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Potential of synthetic chalcone derivatives to prevent marine biofouling

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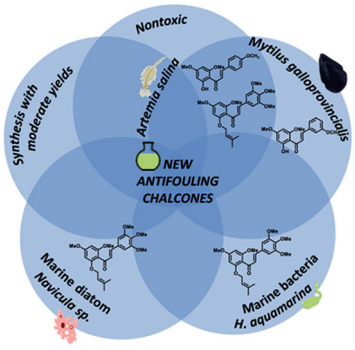
HIGHLIGHTS

- Marine biofouling impacts atmosphere, hydrosphere, and biosphere negatively.
- Antifouling paints in use release toxic and persistent organic pollutants.
- Synthesis of new nontoxic antifoulants was achieved in this work.
- These compounds are able to be obtained in short time and in suitable amounts.

GRAPHICAL ABSTRACT



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ABSTRACT

Biofouling represents a major economic, environmental and health concern for which new eco-friendly solutions are needed. International legislation has restricted the use of biocidal-based antifouling coatings, and increasing efforts have been applied in the search for environmentally friendly antifouling agents. This research work deals with the assessment of the interest of a series of synthetic chalcone derivatives for antifouling applications. Sixteen chalcone derivatives were synthesized with moderate yields (38–85%). Antifouling bioactivity of these compounds was assessed at different levels of biological organization using both anti-macrofouling and anti-microfouling bioassays, namely an anti-settlement assay using mussel (*Mytilus galloprovincialis*) larvae, as well as marine bacteria and microalgal biofilms growth inhibition bioassays. Results showed that three compounds (**11**, **12**, and **16**) were particularly active against the settlement of mussel larvae (EC_{50} 7.24–34.63 μM), being compounds **12** and **16** also able to inhibit the growth of microfouling species (EC_{50} 4.09–20.31 μM). Moreover, the most potent compounds **12** and **16** were found to be non-toxic to the non-target species *Artemia salina* (<10% mortality at 25 μM). A quantitative structure-activity relationship model predicted that descriptors describing the ability of molecules to form hydrogen bonds and encoding the shape, branching ratio and constitutional diversity of the molecule were implied in the antifouling activity against the settlement of mussel larvae. This work elucidates for the first time the relevance of synthesizing chalcone derivatives to generate new non-toxic products to prevent marine biofouling.

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1. Introduction

Both macro and microorganisms are part of the community responsible for the natural process known as biofouling, which occurs when marine species attach to natural or artificial underwater surfaces (Callow and Callow, 2002). Marine biofouling causes not only material and economic loss for the marine sector operations, but also creates a series of environmental and health problems affecting atmosphere, hydrosphere, biosphere due to over-consumption of fuel and carbon dioxide emissions, and also the spread of pathogenic bacteria and indigenous species which contributes to health problems and biodiversity reduction (Qian et al., 2010; Schultz et al., 2011).

Antifouling (AF) paints have been and remain the primary strategy for biofouling control. Biocidal paints based on tributyltin (TBT) have been effective AF agents. Nevertheless, these coatings were banned in several countries since 2008 (IMO, 2008), given its detrimental effect to non-target organisms and whole marine environment (Antizar-Ladislao, 2008). In fact, environmental studies demonstrated that organotin compounds do not readily degrade in the environment, affecting marine organisms and possibly biomagnifying through the food chains (Konstantinou and Albanis, 2004). Booster biocides based on copper, zinc, and several organic compounds were more recently introduced as AF agents, but have also been found to be harmful to many non-target organisms (Thomas and Brooks, 2010). Therefore, there is a high demand for environmentally benign, non-toxic AF agents as an alternative to the biocide-based coatings currently in use (Almeida and Vasconcelos, 2015).

A wide range of diverse natural AF compounds have been identified lately (Qian et al., 2015; Satheesh et al., 2016; Wang et al., 2017). These compounds have been reported as acting against micro and macrofouling species with low toxicity, and were recently considered as models to the synthesis of nature-inspired AF agents namely, synoxazolidinone A (Trepos et al., 2014), 2,5-diketopiperazine (Liao et al., 2015), zosteric acid (Almeida et al., 2017; Catto et al., 2015), batatasin III (Moodie et al., 2018; Moodie et al., 2017a), polygodial (Moodie et al., 2017b).

Chalcones represent one of the major subclasses of flavonoids and have long been recognised for their myriad of biological activities (Singh et al., 2014). Regarding AF properties, the evaluation of their effect is limited to studies using marine bacterial biofilms such as *Vibrio natriegens*, *Bacillus flexus*, and *Pseudomonas fluorescens* (Sivakumar et al., 2010a; Sivakumar et al., 2010b). Moreover, chalcones have been used as anticorrosive agents (Bouklah et al., 2006), alone and combined with iodide ions to synergize the activity of the latter in acid-mediated corrosion of steel (Bouklah et al., 2003; Elayyoubi et al., 2002). Considering the antibacterial and slimicidal activities together with anticorrosive properties, chalcones were proposed as ideal candidates to be used as AF agents in anticorrosive coatings (Sivakumar et al., 2010a). At the best of our knowledge, no studies concerning their effects on other fouling organisms have been conducted.

In this research work, a series of chalcone derivatives was synthesized (Fig. 1, **1**–**16**) and evaluated for their AF activity against both micro and macrofouling species, namely five biofilm-forming marine bacteria (*Cobetia marina*, *Vibrio harveyi*, *Pseudoalteromonas atlantica*, *Halomonas aquamarina and Roseobacter litoralis*), four marine diatom strains (*Cylindrotheca* sp., *Halamphora* sp., *Nitzschia* sp. and *Navicula* sp.) and the adhesive larvae of the macrofouling mussel *Mytilus galloprovincialis*. A quantitative structure-activity relationship (QSAR) model to predict the AF activity against larvae of *Mytilus galloprovincialis* was also developed.

Compounds showing promising AF bioactivity were submitted to complementary assays to evaluate the viability of the selected compounds as AF agents, including the assessment of general ecotoxicity using *Artemia salina* standard ecotoxicity assay, and the evaluation of possible mechanisms of action related with adhesion and neurotransmission pathways.

