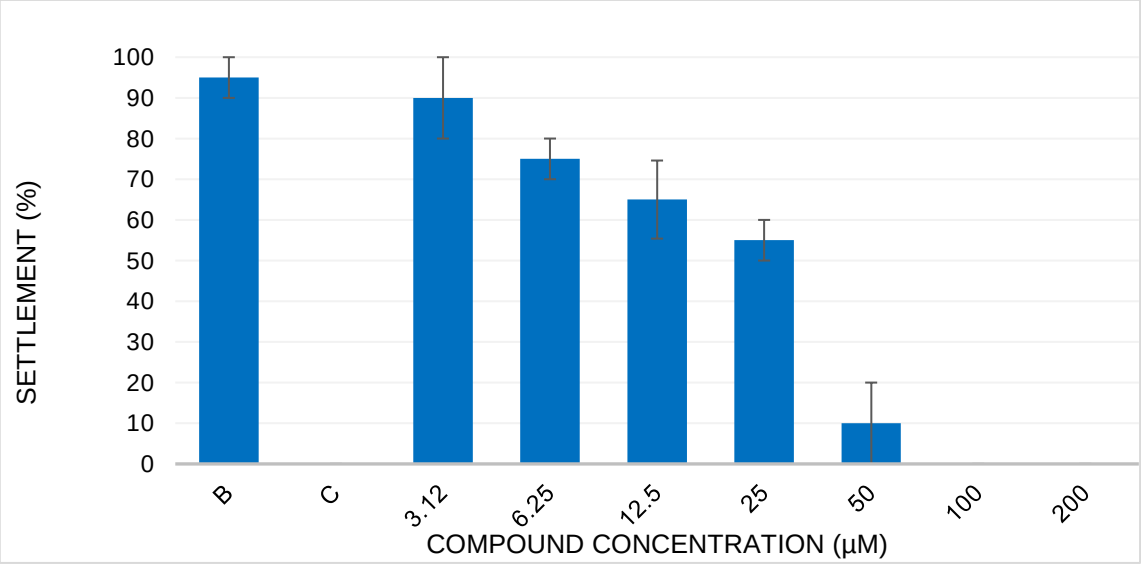


Figure 53. Dose-response activity regarding anti-settlement of compound **41**. B: DMSO (0.01%) as negative control; C: CuSO4 (5µM) as positive control.

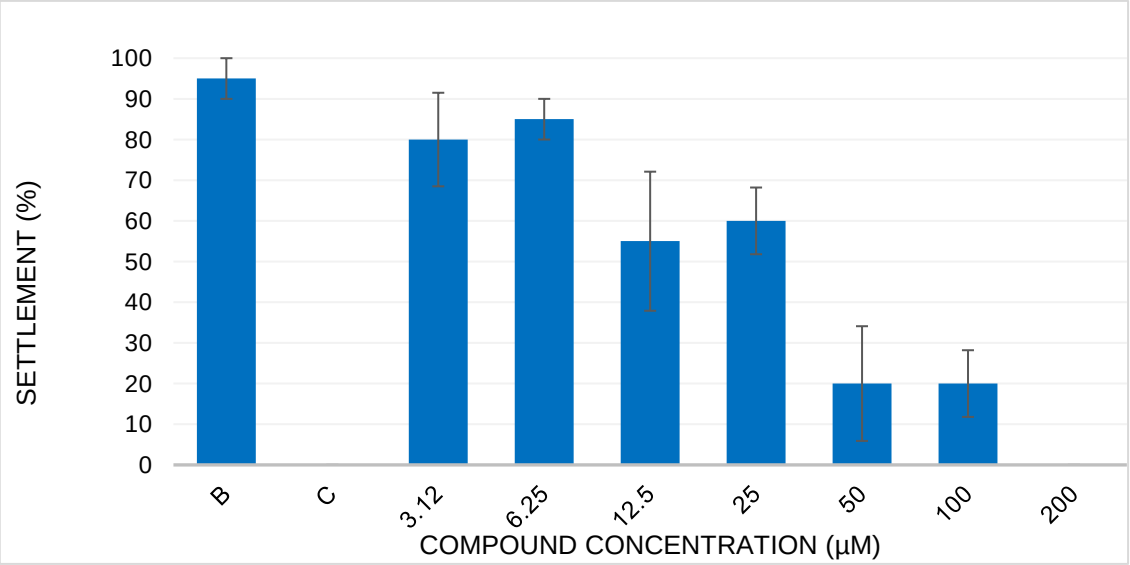


Figure 54. Dose-response activity regarding anti-settlement of compound **42**. B: DMSO (0.01%) as negative control; C: CuSO4 (5µM) as positive control.