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Overcoming environmental problems of biocides: Synthetic bile acid derivatives as a sustainable alternative

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ABSTRACT

Marine biofouling represents a global economic and ecological challenge. Some marine organisms produce bioactive metabolites, such as steroids, that inhibit the settlement and growth of fouling organisms. The aim of this work was to explore bile acids as a new scaffold with antifouling (AF) activity by using chemical synthesis to produce a series of bile acid derivatives with optimized AF performance and understand their structure-activity relationships. Seven bile acid derivatives were successfully synthesized in moderate to high yields, and their structures were elucidated through spectroscopic methods. Their AF activities were tested against both macro- and microfouling communities. The most potent bile acid against the settlement of *Mytilus galloprovincialis* larvae was the methyl ester derivative of cholic acid (**10**), which showed an EC_{50} of 3.7 μM and an $LC_{50}/EC_{50} > 50$ ($LC_{50} > 200$ μM) in AF effectiveness *vs* toxicity studies. Two derivatives of deoxycholic acid (**5** and **7**) potently inhibited the growth of biofilm-forming marine bacteria with EC_{50} values < 10 μM, and five bile acids (**1**, **5**, and **7**–**9**) potently inhibited the growth of diatoms, showing EC_{50} values between 3 and 10 μM. Promising AF profiles were achieved with some of the synthesized bile acids by combining antimacrofouling and antimicrofouling activities. Initial studies on the incorporation of one of these promising bile acid derivatives in polymeric coatings, such as a marine paint, demonstrated the ability of these compounds to generate coatings with anti-macrofouling activity.

1. Introduction

Marine biofouling is a complex biological process involving the accumulation of microorganisms, algae, and animals on a submerged surface (Simone Durr, 2010). When this process occurs on artificial marine surfaces, such as ship hulls, enormous economic and ecological problems can result (Gallardo et al., 2016; Demirel et al., 2017). The attachment of micro- and macroorganisms on the surface of ship hulls decreases speeds, which consequently increases fuel consumption (Schultz et al., 2011; Demirel et al., 2017), ultimately leading to increased air pollution and associated ecological problems. Additionally, marine biofouling promotes the spread of invasive aquatic species, which has well-recognized global effects through the food chains of aquatic environments (Gallardo et al., 2016) and has a negative influence on global biodiversity (Bax et al., 2003).

For a long time, marine biofouling treatments were based on biocides, namely, tributyltin (TBT) and copper (Yebra et al., 2004). However, the toxicities of these biocides against nonfouling marine organisms is now widely recognized (Katranitsas et al., 2003; Schiff et al., 2004; Micael et al., 2007; Zhang et al. 2011, 2013b; Yu et al., 2013; Zhang et al., 2016). After the banning of TBT in Europe in 2008, booster biocides were introduced in the market. Some of them,

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including Irgarol® 1501 and diuron, act as herbicides/pesticides and are used in conjunction with copper-based antifouling (AF) agents. However, several studies have shown that these compounds are also toxic to marine ecosystems and nonfouling marine organisms (Okamura et al., 2000; Owen et al., 2002; Negri et al., 2005; Thomas and Brooks, 2010; Bao et al., 2011; Mai et al., 2013; Jung et al., 2017; Carvalhal et al., 2018). Therefore, the development of new environmentally friendly alternatives to these treatments is of great importance.

One of the most environmentally friendly AF strategies to combat marine biofouling is the use of chemical defenses of sessile marine organisms that naturally remain free of fouling (Fusetani, 2011). This strategy has been explored by researchers, and hundreds of compounds with AF activities have been isolated from marine organisms and reviewed in the literature (Dobretsov et al., 2006; Fusetani, 2011; Qian and Ying Xu, 2012; Qi and Ma, 2017; Vilas-Boas et al., 2017). AF activities have been described for diverse steroid classes, such as sulfated steroids, polyhydroxy sterols, pentacyclic steroids, secosteroids, and A-nor steroids (Nakatsu et al., 1983; Iorizzi et al., 1995; Mizobuchi et al., 1996; Tsukamoto et al., 1997; Sera et al., 1999; Tomono et al., 1999a, 1999b; Qiu et al., 2008; Qi et al., 2010; Cho, 2012; Liao et al., 2012; Zhang et al., 2013a; Zhang et al., 2014; Chen et al., 2015; Carvalhal et al., 2018). Although natural compounds represent an interesting platform for the development of environmentally friendly AF agents, the yields of natural compounds from marine organisms are generally poor, hindering their development as AF agents (Nogata and Kitano, 2006). Our group has been focused on the synthesis of bio-inspired AF molecules as alternatives to circumvent the abovementioned limitations (Almeida et al., 2017; Almeida et al., 2018).

Compounds possessing a carboxylic acid in their structure have been reported as promising AF agents (Catto et al., 2015; Almeida et al., 2017).

In this work, bile acids were hypothesized to be an interesting platform for the development of new AF agents due to the presence of both steroid cores and carboxylic acids in their structure. To the best of our knowledge, bile acids are an unexplored scaffold with respect to AF activity.