2. Material and methods

2.1. Synthesis and structure elucidation

Microwave (MW) reactions were performed using a glassware setup for atmospheric pressure reactions and a 100 mL Teflon reactor (internal reaction temperature measurements with a fiber-optic probe sensor), and were carried out in an Ethos MicroSYNTH 1600 Microwave Labstation from Milestone. The reactions were monitored by thin-layer chromatography (TLC). Compounds purification was performed by flash column chromatography using Macherey-Nagel silica gel 60 (0.04–0.063 mm), and preparative thin-layer chromatography (TLC) using Macherey-Nagel silica gel 60 (GF254) plates. Melting points were obtained in a Köfler microscope and are uncorrected. ^{1}H and ^{13}C NMR spectra were taken in $CDCl_3$ at room temperature, on Bruker Avance 300 instrument (300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C). Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as an internal reference; ^{13}C NMR assignments were made by 2D (HSQC and HMBC) NMR experiments (long-range C, H coupling constants were optimized to 7 Hz). HRMS mass spectra were recorded at C.A.C.T.I.-University of Vigo, Spain. Experiments were performed on an APEXQe FT-ICR MS (Bruker Daltonics, Billerica, MA), equipped with a 7 T actively shielded magnet. Ions were generated using a Combi MALDI-electrospray ionization (ESI) source. Ionization was achieved by electrospray, using a voltage of 4500 V applied to the needle, and a counter voltage of 300 V applied to the capillary. Samples were prepared by adding a spray solution of 70:29.9:0.1 (v/v/v) CH_3OH/water/formic acid or 70:29.9:0.1 (v/v/v) CH_3CN/water/formic acid to a solution of the sample at a v/v ratio of 1 to 5% to give the best signal-to-noise ratio. Data acquisition was performed using the ApexControl software version 3.0.0, and data processing was performed using the DataAnalysis software, version 4.0 both from Bruker Daltonics. 2-Hydroxy-4,6-dimethoxyacetophenone and benzaldehydes were purchased from Sigma Aldrich. Chalcone derivatives **1**–**6** (33–42%) (Pereira et al., 2016), **8** (46%) (Detsi et al., 2009), **9** (60%) (Kadival et al., 1962), **10** (47%) (Boeck et al., 2006), **11** (40%) (Boeck et al., 2006), **12** (77%) (Thieury et al., 2017), **13** (39%) (Detsi et al., 2009), **14** (38%) (Alvim et al., 2010), **15** (71%) (Mateeva et al., 2002), and **16** (73%) (Neves et al., 2012) were synthesized as described elsewhere. The NMR data of compound **9** was described for the first time, as indicated below. The new chalcone derivative **7** was synthesized and purified by the following procedures.

2.1.1. Synthesis of chalcone 7

2-Hydroxy-4-methoxy-3-propylacetophenone was synthesized (quantitative yield) and characterized according to a previous described procedure (Pereira et al., 2016). Then, to a solution of 2-hydroxy-4-methoxy-3-propylacetophenone (1.000 mmol, 0.208 g) in methanol was added an aqueous solution of 40% sodium hydroxide until pH 13–14. Then, a solution of 2 mmol of 4-chlorobenzaldehyde in methanol was slowly added to the reaction mixture. The reaction was submitted to successive 15 min periods of MW irradiation at 180 W. Total irradiation time was 30 min and the final temperature was 75 °C. The solution was extracted with chloroform (3 × 50 mL). The combined organic layers were rinsed with brine and water, dried over anhydrous sodium sulfate and evaporated under reduced pressure. The obtained residue was purified by flash column chromatography (SiO_2; n-hexane/ethyl acetate 8:2) affording compound **7** as orange crystals in 57% yield.

(***E***)-**1**-(**2-hydroxy-4-methoxy-3-propylphenyl**)-**3**-(**4-Clorophenyl**) **prop-2-en-1-one** (**7**). mp (ethyl acetate): 109–112 °C; ^{1}H NMR ($CDCl_3$, 300.13 MHz): δ 13.30 (1H, s, 2′-OH), 7.82 (1H, d, J = 15.0 Hz, H-β), 7.78 (1H, d, J = 9.2 Hz, H-6′), 7.58 (1H, d, J = 15.0 Hz, H-α), 7.57 (2H, d, J = 8.5 Hz, H-2,6), 7.40 (2H, d, J = 8.5 Hz, H-3,5), 6.50 (1H, d, J = 9.2 Hz, H-5′), 3.90 (3H, s, 4′-OCH_3), 2.66 (2H, t, J = 7.7 Hz, H-1″), 1.59–1.51 (2H, m, H-2″), 0.96 (3H, t, J = 7.4 Hz, H-3″); ^{13}C NMR ($CDCl_3$,

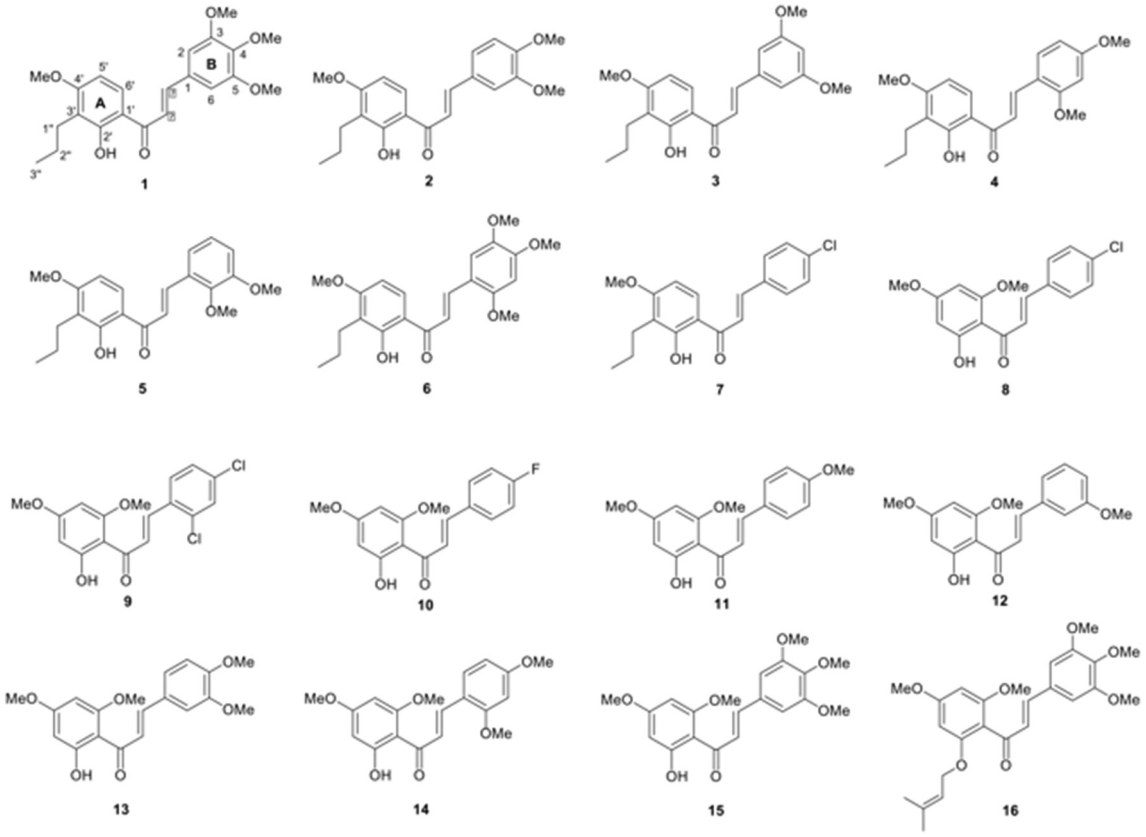


Fig. 1. Structure of chalcone derivatives **1**–**16**.