A series of deoxycholic acid (**1**) derivatives with diverse polarities and with the ability to form both hydrophobic and electrostatic interactions was designed (Fig. 1). After validation by performing AF bioassays, the modifications resulting in the best outcomes were also applied to other two bile acids, chenodeoxycholic acid (**2**) and cholic acid (**3**) (Fig. 1). The AF effects of the synthesized derivatives as well as of the three parent bile acids - deoxycholic acid (**1**), chenodeoxycholic acid (**2**), and cholic acid (**3**) - were assessed through a set of AF bioassays, including antisettlement bioassays against the mussel macrofouler species *Mytilus galloprovincialis* and antimicrofouling tests against five biofilm-forming marine bacteria and four representative biofouling microalgae species.

The marine ecotoxicity was evaluated using *Artemia salina* ecotoxicity tests and bioaccumulation potential was also predicted for the most promising compounds. Finally, the AF activity of one of the most promising synthesized bile acid derivatives was also evaluated after being incorporated in a marine paint.

2. Materials and methods

2.1. General methods

All experiments were conducted in accordance with the ethical guidelines of the European Union Council (Directive, 2010/63/EU) and the Portuguese Agricultural Ministry (Portaria nr. 1005/92 of 23 October 2010) for the protection of animals used for experimental and other scientific purposes. Deoxycholic acid (**1**, D2510), triethylamine-sulfur trioxide complex (TEA·SO_3, S5139), and ethylenediamine (EDA, 03550) were purchased from Sigma-Aldrich (Spain); chenodeoxycholic acid (**2**, C0750) was purchased from TCI (Zwijndrecht, Belgium); and cholic acid (**3**, 159110250) was purchased from Acros Organics (Geel, Belgium). Solvents were of analytical grade and were purchased from Sigma-Aldrich. Spectra/Por Dialysis membranes (MWCO 1000) were purchased from Spectrum Laboratories, Inc. (California, USA). Sodium hydroxide (NaOH, 18244.295) and hydrochloric acid (HCl, 20252.290) were purchased from VWR Chemicals (Portugal). Microwave (MW) reactions were performed using a MicroSYNTH 1600 synthesizer from Millestone (ThermoUnicam, Portugal) in open reaction vessels (30 mL). TLC separations were performed using Merck silica gel 60 (GF254) plates, and flash column chromatography separations were performed

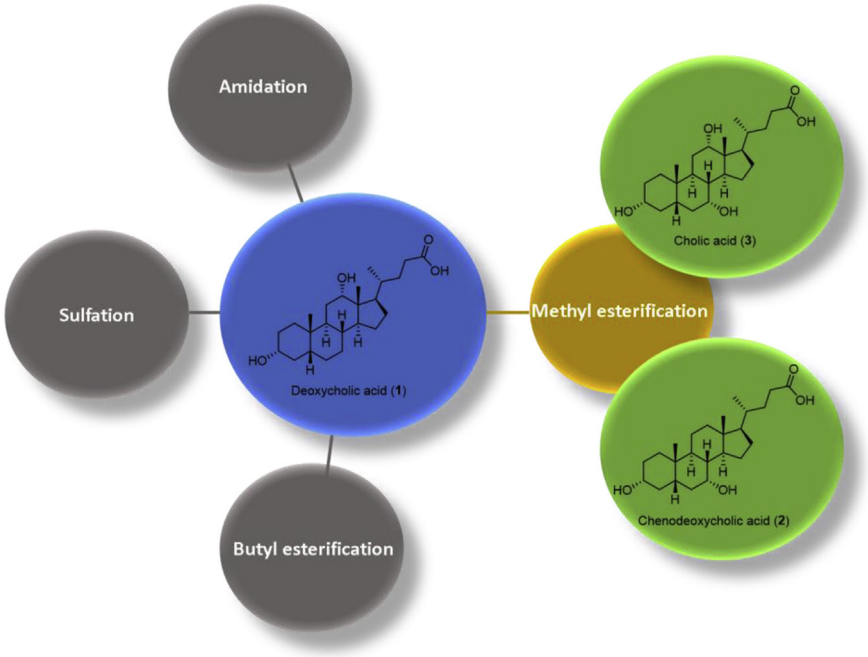


Fig. 1. Chemical structures of deoxycholic acid (**1**), chenodeoxycholic acid (**2**), and cholic acid (**3**) and modifications performed to generate the corresponding derivatives. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

using Fluka silica gel 60 (0.04–0.063 mm). Compounds were visually detected after developing the TLC plates with 80% H_2SO_4 in methanol (MeOH) or 3 mg mL^{-1} ninhydrin in ethanol followed by heat activation. Melting points were obtained using a Köfler microscope and are uncorrected. Infrared (IR) spectra were recorded on a PerkinElmer 257 instrument using KBr for bile acids and derivatives. For the bile acid-based coatings, Fourier transform infrared (FTIR) spectroscopy was conducted on a Nicolet Magna FTIR 550 spectrometer coupled to an attenuated total reflectance unit (Smart MiracleTM-Pike Technologies) with a ZnSe single crystal. Studies were carried out over the frequency range of 500–4000 cm^{-1} with 4 cm^{-1} resolution. ^1H and ^{13}C Nuclear magnetic resonance (NMR) spectra were acquired in $CDCl_3$ or DMSO-d_6 at room temperature on a Bruker Avance 300 instrument (300.13 MHz for ^1H and 75.47 MHz for ^{13}C). Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as an internal reference. High-resolution mass spectrometry (HRMS) data were recorded in ESI (electrospray ionization) mode on an APEXQe FT-ICR MS instrument (Bruker Daltonics) equipped with a 7 T actively shielded magnet at C.A.C.T.I.-University of Vigo, Spain.