75.47 MHz): δ 191.7 (CO), 163.5 (C-4′), 163.1 (C-2′), 142.3 (C-β), 136.1 (C-4), 133.1 (C-1), 129.3 (C-2,-6), 129.0 (C-3,-5), 128.8 (C-6′), 120.8 (C-α), 118.4 (C-3′), 114.1 (C-1′), 101.8 (C-5′), 55.5 (4′-OCH_3), 24.2 (C-1″), 21.6 (C-2″), 14.0 (C-3″); ESI-TOF-HRMS (+) *m/z*: Anal. Calc. for $C_{19}H_{20}ClO_3$ ($M + H^+$): 331.10955; found: 331.10862.

(***E***)-**3**-(**2,4-dichlorophenyl**)-**1**-(**2-hydroxy-4,6-dimethoxyphenyl**) **prop-2-en-1-one** (**9**): mp (ethanol): 182–183 °C; ^1H NMR ($CDCl_3$, 300.13 MHz): δ 14.15 (s, 2′-OH), 8.05 (d, *J* = 15.6 Hz, H-β), 7.85 (d, *J* = 15.6 Hz, H-α), 7.62 (d, *J* = 8.7 Hz, H-6), 7.45 (d, *J* = 2.1 Hz, H-2), 7.28 (dd, *J* = 8.7, 2.1 Hz, H-5), 6.12 (d, *J* = 2.3 Hz, H-5′), 5.96 (d, *J* = 2.3 Hz, H-3′), 3.90 (s, 3H, 4′-OCH_3), 3.84 (s, 3H, 6′-OCH_3); ^{13}C NMR ($CDCl_3$, 75.47 MHz) δ: 192.0 (CO), 168.5 (C-4′), 166.5 (C-6′), 162.4 (C-2′), 136.6 (C-β), 135.9 (C-4), 132.5 (C-1), 130.4 (C-2), 130.1 (C-3), 130.0 (C-α), 128.5 (C-6), 127.5 (C-5), 106.2 (C-1′), 93.8 (C-3′), 91.3 (C-5′), 55.9 (4′-OCH_3), 55.6 (6′-OCH_3); ESI-TOF-HRMS (+) *m/z*: Anal. Calc. for $C_{17}H_{15}Cl_2O_4$ ($M + H^+$): 353.03419; found: 353.03380.

2.2. Mussel adhesive larvae sampling and processing

Mussel (*Mytilus galloprovincialis*) juvenile aggregates were sampled during low neap tides in intertidal pools, at Memória beach, Matosinhos, Portugal (41° 13′ 59″ N; 8° 43′ 28″ W). In the laboratory, mussel plantigrade adhesive larvae (0.5–2 mm) were screened among the mussel aggregates samples in a binocular magnifier (Olympus SZX2-ILLT), isolated in a petri dish with filtered seawater and gently washed to remove adhered organic particles immediately before the bioassays.

2.3. Mussel larvae anti-settlement bioassays

M. galloprovincialis plantigrade larvae showing exploring behaviour were selected and exposed to the series of chalcone derivatives **1**–**16** at 50 μM in 24-well microplates with 4 replicates for 15 h, at 18 ± 1 °C, in the darkness (Almeida et al., 2017). Solutions of compounds **1**–**16** were prepared in filtered seawater and obtained by dilution of the compounds stock solutions (50 mM) in DMSO. A negative control (0.01% DMSO) and a positive control (5 μM $CuSO_4$) were included in all bioassays. The anti-settlement response of ECONEA® (50 μM), a commercial AF agent, was also included for comparative purposes. After exposure, the anti-settlement activity was determined by the presence/absence of efficiently attached byssal threads produced by each individual for all the tested conditions.

Compounds causing significant settlement inhibition ($p < 0.01$) at 50 μM in the screening bioassay compared to negative control were considered bioactive. The bioactive compounds were selected for further testing and serial dilutions of compounds stock solutions (200, 100, 50, 25, 12.5, 6.25, and 3.12 μM) were performed to determine the semi-maximum response concentrations that inhibited 50% larval settlement (EC_{50}) and the median lethal dose (LC_{50}), if applicable.

2.4. QSAR model construction

The 3D structures of the 16 chalcones and positive control Tralopyril were drawn using Hyperchem 7.5 (Hypercube, FL, USA) (Froimowitz, 1993), being minimized by the AM1 semi-empirical method with the Polak-Ribiere conjugate gradient algorithm (RMS < 0.1 kcal·$Å^{-1}$·-mol^{-1}) (Zhang et al., 2006). Twelve molecules were used to construct QSAR models using the biological data obtained *in vitro* studies for AF activity (AF = −log(incrustration / Maximal_incrustration)). AF activity was adopted as a dependent variable in the QSAR analysis. The 17 molecules were randomly distributed into a training set (12 molecules) and a test set (5 molecules). CODESSA software (version 2.7.10, University of Florida, USA) was used to calculate >500 constitutional, topological, geometrical, electrostatic, quantum-chemical, and thermodynamical molecular descriptors (Katritsky et al., 2004). The heuristic multilinear regression procedures available in the framework of the CODESSA program was used to perform a complete search for the best

multilinear correlations with a multitude of descriptors of the training set (Katritsky et al., 2004). The 2D-QSAR model with the best correlation coefficient (R^2), F-test (F), standard error (s), as cross-validation (Q^2) was selected. The final model was further validated using the test set.

2.5. In vitro evaluation of acetylcholinesterase and tyrosinase activities

Acetylcholinesterase (AChE) and tyrosinase (Tyr) inhibitory activity were determined as potential mechanisms of action of AF compounds **11**, **12**, and **16**, as described previously (Almeida et al., 2017). The potential of compounds **11**, **12**, and **16** to inhibit AChE activity was evaluated using Electrophorus electric AChE Type V-S (SIGMA C2888, E.C. 3.1.1.7), according to Ellamn et al. (Ellman et al., 1961) with some modifications (Almeida et al., 2015). Briefly, to a reaction solution containing 10 mM of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in 1 M of phosphate buffer pH 7.2 (with sodium hydrogen carbonate) and 75 mM of acetyltiocholine iodide, pure AChE enzyme (0.25 U/mL) and each tested compound (final concentration of 50 μM) was added. The optical density was measured at 412 nm in a microplate reader during 5 min at 25 °C. A negative control (B) with 1% DMSO and a positive control with 200 μM eserine was also included.