2.2. General method for the synthesis of ester derivatives

To understand the importance of the carboxyl group in bile acids on the AF activity, this functional group was modified via esterification with different alkyl chains. The synthesis of the ester derivatives was adapted from Lee et al. (2007). Generally, the starting materials, deoxycholic acid (**1**), chenodeoxycholic acid (**2**), and cholic acid (**3**) (0.5–5.1 mmol), were dissolved in the appropriate alcohol, MeOH or *n*-butanol (BuOH) (3–10 mL) and acidified with conc. HCl (0.1–0.2 mL). The mixtures were stirred and heated to reflux for 1 h. The solvent was evaporated under reduced pressure, and the crude products were purified either by flash column chromatography (for methyl deoxycholate (**5**), butyl deoxycholate (**6**), and methyl chenodeoxycholate (**9**)) or by crystallization from MeOH (methyl cholate (**10**)). The NMR data were in accordance with the literature for compounds **5** (Pore et al., 2006), **6** (Marples and Stretton, 1990), **9** (Stoltz et al., 2017), and **10** (Pore et al., 2006).

Methyl deoxycholate (5): White solid (0.54 g, 1.3 mmol, 51% yield). mp 66–68 °C (Chloroform ($CHCl_3$):MeOH); lit. 82–105 °C (Pore et al., 2006); HRMS (ESI^+) m/z calcd for $C_{25}H_{42}O_4Na$ 429.29753, found 429.29836.

Butyl deoxycholate (6): White solid (0.092 g, 0.21 mmol, 40% yield). mp 106–109 °C ($CHCl_3$:MeOH); HRMS (ESI^+) m/z calcd for $C_{28}H_{49}O_4$ 449.36254, found 449.36129.

Methyl chenodeoxycholate (9): White solid (1.3 g, 3.2 mmol, 63% yield). mp 69–70 °C ($CHCl_3$:MeOH); HRMS (ESI^+) m/z calcd for $C_{25}H_{42}O_4Na$ 429.29753, found 429.29847.

Methyl cholate (10): White solid (1.99 g, 4.7 mmol, 96% yield). mp 154–155 °C (MeOH); lit. 155–156 °C (Pore et al., 2006); HRMS (ESI^+) m/z calcd for $C_{25}H_{42}O_5Na$ 445.29245, found 445.29337.

2.3. General method for the synthesis of sulfated derivatives

In nature, sulfation is used by organisms to decrease the toxicity of xenobiotics; thus, it was assumed that the synthesis of sulfated derivatives could lead to nontoxic antifoulants (Almeida et al., 2017). Additionally, some marine sulfated steroids have been reported to have AF activities (Carvalhal et al., 2018). The synthesis of the sulfated derivatives was adapted from Correia-da-Silva et al. (2011b) Generally, the starting materials, deoxycholic acid (**1**) and methyl deoxycholate (**5**) (1.2–1.3 mmol), were dissolved in dimethylacetamide (DMA, 12–14 mL), and TEA·SO_3 (3 eq./OH) was added. The mixtures were kept for 2–3 h under MW irradiation (200 W) at 100 °C (compound **1**), or conventional heating (compound **5**). After cooling, diethyl ether was added, and the solutions were kept at 4 °C overnight. The formed solids were separated by filtration and washed several times with ether and then dissolved in a methanolic solution of NaOH (0.1 N). The obtained insoluble sodium sulfate was removed by filtration, and the filtrate was concentrated to dryness to afford the desired compound, deoxycholic acid disulfate (**4**). In the case of methyl deoxycholate monosulfate (**8**), the filtrate was further purified by flash column chromatography ($CHCl_3$:MeOH - gradient from 85:15–100% MeOH).

Deoxycholic acid disulfate (4): Pearl white solid (0.704 g, 1.18 mmol, 93% yield). mp: 200–203 °C (MeOH); 240 °C dec. (ether) (Pageaux et al., 1979); HRMS (ESI^+) m/z calcd for $C_{24}H_{38}O_{10}S_2Na_3$ 619.15940, found 619.16080.

Methyl deoxycholate monosulfate (8): White solid (0.0199 g, 0.039 mmol, 3% yield). IR (KBr) υ_{max}: 2940, 2869, 1733, 1210, 1072, 946, 872 cm^{-1}; ^1H NMR (DMSO-d_6, 300.13 MHz) δ: 4.23 (1H, d, J = 6.0 Hz, OH), 3.97 (1H, brs, H-3), 3.78 (1H, m, H-12), 3.57 (3H, s, O–CH_3), 2.37–0.99 (27H, m, H-steroid), 0.90 (3H, d, J = 6.0 Hz, 21-CH_3), 0.85 (3H, s, 19-CH_3), 0.59 (3H, s, 18-CH_3) ppm; ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 173.8 (C-24), 75.8 (C-3), 71.0 (C-12), 51.2–23.5 (18C, steroid), 23.0 (C-19), 16.9 (C-21), 12.4 (C-18) ppm; HRMS (ESI^-) m/z calcd for $C_{25}H_{42}O_7S$ 486.26458, found 486.26501.

2.4. Synthesis of N-deoxycholylethylamine

Alkyl amines are known for their antifungal, disinfectant, antiseptic and AF properties (Finlay and Callow, 1996). The introduction of an alkyl amine on a steroid core could generate compounds with AF properties; therefore, the influence of a primary alkyl amine on AF activity was studied. Methyl deoxycholate (**5**, 0.5 g, 1.02 mmol) was dissolved in EDA (5 mL), and the mixture was kept under reflux for 6 h. The obtained solution was poured into ice, and the solid was separated by filtration to afford *N*-deoxycholylethylamine (**7**) as a white solid (0.43 g, 0.99 mmol, 97% yield) (Lee et al., 2007). mp 120–123 °C (water); lit. 98 °C dec (ethanol/ethyl acetate) (Hill et al., 1981); HRMS (ESI^+) m/z calcd for $C_{26}H_{47}N_2O_3$ 435.35812, found 435.35609.

2.5. In vivo antisettlement bioassay withMytilus galloprovincialis larvae

The problems associated with the fouling of man-made surfaces are caused mostly by the colonization of macrofoulers, such as mussels. Although the Mediterranean mussel (*Mytilus galloprovincialis*) is native to the Mediterranean, Black, and Adriatic Seas, this species has spread (mostly via ballast water and ship hull fouling) to many other regions (Branch and Nina Steffani, 2004). Accordingly, the IUCN/SSC* Invasive Species Specialist Group has listed *Mytilus galloprovincialis* among the 100 “World's Worst” invaders (Lowe et al., 2000). Additionally, the increase in ship weight caused by mussels negatively impacts the marine industry.

In this direction, the AF activity of the three bile acids (**1–3**) and the seven synthesized bile acid derivatives (**4–10**) was firstly evaluated using *Mytilus galloprovincialis* larvae. Aggregates of juvenile *Mytilus galloprovincialis* were collected from intertidal mussel beds during low neap tides at Memória beach, Matosinhos, Portugal (41°13′59″N; 8°43′28″W), and immediately transported to the laboratory. *Mytilus galloprovincialis* plantigrade larvae (0.5–2 mm) were screened from the mussel samples with a binocular magnifier (Olympus SZX2-ILLT), cleaned and isolated in petri dishes with filtered seawater for the bioassays. *In vivo* antisettlement assays with *Mytilus galloprovincialis* plantigrade larvae were performed according to a previously validated methodology (Almeida et al., 2017). Briefly, competent *Mytilus galloprovincialis* plantigrades showing foot exploring behavior were selected for exposure to the test compounds at 50 μM in 24-well microplates for 15 h at 18 ± 1 °C in darkness. The test solutions were prepared by diluting the stock solutions of the compounds (50 mM) in DMSO. Four well replicates were used per set of conditions with five plantigrades per well. A negative control with DMSO was included in all bioassays, as was a positive control with 5 μM $CuSO_4$ (a potent AF agent). At the end of the exposure period, the antisettlement activity was determined

by the presence/absence of efficiently attached byssal threads by each individual for all the conditions tested. Compounds that showed anti-settlement efficacy at 50 μM were selected for further testing at higher and lower concentrations (50, 25, 12.5, 6.25, and 3.12 μM) to determine the half-maximal response concentrations (the concentration required to inhibited 50% of larval settlement (EC_{50})), and the eventual mortality was recorded.

2.6. In vitro determination of AChE activity

Inhibitors of acetylcholinesterase (AChE), the enzyme responsible for the hydrolytic metabolism of acetylcholine, can inhibit invertebrate larval settlement (Almeida et al., 2015). The cholinergic system is responsible for modulating motor and sensor functions in synaptic neurons by the maintenance of the neurotransmitter acetylcholine. Therefore, AChE activity determination is useful as a potential target for AF compounds.

Thus, AChE inhibition was tested as a potential AF mechanism for compounds **5**, **8–10** (EC_{50} (Mytilus) < 10 μM). AChE activity was evaluated using *Electrophorus electricus* AChE Type V–S (SIGMA C2888, E.C. 3.1.1.7) according to the procedure described by Ellman et al. (1961) with some modifications (Almeida et al., 2015). Briefly, the reaction solution containing phosphate buffer (1 M, pH 7.2), dithiobisnitrobenzoate (DTNB, 10 mM dithiobisnitrobenzoic acid and sodium hydrogen carbonate in phosphate buffer) and acetylcholine iodide (0.075 M) were added to pure AChE enzyme (0.25 U mL^{-1}) and each compound was tested in quadruplicate. The optical density was measured at 412 nm using a microplate reader for 5 min at 25 °C. A negative control (B) with DMSO and a positive control (C) with eserine at 200 μM were included.

2.7. Antibacterial bioassay

Marine biofouling includes primary colonization of the substrate by microorganisms that form biofilms, and can modulate the settlement of macrofouling organisms (Qian et al., 2007; Antunes et al., 2018). Therefore, biofilm inhibition together with the inhibition of macrofouling organisms settlement is considered necessary when developing efficient AF compounds (Cho, 2012; Antunes et al., 2019).