Tyr inhibition assays were conducted using *Agaricus bisporus* Tyr (EC 1.14.18.1) according to Adhikari et al. (2008) with slight modifications (Almeida et al., 2017). The enzymatic reaction follows the catalytic conversion of L-Dopa to dopaquinone and the formation of dopachrome by measuring the absorvance at 475 nm. Briefly, Tyr (25 U/mL) is added to 50 mM fosfate buffer pH 6.5 and the tested compound at 50 μM. The enzymatic activity was triggered by the addition of L-dopa (25 mM). Kojic acid (1.4 mM) was included as positive control and 1% DMSO as negative control.

2.6. Antibacterial and anti-diatom bioassays

For antibacterial activity screening, five strains of marine biofilm-forming bacteria *Cobetia marina* CECT 4278, *Vibrio harveyi* CECT 525, *Halomonas aquamarina* CECT 5000, *Pseudoalteromonas atlantica* CECT 570 and *Roseobacter litoralis* CECT 5395 from the Spanish Type Culture Collection (CECT) were inoculated and incubated for 24 h 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 compounds **11**, **12**, and **16** at 9.7, 9.7 and 6.9 μM, respectively (corresponding to 3 $\mu g \cdot mL^{-1}$) test concentration. Bacterial growth inhibition in the presence of the tested compounds was determined in quadruplicate at 600 nm using a microplate reader (Biotek Synergy HT, Vermont, USA). Marine broth with 0.1% DMSO and 1:100 penicilin-streptomycin-neomycin stabilized solution (Sigma P4083) were used as negative (B) and positive (C) controls, respectively.

For the anti-microalgal screening, four strains of benthic marine diatoms, *Cylindrotheca* sp., *Halamphora* sp., *Nitzschia* sp. and *Navicula* sp. were purchased from the Spanish Collection of Algae and inoculated in f/2 medium (Sigma) at an initial concentration of $2–4 \times 10^6$ cells mL^{-1}, and grown in 96 well flat-bottom microtiter plates for 10 days at 20 °C. Diatoms growth inhibition in the presence of chalcones **11**, **12** and **16** at 9.7, 9.7 and 6.9 μM, respectively, was determined in quadruplicate, and quantified by the difference in cell densities among treatments, counted using a Neubauer counting chamber. f/2 medium with 0.1% DMSO and cycloheximide (3.55 μM) were used as negative and positive controls, respectively. The anti-bacterial and anti-microalgal activity of the comercial AF agent ECONEA® was also included in the same bioassay conditions (8.7 μM) as reference. Compounds producing <25% growth inhibition were considered not active. Compounds that showed significant inhibitory activity at screening assays were selected for further determination of inhibitory effect concentrations (EC). A serial dilution of the stock solution was used to obtain final concentrations from 27.6 μM to 1.7 μM.

2.7. Artemia salina ecotoxicity bioassay

Bioactive chalcones **11**, **12**, and **16** were tested for general ecotoxicity using the brine shrimp (*Artemia salina*) nauplii lethality test (Almeida et al., 2017; Meyer et al., 1982). *A. salina* eggs were allowed to hatch in nutrient-enriched seawater for approximately 48 h at 25 °C. Test solutions of the selected compounds were prepared in filtered seawater. Test concentrations were 50 and 25 μM and a concentration correspondent to the anti-settlement EC_{50} value for each compound, when applicable. Toxicity tests were performed in the darkness in 96-wells microplates with eight replicates per condition and 15–20 nauplii per well. 13.6 μM $K_2Cr_2O_7$ was included as positive control and 1% DMSO as negative control. Percentage of mortality was assessed as endpoint at the end of the exposure period.

2.8. Data analysis

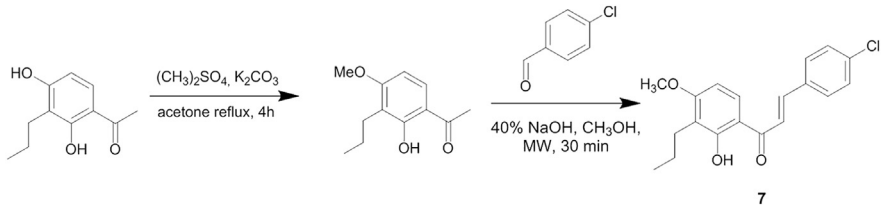
One-way analysis of variance (ANOVA) followed by a Dunnett test against the DMSO control ($p < 0.01$) was used to analyse anti-settlement, antibacterial and anti-microalgal screening data as well as *Artemia salina* ecotoxicity data. Semi-maximum response concentration that inhibited 50% mussel larval settlement (EC_{50}), response concentration that inhibited bacterial growth (EC) and the median lethal dose (LC_{50}) for each bioactive compound were assessed using Probit regression analysis, when applicable. Pearson Goodness-of-fit (Chi-Square) significance was considered at $p < 0.01$ for this analysis, and 95% lower and upper confidence limits (95% LCL; UCL) were presented. Therapeutic ratio (LC_{50}/EC_{50}) was used as a measure of effectiveness *vs* toxicity of compounds (Almeida and Vasconcelos, 2015; Qian et al., 2010; Rittschof, 1999). The software IBM SPSS Statistics 25 was used for statistical analysis.

3. Results and discussion

3.1. Synthesis and structure elucidation

A series of structure-related chalcone derivatives with different substitution patterns in A and B rings were synthesized (Fig. 1, **1**–**16**) and evaluated for their AF activity. The chemical diversity was selected in order to allow structure-activity relationship (SAR) studies. Chalcones **1**–**15** were prepared by base-catalysed aldol reaction of appropriately substituted acetophenones with benzaldehydes with moderate yields. Chalcone **16** was synthesized by the *O*-alkylation of the non prenylated chalcone **15** with prenyl bromide in presence of tetrabutylammonium hydroxide at room temperature leading to prenyloxychalcone **16** (73% yield) as previously reported (Neves et al., 2012). The synthetic approach followed for the synthesis of the new chalcone **7** is described in Scheme 1. Firstly, 2-hydroxy-4-methoxy-3-propylacetophenone was prepared by the methylation of 2,4-dihydroxy-3-propylacetophenone with dimethylsulfate in presence of anhydrous potassium carbonate and anhydrous acetone with quantitative yield. After, compound **7** was prepared by base-catalysed aldol reaction of 2-hydroxy-4-methoxy-3-propylacetophenone with 4-chlorobenzaldehyde by MW assisted organic synthesis, according to the strategy illustrated on Scheme 1.