The antibacterial activities of the three bile acids (**1–3**) and the seven synthesized bile acid derivatives (**4–10**) were evaluated against 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, all from the Spanish Type Culture Collection (CECT), according to Almeida et al. (2018). The bacteria were inoculated and incubated for 24 h at 26 °C in marine broth (Difco) at an initial density of 0.1 (OD600) in 96-well flat-bottom microtiter plates. Stock solutions of the test compounds were prepared in ultra-pure water. For the initial screening by this bioassay, final concentrations of 12.5 μM were used given that the bacteria are more sensitive than the mussel plantigrades. Bacterial growth inhibition in the presence of the tested compounds (**1–10**) was measured in quadruplicate by reading the optical density at 600 nm using a microplate reader (Biotek Synergy HT, Vermont, USA). Marine broth with ultra-pure water and 1:100 diluted penicillin-streptomycin-neomycin-stabilized solution (P4083, Sigma-Aldrich, St. Louis, MO, USA) were used as negative (B) and positive (C) controls, respectively. Compounds that showed significant inhibitory activities in this initial screening were selected for further bioassays. To determine the effective inhibitory concentrations (EC_{50} values), a stock solution was serially diluted to obtain final concentrations from 1 μM to 32 μM, and the same procedure was applied. For ECONEA®, only the EC_{30} value against *Halomonas aquamarina* could be determined; therefore, the results obtained for the bile acids cannot be compared with the results of this AF compound.

2.8. Antimicroalgal bioassay

Along with bacteria, benthic diatoms are among the first microorganisms to colonize submerged surfaces. Thus, diatoms also contribute to the problems associated with marine biofouling by initiating the process that leads to the attachment of macrofoulers (Salta et al., 2013).

The antimicroalgal activities of the three bile acids (**1–3**) and the seven synthesized bile acid derivatives (**4–10**) were evaluated against four strains of benthic marine diatoms, *Cylindrotheca* sp., *Halamphora* sp., *Nitzschia* sp., and *Navicula* sp., purchased from the Spanish Collection of Algae (BEA). The diatoms were inoculated in f/2 medium (Sigma) at an initial concentration of 2–4 × 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 each compound at 12.5 μM was determined in quadruplicate and quantified based on the difference in cell densities among the treatments, and cells were counted using a Neubauer counting chamber. The negative control was f/2 medium with 0.1% DMSO, and the positive control was cycloheximide (3.55 μM). Compounds producing < 25% growth inhibition were considered inactive. Compounds that showed significant inhibitory activities in the screening assays were selected for further determination of their effective inhibitory concentrations (EC_{50} values). The stock solutions of the test compounds were serially diluted to obtain solutions with concentrations from 1 μM to 32 μM.

2.9. Ecotoxicity assay with Artemia salina

Artemia salina is one of the most popular test organisms available for ecotoxicity research due to its sensitivity, ease of culture, short generation time, cosmopolitan distribution and the commercial availability of its dormant eggs (cysts) (Libralato et al., 2016).

Compounds with both macro and micro AF activities (**5** and **8–10**) were selected for ecotoxicity investigation using the brine shrimp (*Artemia salina*) nauplii lethality test (Meyer et al., 1982; Almeida et al., 2017). *Artemia salina* eggs were placed in nutrient-enriched seawater for approximately 48 h at 25 °C to allow them to hatch. Two test solutions (25 and 50 μM) were prepared in filtered seawater for each of the selected compounds. When applicable, a test solution at the concentration corresponding to the antisettlement EC_{50} value for each compound was also prepared. Toxicity tests were performed in darkness, in 96-well microplates, with eight replicates per set of conditions, and with 15–20 nauplii per well. A positive control, $K_2Cr_2O_7$ (13.6 μM), was included, and 1% DMSO was used as a negative control. The percentage of mortality was assessed at the end of the exposure period.

2.10. In silico prediction of $LogK_{ow}$

Bioaccumulation is one of the factors to consider when evaluating the hazards posed by any substance released into the environment. The theoretical $LogK_{ow}$ value is used as an indicator of the bioaccumulation potential of AF compounds. KOWWIN™ v1.68 (a Log octanol-water partition coefficient calculation program) (EPA, 2016), developed jointly by Syracuse Research Cooperation and the Environmental Protection Agency (EPA), was used for the *in silico* calculation of $LogK_{ow}$ (octanol-water partition coefficient) of the compounds with both macro and micro AF activity (**5** and **8–10**).

2.11. Statistical analysis

The data obtained from the antisettlement, antibacterial, antimicroalgal, and ecotoxicity assays were analyzed using a one-way analysis of variance (ANOVA) followed by a Dunnett's test against the DMSO control ($p < 0.05$). When applicable, a probit regression analysis was applied to assess the half-maximal response concentration (concentration that inhibited 50% of mussel larval settlement, EC_{50}),

the concentration that inhibited 50% of bacterial and microalgal growth (EC_{50}) and the median lethal concentration (LC_{50}) for each bioactive compound. A Pearson goodness-of-fit (chi-square) significance was considered at $p < 0.01$, and 95% lower and upper confidence limits (95% LCL; UCL) are presented. The therapeutic ratio (LC_{50}/EC_{50}) was used as a measure of the effectiveness *vs* toxicity of the compounds (Qian et al., 2010; Almeida and Vasconcelos, 2015). The statistical analyses were performed using the software IBM SPSS Statistics 25.

2.12. Bile acid derivative-based coatings

Compound **5** was selected as the most promising bile acid derivative based on its AF *versus* toxicity performance. To assess the application potential of a methyl ester bile acid derivative (**5**) as an AF agent for protective coating systems, the bioactive derivative was incorporated for the first time into two representative coating systems, a polyurethane (PU)-based marine paint, generously provided by Hempel A/S (PU, Ref. F0032/95580), and a polydimethylsiloxane (PDMS) coating system (RTV11, Momentive™). Both systems have two components, a base component and a curing agent component. The bioactive derivative was incorporated into the coating formulations as an additive following the preparation instructions provided by the paint components suppliers, and this process may require additional component optimization depending on the bioactive derivative content. For both polymeric coating systems, the optimized formulations allowed the incorporation of the bioactive derivative at contents as high as 0.58 wt% relative to the total weight of the uncured formulation. For the preparation of the optimized PU-based formulations, the bioactive derivative was dissolved in *N*-methylpyrrolidone (99.5%, Acros Organics) in a bioactive derivative/solvent weight ratio of 13.95, and this solution was blended into the paint components, the base and curing agent, which were mixed at a base/curing agent ratio of 9/1.

For the PDMS coating formulations, the preparation methodology was similar and involved the prior dissolution of the bioactive derivative in *N*-methylpyrrolidone at a bioactive derivative/solvent weight ratio of 17.17, and this solution was blended into the paint components, which were then mixed at a base/curing agent ratio of 200/1.

The optimized formulations were then used to coat 24-well microplates for the antimacrofouling evaluation of the bile acid-based coatings. Each coating formulation was used to coat four replicate (wells) per microplate. In addition, for comparison, a commercial biocide, ECONEA®, was also incorporated into the polymeric coatings using prior optimized formulations (Silva et al., 2019).

2.13. Antimacrofouling activity with the optimized coating formulations

The previously coated 24-well microplates were used for a preliminary evaluation of the antimacrofouling activities under laboratory conditions. For the initial antisettlement assessment, competent *Mytilus galloprovincialis* plantigrades showing exploring behavior were selected and transferred to the test-coated microplates. All the test wells were filled with clean filtered seawater to evaluate only the effect of the coating content. Each coating was tested in four replicates (wells) with five plantigrades per well. A negative control (AF agent-free coating system) was included, as well as an ECONEA®-based coating for comparison. After 15 and 40 h, for all the conditions tested, the antisettlement activity was determined by the presence/absence of efficiently attached byssal threads produced by each individual (settlement).

3. Results

3.1. Synthesis of bile acid derivatives

In total, seven derivatives, namely, five deoxycholic acid derivatives (**4–8**) (Scheme 1 A), one chenodeoxycholic acid derivative (**9**), and one cholic acid derivative (**10**) (Scheme 1 B) were synthesized.

Ester derivatives **5** and **6** were synthesized through Fisher esterifications with methanol or *n*-butanol, respectively, with conc. HCl, according to Lee et al. (2007), in 40–51% yield (Scheme 1 A).

Sulfated derivatives were obtained according to Correia-da-Silva et al. (2011a, 2011b, 2011c). The use of microwave irradiation in sulfation of bile acid **1**, allowed to obtain the sulfated derivative in shorter reaction times and higher yield than previously described (Pageaux et al., 1979).

N-Deoxycholylethylamine (**7**) was obtained by refluxing compound 5 in ethylenediamine according to Lee et al. (2007), in 97% yield (Scheme 1 **A**).

Following, due to the better AF profile exhibited by the methyl ester derivative, bile acids **2** and **3** were methyl esterified using the same procedure and obtained in 63–96% yields (Scheme 1 **B**).

The nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS) data of compounds **4–7**, **9**, and **10** were in accordance with the literature (Pageaux et al., 1979; Hill et al., 1981; Marples and Stretton, 1990; Gao and Dias, 1998; Kim et al., 2004; Pore et al., 2006; Lee et al., 2007; Yasukawa et al., 2009; Afzal et al., 2011; Lofman et al., 2011; Stoltz et al., 2017). Compound **8** was obtained for the first time, and its structure was elucidated for the first time. The IR spectrum of compound **8** showed important bands at 1210 cm^{-1} (S═O) and 1072 cm^{-1} (C–O–S) and a band at 1733 cm^{-1} (C═O stretching), suggesting the presence of sulfate and ester groups. The ^{1}H NMR spectrum of compound **8** showed that the proton (H-3) *ipso* to the strongly electron-withdrawing sulfate group was substantially deshielded relative to the same proton in compound **5**. The ^{1}H NMR spectrum of **8** also showed only one signal corresponding an alcohol group (OH-12), unlike the spectrum of **5**, which showed two alcohol signals (OH-3 and OH-12). The ^{13}C NMR spectrum of compound **8** showed that the carbon directly bound to the strongly electron-withdrawing sulfate group (C-3) was substantially deshielded with respect to the same carbon of compound **5**.