The structure elucidation of all compounds was established on the basis of NMR and HRMS techniques. The HRMS allowed to confirm the molecular formula and the purity of compounds. The characterization of compound **9** was performed for the first time by ^1H, ^{13}C NMR and HRMS. ^{13}C NMR assignments were determined by 2D heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments. The coupling constants of the vinylic system ($J_{H\alpha\text{-}H\beta} = 16.0–15.0$ Hz) confirm the (*E*)-configuration for all synthesized chalcones.



Scheme 1. Synthesis of chalcone **7**.

3.2. Anti-settlement activity

Mytilus spp. are among the most common macrofouling species with worldwide representatives and studies focusing on anti-settlement bioassays with *Mytilus galloprovincialis* adhesive plantigrade larvae have produced consistent results (Almeida et al., 2017; Almeida and Vasconcelos, 2015; Bers and Wahl, 2004; Carl et al., 2011; Dobretsov, 1999; Rajagopal et al., 2003). Therefore, the anti-settlement of *M. galloprovincialis* larvae was selected as the first screening assay for the evaluation of the AF activity of the synthesized compounds.

From the series of chalcone derivatives tested, polymethoxylated chalcones **11**, **12**, and **16** presented highly significant anti-settlement responses (>80% inhibition) at 50 μM ($p < 0.01$) against the settlement of mussel *M. galloprovincialis* larvae when compared to the negative control (Fig. 2). When comparing the results of chalcones **1**–**7** with the same A ring substitution pattern, the results revealed that the presence of three methoxy groups on B ring at positions 3,4,5 (compound **1**) and 2,4,5 (compound **5**) is associated with a more favourable anti-settlement activity than the presence of a lower number of methoxy groups or a chlorine atom. In contrast, for chalcones **8**–**16** the best activity was obtained for compounds with only one methoxy group at B ring (compounds **11** and **12**). The presence of a 3,4,5-trimethoxyphenyl B ring is only associated with an enhancement of activity for the 2′-prenyloxychalcone **16**. In fact, when comparing the results of prenylated derivative **16** (55% inhibition at 50 μM) with the non prenylated chalcone **15** (10% induction at 50 μM) it seems that the presence of a prenyloxy group at C-2′ is associated with a significant ($p < 0.01$) enhancement of the anti-settlement activity. Together, these results may suggest the importance of the presence of a trimethoxyphenyl B ring associated with a lipophilic side chain in A ring, such as a propyl or a prenyl group, to induce bioactivity. It is noteworthy to point out that compound **16** is structure-related with 2′-methoxykurarinone, isolated from the Chinese medicinal plant *Sophora flavescens*, a AF flavonoid with a prenyl side chain also in A ring which has revealed to inhibit the settlement of cyprids of *B. albicostatus*, with low toxicity (Feng et al., 2009).

For compounds **11**, **12**, and **16** the anti-settlement activity was evaluated at lower and higher concentrations to determine the EC_{50} values (Fig. 3). Chalcone **12** (EC_{50} = 7.24 μM) showed to be the most effective larval settlement inhibitor, followed by compounds **16** (EC_{50} = 16.48 μM) and **11** (EC_{50} = 34.63 μM) (Table 1). Considering toxicity, none of these compounds caused mortality to the target species *M. galloprovincialis* plantigrades at the maximum tested concentration (200 μM), while the commercial AF agent ECONEA® was found to exert some toxicity (LC_{50} = 107.89 μM) (Table 1) (Almeida et al., 2017). Among these chalcones, the lower levels of toxicity and higher effectiveness to mussel larvae was observed for **12** with LC_{50} levels higher than 200 μM and LC_{50}/EC_{50} higher than 27.6, respectively. These results indicate that **12** had the best performance as promising AF agent against this target species, given the higher AF effectiveness, no toxicity until 200 μM, and therapeutic ratios higher than 25 (Fig. 3; Table 1). Noteworthy, although **12** presented lower levels of effectiveness than ECONEA® (Table 1), this compound has evidenced more potential as ecofriendly AF agent considering the non-toxic effects found.

3.3. QSAR model

QSAR have been successfully established to predict different types of biological activities and/or properties (Dudek et al., 2006). Accordingly, with the overall anti-settlement activity against *M. galloprovincialis* larvae results, a QSAR model was built to establish a relationship between the chalcones structures and their AF properties, which will which will allow the design of new active compounds. In this work, a 2D-QSAR model was built using Comprehensive Descriptors for Structural and Statistical Analysis (CODESSA) software package (CODESSA software version 2.7.2, University of Florida, USA). A large number of molecular descriptors divided into five categories (constitutional, topological, geometrical, electrostatic and quantum-chemical) were generated. The heuristic method proceeds with a preselection of descriptors by eliminating those descriptors that are not available for each structure, descriptors having a small variation in magnitude for all structures, descriptors found to be correlated pairwise, and descriptors found to be of no statistical significance. The heuristic method is a very useful tool for searching the best pool of descriptors. It is a quick method and

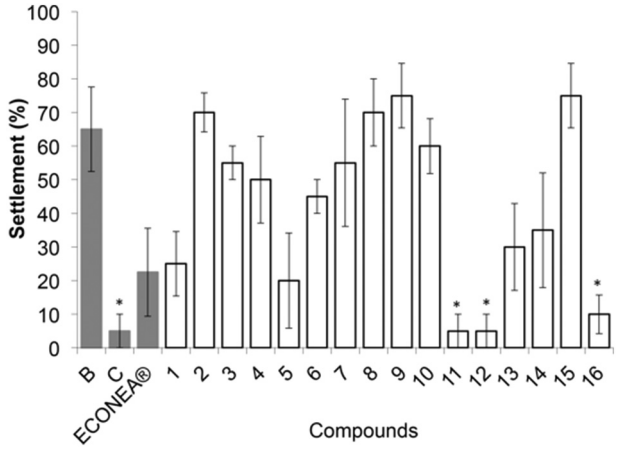


Fig. 2. Anti-settlement activity of compounds **1**–**16** at 50 μM towards plantigrade larvae of the mussel *Mytilus galloprovincialis*. * means significant differences at $p < 0.01$ (Dunnett test); B: DMSO control (0.01%); C: 5 μM $CuSO_4$ as positive control; ECONEA® (50 μM) was used for comparative purposes.

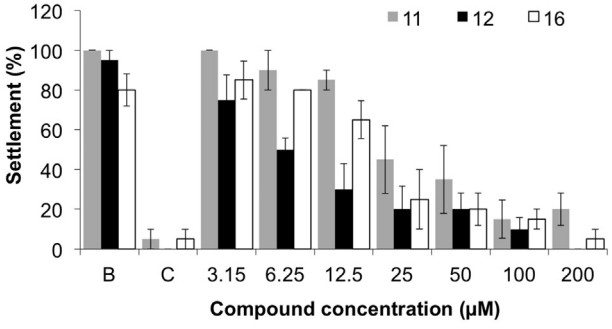


Fig. 3. Dose-response activity regarding anti-settlement of the promising compounds **11**, **12**, and **16**. B: DMSO control (0.01%); C: 5 μM $CuSO_4$ as positive control.