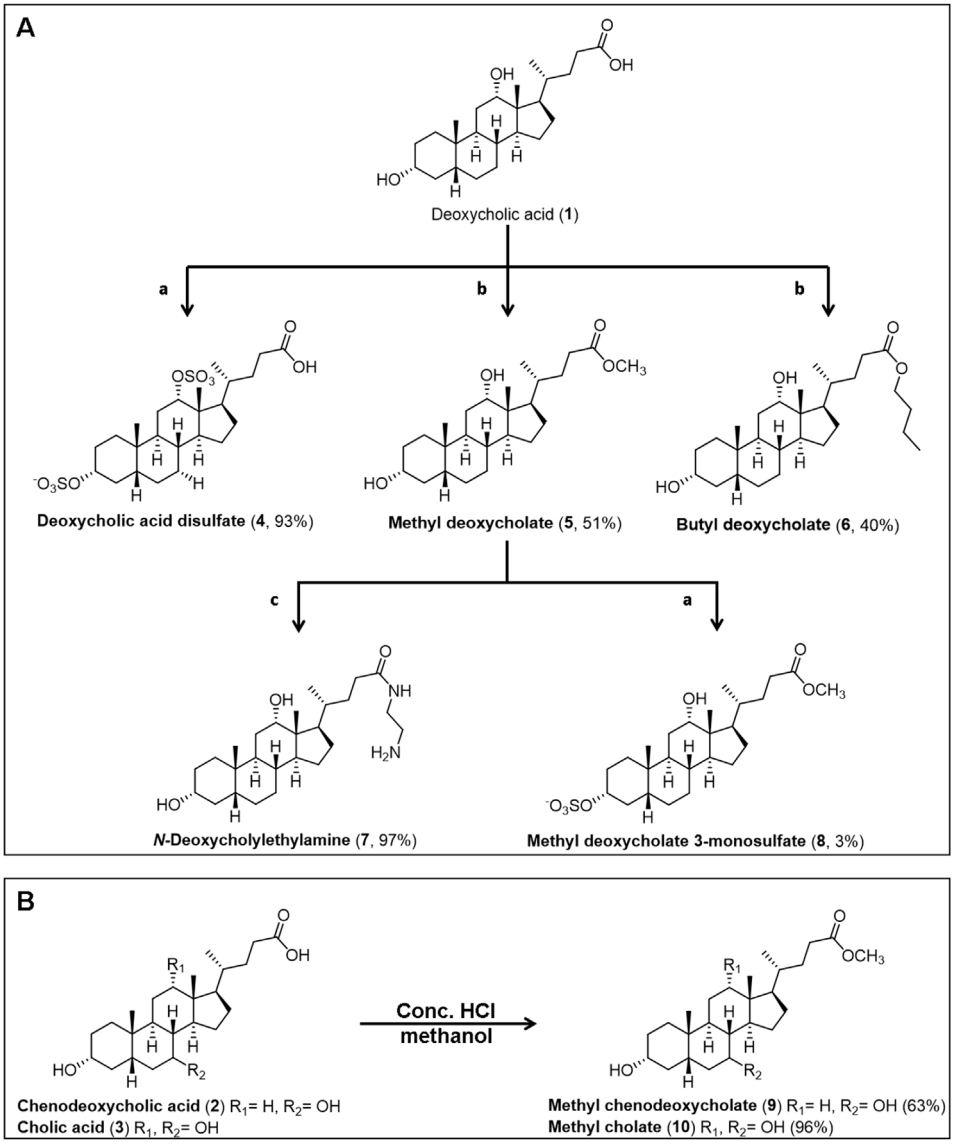
3.2. Antifouling activity of the bile acid derivatives

3.2.1. Antimacrofouling activity

3.2.1.1. Settlement inhibition of Mytilus galloprovincialis larvae. Significant differences ($p \leq 0.05$ at 50 μM) in the percentage of settled larvae relative to the control (filtered seawater) were observed for the bile acid chenodeoxycholic (**2**) and for derivatives **5**, **6**, and **8–10** (Fig. S1). In contrast to bile acid **2**, deoxycholic acid (**1**) and cholic acid (**3**) did not significantly affect the settlement of mussel larvae (55% of settlement inhibition at 50 μM). The compounds methyl chenodeoxycholate (**9**) and methyl cholate (**10**) were found to induce some toxicity to mussel larvae at 50 μM (Fig. S1), however even at 200 μM these compounds did not cause more than 50% of lethality. In Fig. S2 and Table 1 are the results of the AF efficacy *versus* toxicity studies performed for the most promising compounds.

The results revealed that all compounds showed EC_{50} (minimum concentration that inhibited 50% of larval settlement) values lower than the U.S. Navy recommendation ($EC_{50} < 25\ \mu g\ mL^{-1}$), with compounds **5 and 8–10** showing LC_{50}/EC_{50} (therapeutic ratio) values higher than 15. When exploring nontoxic antifoulants, the therapeutic ratio, LC_{50}/EC_{50}, is often used to evaluate the effectiveness of an AF compound in relation to its toxicity. Usually, a compound is considered a nontoxic antifoulant when it presents an LC_{50}/EC_{50} ratio > 15 (Qian et al., 2010).

3.2.1.2. Insights into the mechanism of action of bile acid derivatives: AChE activity. The *in vitro* inhibition of AChE, which has a metabolic role in the settlement of macrofouling organisms (Faimali et al., 2003), was determined to elucidate the mechanism of action of derivatives that presented a EC_{50} value lower than l0 μM in the settlement of *M. galloprocincialis* (**5** and **8–10**) (Table 2).



Scheme 1. General synthesis of bile acid derivatives. **A** – deoxycholic acid derivatives **4–8**; **B** – methyl ester derivatives of chenodeoxycholic acid (**2**) and cholic acid (**3**). a) TEA·SO_3, DMA, b) MeOH/*n*-BuOH, HCl, c) $NH_2CH_2CH_2NH_2$.

The AChE activity was slightly decreased in the presence of derivative **9**, and none of the other tested compounds significantly affected the activity of this enzyme.