Table 1
Antifouling effectiveness and toxicity parameters of compounds **11**, **12**, and **16** towards mussel plantigrades.

Compound	EC_{50} (μM)	LC_{50} (μM)	LC_{50}/EC_{50}
11	34.63 (95% CI: 20.47–60.48)	>200	>5.78
12	7.24 (95% CI: 3.75–11.24)	>200	>27.61
16	16.48 (95% CI: 11.10–23.53)	>200	>12.14
ECONEA®	4.012 (95% CI: 0.38–9.54)	107.78	26.86

EC_{50}, minimum concentration that inhibited 50% of larval settlement; LC_{50}, the median lethal dose; LC_{50}/EC_{50}, therapeutic ratio; CI, confidence interval. Note: a therapeutic ratio (LC_{50}/EC_{50}) higher than 15 is recommended (Wang et al., 2017).

presents no restrictions on the size of the data set (Dunn and Hopfinger, 2002).

The correlation coefficient (R2) (a statistical measure of how close the data are to the fitted regression line), standard error (s) (which consists of an absolute measure of the quality of fit), Fisher's value (F) (which represents the F-ratio between the variance of actual and predicted activity), and cross-validation (Q2) (which measures the goodness-of-prediction) were employed to judge the validity of regression equation (Kubinyi, 1993). A major point in developing QSAR model is the number of descriptors used to elaborate the equation. Laws of QSAR establish that it should be one descriptor for each five molecules (Kubinyi, 1993); therefore, as the training set was composed of 12 molecules, 2 descriptors were used to build the QSAR models. The multilinear regression analysis using Heuristic method for 12 compounds in the 2-parameter model is represented by:

$$\begin{aligned} &AF = -0.14079\,HASA2 - 1.0969\,AIC2 + 6.0319 \\ &\left(R^2 = 0.8595, s^2 = 0.0118, F = 27.52, Q^2 = 0.8020\right) \end{aligned} \quad (1)$$

where HASA2 is the area-weighted surface charge of hydrogen bonding acceptor atom and AIC2 is Average Information content (order 2) (Fig. 4).

The best training model had a quality (R^2) of 0.8595, Fisher value (F) of 27.52, $s^2 = 0.0118$, and cross-validation (Q^2) of 0.8020, which demonstrate that the proposed model has statistical stability and validity (Bewick et al., 2003). The squared correlation coefficient R^2 is a relative measure of quality of fit by regression equation. Correspondingly, it represents >85% of the total variance ($R^2 = 0.8595$) in AF activity exhibited by the test molecules. Its value is close to 1.0 which represents the better fit to the regression line (Alexander et al., 2015). The F-test reflects the ratio of the variance explained by the model and the variance due to the error in the regression. High value of the F-test indicates that the model is statistically significant. The QSAR model is significant at 95% level as shown by their Fischer ratio values which exceed the tabulated values (4.25) as desired for a meaningful correlation (Liu and Long, 2009). Standard deviation s^2 is 0.0118 and this value expresses the variation of the residuals or the variation about the regression line. Thus, standard deviation is an absolute measure of quality of fit and should have a low value for the regression to be significant (Gramatica, 2013). The cross-validated R^2 (Q^2) process repeats the regression many times on subsets of data and R is computed using the predicted values of the missing molecules. Q^2 (0.8020) is smaller than the overall R^2 (0.8595), as expected, but still the difference between R^2 and Q^2 is lower than 0.3, which indicates that the model has good predictive power (Gramatica, 2013). External (test set) predictivity was used as validation criteria, and the model was able to predict the AF activity with an average difference of 0.28 from the experimental value (Golbraikh et al., 2003). From all the above, it can be concluded that the QSAR model is applicable for AF activity, which suggests that the model may have predictive capacity for more AF hits.

Descriptors selected as influential are those describing the ability of molecules to form hydrogen bonds (Area-weighted surface charge of hydrogen bonding acceptor atom - HASA2) and encoding the shape, branching ratio and constitutional diversity of the molecule (Average Information content (order 2) – AIC2).

Geometric descriptors affecting the shape of chalcones as well as relative charged surface area descriptors have already been described as important for antibiofouling activity (Sivakumar et al., 2010b). Moreover, an appropriate balance between hydrophobicity and hydrogen bond forming capacity has also been described as an influential physicochemical factor for the settlement inhibition (Moodie et al., 2017a). Very low logP values originate weak antifouling activity (compound **15** has the lowest logP value and the lowest antifouling activity) (Moodie et al., 2017a).

3.4. Acetylcholinesterase and tyrosinase activities

Molecular mechanisms of the AF compounds are largely source of speculation in the literature (Qian et al., 2013). Given the lack of precise information about the genomic and proteomic background of major fouling organisms, identifying molecular targets and mapping the mechanisms associated with AF activity remains a challenge. AF compounds described in literature with known molecular targets mainly act on ion channels, enzymes, adhesive production and neurotransmission blocking (Qian et al., 2013). The mechanisms of action of the most promising non-toxic AF compounds was investigated, concerning neurotransmission and adhesive metabolism, namely inhibition of AChE and Tyr activities. These functions modulation is known to have effects on organism settlement and thus are described as a mode of action of some AF bioactive compounds (Chen and Qian, 2017). In fact, it has been reported that AChE may be involved in the settlement of marine organisms, and that Tyr is a phenoloxidase enzyme with an important role in the formation of the DOPA-containing adhesive plaques of mussels (Faimali et al., 2003; Hellio et al., 2000; Mansueto et al., 2012).

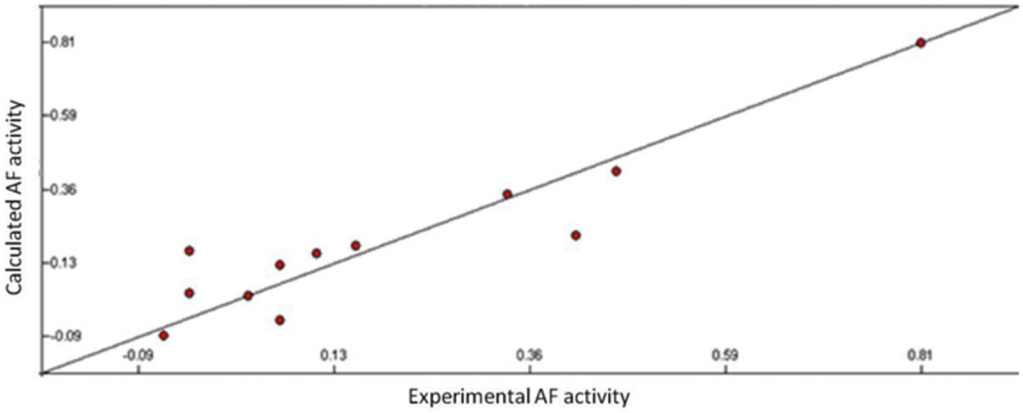


Fig. 4. Plot of experimental *versus* calculated AF activity according to QSAR model.

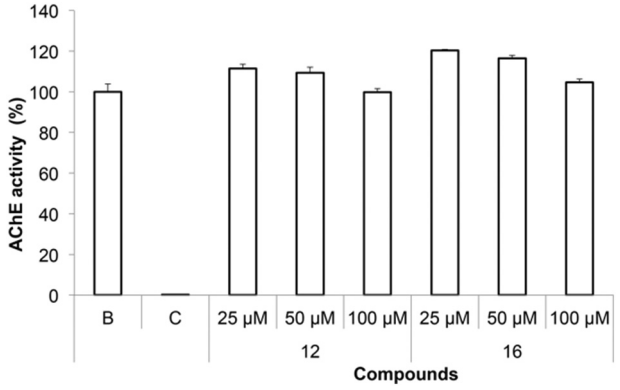


Fig. 5. Acetylcholinesterase activity of the most promising AF chalcones **12**, and **16**. B: 1% DMSO. C: Eserine (200 μM).

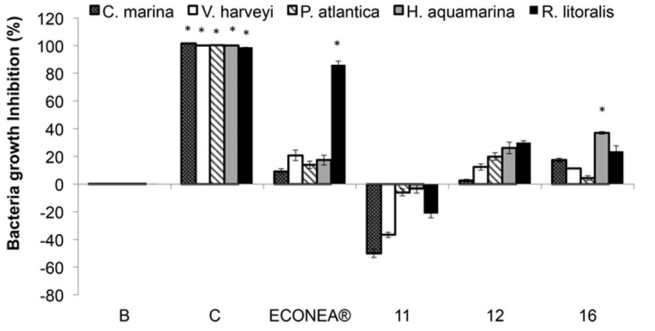


Fig. 7. Antibacterial activity of compounds **11**, **12**, and **16** at 9.7, 9.7 and 6.9 μM, respectively towards five biofilm-forming marine bacteria *Cobetia marina*, *Vibrio harveyi*, *Pseudoalteromonas atlantica*, *Halomonas aquamarina and Roseobacter litoralis*. *indicates significant diferences ($p < 0.01$, Dunnett test) against the negative control (B: 0.1% DMSO); 1:100 penicilin-streptomycin-neomycin stabilized solution (Sigma P4083) was used as positive control (C); ECONEA® was used for comparative purposes.

However, AChE (Fig. 5) and Tyr (Fig. 6) inhibition was not found for any of the tested compounds, indicating that the observed AF activity seems to be not related with these metabolic functions.

3.5. Antibacterial and anti-microalgal activities

Marine biofilms are known to modulate in some degree the succession of colonization of macrofouling species by producing specific chemical cues that attract or repulse the successive fouling species (Hadfield, 2011; Hadfield and Paul, 2001). For that reason, efforts on AF screenings are often based only on anti-biofilm properties (Ding et al., 2018; Salta et al., 2013). However, the AF response at different levels of biological organization, including both macrofoulers and biofilm-forming species is ideal to draw the full picture of promising AF agents (Martin-Rodriguez et al., 2015). In this study, marine biofilm-forming bacterial and microalgae (diatoms) were used to broad the AF effectiveness assessment and better understand the selectivity of the promising compounds towards microfouling species.

Among the three compounds selected from the initial screening as potential AF compounds (**11**, **12**, and **16**), compound **11** was not able to prevent microfouling (biofilms), causing an induction of growth of all the strains tested in both antibacterial and antidiatom screenings (Figs. 7 and 8). In contrast, compounds **12** and **16** showed inhibitory activity against bacterial growth of *H. aquamarina* (EC_{50} = 18.67 and 18.78 μM, respectively), in higher levels than observed for ECONEA (EC_{30} = 15.31 μM) and also against *Roseobacter litoralis* (EC_{50} = 4.09 and 12.34 μM, respectively) (Fig. 7, Table 2), being however this last strain less sensitive to compounds **12** and **16** than to ECONEA® (EC_{50} = 0.22 μM). Compounds **12** and **16** showed lower bioactivity against all the other bacteria tested (<25%).

Compounds **12** and **16** also showed a promising inhibitory effect of microalgal growth. Particularly, compound **16** significantly ($p < 0.01$) inhibited the growth of the biofilm-forming marine diatom *Navicula* sp. (EC_{50} = 6.75 μM), showing also some inhibitory effect against the growth of other tested diatoms (EC_{50} 7.04–20.31 μM) (Fig. 8, Table 2). Chalcone **12** displayed a low growth inhibition towards *Cylindrotheca* sp., *Nitzschia* sp. and *Navicula* sp. (<25% inhibition), and any effect on *Halamphora* sp. (Fig. 8, Table 2).

Concerning the anti-microalgal bioassays, the observed bioactivity for chalcones is below the levels of efficacy obtained for ECONEA® (~50% growth inhibition at the same concentration). Thus, these two compounds seem to affect in some way the marine biofilm community, however being not as potent as ECONEA® concerning anti-microalgal properties. Nevertheless, compound **12** and **16** were able to inhibit the growth of microfouling biofilms (EC_{50} 4.09–20.31 μM) allied with the previously mentioned *Mytillus* larval anti-settlement activity, being these characteristics together very promising to take these compounds in consideration as AF agents. The broad AF responses of compounds **12** and **16** towards different levels of biological organization (micro and macro species) highlights that the target of these compounds is most probably acting on the biofouling colonization succession, rather than on a specific mechanism related with invertebrate adhesion. This is also confirmed by the negative responses towards the modulation of the studied enzymes AChE and Tyr.

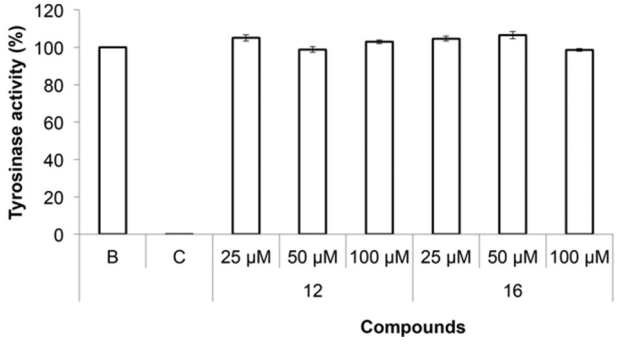


Fig. 6. Tyrosinase activity of the most promising AF chalcones **12**, and **16**. B: 1% DMSO. C: Kojic acid (1.4 mM).

3.6. Ecotoxicity assessment

For the most promising AF compounds (**11**, **12**, and **16**) the potential ecotoxicity was assed using the nauplii of the marine crustacean *Artemia salina* at concentrations ranging from 50 μM and the concentration

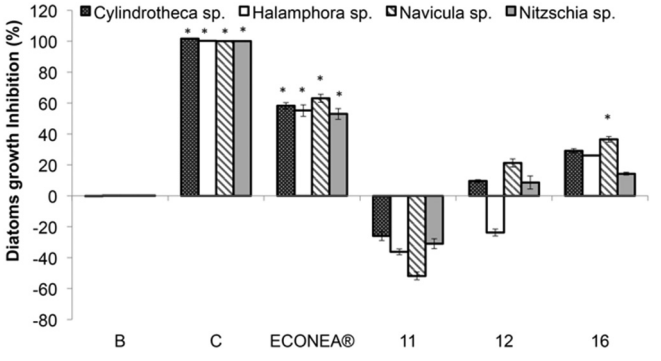


Fig. 8. Anti-microalgal activity of compounds **11**, **12**, and **16** at 9.7, 9.7 and 6.9 μM, respectively, towards four biofilm-forming marine diatoms *Cylindrotheca* sp., *Halamphora* sp., *Nitzschia* sp. and *Navicula* sp. *indicates significant diferences ($p < 0.01$, Dunnett test) against the negative control (B: 0.1% DMSO); Cycloheximide (3.55 μM) was used as positive control (C); ECONEA® was used for comparative purposes (8.7 μM).

Table 2
Bacteria and microalgal growth inhibition of compounds **12** and **16** *vs* ECONEA®.

		ECONEA®		**12**	**16**
		EC_{30} (μM)	EC_{50} (μM)	EC_{50} (μM)	EC_{50} (μM)
Bacteria	*Halomonas aquamarina*	15.31 (95% CI: 7.3–70.4)	–	18.67 (95% CI: 8.3–98.6)	18.78 (95% CI: 13.4–31.1)
	Roseobacter litoralis	–	0.22 (95% CI: 0.004–0.517)	4.09 (95% CI: 0.9–8.2)	12.34 (95% CI: 9.8–16.4)
Diatoms	*Cylindrotheca* sp.		5.22 (95% CI: 2.9–10.8)		7.04 (95% CI: 6.1–8.2)
	Halamphora sp.		6.04 (95% CI: 5.1–7.2)		14.65 (95% CI:9.7–60.9)
	Nitzschia sp.		4.97 (95% CI: 2.6–11.0)		20.31 (95% CI:13.4–60.6)
	Navicula sp.		5.65 (95% CI: 2.8–15.9)		6.75 (95% CI: 5.3–8.7)

EC_{30} and EC_{50}, minimum concentration that inhibited 30 and 50% of bacterial and diatoms growth, respectively; CI, confidence interval.

correspondent to the anti-settlement EC_{50} value for each compound. For compounds **12** and **16** no significant differences were found at the concentrations tested against the negative control ($p < 0.01$), however results showed slightly >10% mortality at the concentration of 50 μM for both compounds (13.92% and 14.03%, respectively) (Fig. 9). The observed lethality is not significantly different ($p < 0.01$) from the negative control (filtered seawater) and thus these compounds are considered non-toxic to this non-target species. Compound **11** showed significant toxicity even with a concentration (25 μM) below the EC_{50} value for mussel larvae anti-settlement activity (34.63 μM) (Fig. 9). Overall results suggest that compounds **12** and **16** are more suitable as AF agents considering the lower toxicity to the environment.

4. Conclusions

The present scenario of marine antifoulants calls for more environmental compatible alternatives than the active principles now in use. Natural products are one of the most promising sources of nontoxic AF agents. Nevertheless, the development of suitable AF agents requires the compounds produced in high amounts, and this is often not possible to reach by isolation from natural sources. Alternatively, new agents obtained from chemical synthesis can mimic promising non-toxic natural products with the advantage of being produced in large scale, in a short period of time, avoiding the exhaustion of the natural resources.

In this study synthetic chalcone derivatives were obtained with good yields and their potential for AF application was disclosed, particularly for polymethoxylated chalcones **11**, **12**, and **16**. Chalcones **12** and **16** showed high anti-settlement potential ($EC_{50} < 25\ \mu M^{-1}$) towards larvae of *Mytilus galloprovincialis*, and some inhibitory effect on bacteria and diatoms growth, suggesting their potential in the suppression of biofouling colonization succession. The ecotoxicity study on both target and non-target species revealed that these compounds did not show toxicity at environmentally relevant concentrations. This research describes for the first time the potential application of the synthesized chalcone derivatives to prevent biofouling, giving inputs to their consideration as promising candidates to a new generation of non-toxic AF agents, and contributing to minimize the economic, environmental, and health problems associated with biofouling.

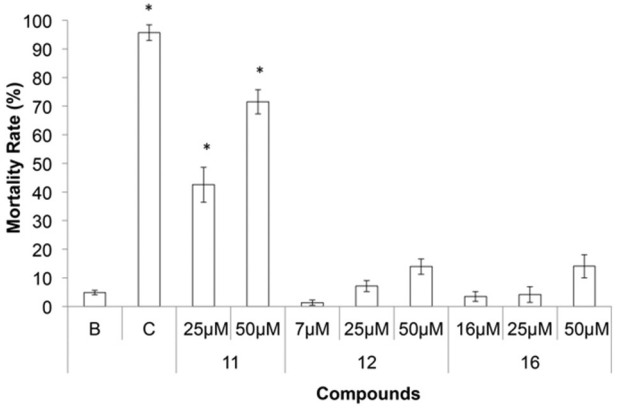


Fig. 9. Mortality rate of *Artemia salina* nauplii after 48 h of exposure to the promising chalcones **11**, **12**, and **16**. B: 1% DMSO in filtered seawater. C: $K_2Cr_2O_7$ at 13.6 μM. * means significant differences at $p < 0.01$ (Dunnett test).

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Author contributions

MCS planned all the aspects of this study. Synthesis procedures were performed by JM and DP. HC and MP designed the experimental work concerning the synthesis. QSAR model was constructed by AP. *In vivo* AF bioassays were performed by JRA and SP and antibacterial and anti-microalgal experiments were performed by JA and JRA. *Artemia salina* bioassays were performed by JRA and SP. *In vitro* enzymatic assays were executed by JRA. JRA and VV designed the experimental work concerning the biological activity. JRA and HC wrote this manuscript. MCS, VV, and MP had an important contribution in the revision of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.06.169.

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