3.2.2. Antibacterial activity

Significant bacterial growth inhibition was observed for bile acid **1** and derivatives **5–9** against some of the tested species, with inhibitory values of approximately 40% at concentrations of 12.5 μM (Fig. S3) and EC_{50} values were determined for these compounds (Table 3).

Roseobacter litoralis was the most sensitive species to the presence of these compounds, namely to compounds **5** and **7**. While bile acid derivatives **5** and **7–9** showed a broad spectrum of activity inhibiting the growth of three or four biofilm-forming marine bacteria, bile acid **1** and derivative **6** were species specific, only inhibiting the growth of one species.

Table 1
Effectiveness *vs* toxicity of the most promising compounds (**2**, **5**, **6**, and **8–10**) against *Mytilus galloprovincialis* larvae.

Compound	EC_{50} (μM)	EC_{50} (μg.mL^{-1})	LC_{50} (μM)	LC_{50}/EC_{50}
2	27.42 (95% CI: 11.578–73.499)	10.70	> 200	> 7
5	7.47 (95% CI: 2.634–13.470)	3.03	> 200	> 27
6	29.75 (95% CI: 15.763–60.372)	13.30	> 200	> 7
8	8.88 (95% CI: 3.58–14.44)	4.32	> 200	> 24
9	4.16 (95% CI: 2.796–5.388)	1.69	> 200	> 48
10	3.72 (95% CI: 2.603–4.452)	1.57	> 200	> 54
ECONEA®	4.01 (95% CI: 0.38–9.54)	1.40	107.78	27

EC_{50} – minimum concentration that inhibited 50% of larval settlement; LC_{50} – the median lethal concentration; LC_{50}/EC_{50} – therapeutic ratio; CI – confidence interval. Note: reference values for EC_{50} < 25 μg mL^{-1} (U.S. Navy program) and therapeutic ratio (LC_{50}/EC_{50}) > 15.

Table 2
Inhibition of AChE activity by compounds **5** and **8–10**.

Concentration (μM)	AChE activity (%)			
	5	8	9	10
25	104.6 ± 7.7	115.7 ± 0.1	98.1 ± 4.3	131.2 ± 1.0
50	–	108.0 ± 6.3	83.7 ± 2.2	105.7 ± 11.7
100	–	109.9 ± 20.3	63.7 ± 7.2	108.4 ± 5.1

B (negative control with DMSO): 100 ± 3.9%; C (Eserine at 200 μM): 0.1 ± 0.1%.

3.2.3. Antimicroalgal activity

The inhibitory effects of the three bile acids (**1–3**) and the seven synthesized bile acid derivatives (**4–10**) on the growth of four strains of benthic marine diatoms are shown in Fig. S4. The concentrations that inhibited 50% of diatom growth (EC_{50} values) were calculated for compounds **1**, **2**, and **5–10** (Table 4).

Bile acids **1** and **2** and derivatives **5**, **8**, and **9** were able to inhibit the growth of all the diatoms species. Compound **6** was species specific, only inhibiting the growth of *Halamphora* sp. Compounds **1**, **5**, and **7–9** were the most potent compounds, presenting EC_{50} values < 10 μM for all the tested species except for *Navicula* sp.

3.2.4. Marine ecotoxicity

The mortalities of a marine brine shrimp (*Artemia salina*) observed in the presence of the tested compounds (**5** and **8–10**) were not significantly different from that of the negative control, even at 50 μM (Fig. S5). In contrast, ECONEA® caused 100% of mortality at the same concentration range.

3.2.5. In silico evaluation of bioaccumulation potential

The $LogK_{ow}$ values calculated for compounds **5** and **8–10** are shown in Table S1. Compound **8** showed a $LogK_{ow}$ value < 3, compounds **5**, **9**, and **10** showed $LogK_{ow}$ values > 3. Compounds **8** and **10** showed $LogK_{ow}$ values lower than ECONEA®.

3.3. Bile acid-based antifouling coatings

The representative methyl ester bile acid derivative **5** showed good compatibility with PU marine paint and PDMS coatings at contents up to 0.58 wt%. Fig. 2 shows a 24-well microplate with the developed coatings.

The marine PU coating containing compound **5** (5 PU) was shown to be more effective in inhibiting the larvae settlement than the ECONEA®-containing coating at 40 h of exposure (Fig. 2). Considering PDMS coatings containing ECONEA® or compound **5**, no mussel larvae settlement could be observed at 40 h of exposure, despite the inherent repellent characteristics of the PDMS coating (negative control).

4. Discussion

The fouling of submerged surfaces is a natural and complex process that compromises various human activities. Substantial effort has been devoted to the development of effective solutions for this problem, although avoiding significant hazards to the environment remains challenging.

In this study, seven diverse bile acid derivatives were obtained by one or two step syntheses with simple methods, from available and inexpensive raw materials, in moderate to high yields.

Considering the three parent bile acids **1–3**, while the settlement of mussel larvae did not decrease in the presence of deoxycholic acid (**1**), this compound was found to inhibit the growth of the marine bacteria *R. litoralis* (EC_{50} = 16.81 μM) and marine diatoms *Cylindrotheca* sp., *Halamphora* sp., *Nitzschia* sp., *Navicula* sp. (EC_{50} around 10 μM). In contrast, chenodeoxycholic acid (**2**) was able to inhibit the settlement of mussel larvae (EC_{50} = 27.42 μM) without influencing the growth of marine bacteria. Similar to bile acid **1**, bile acid **2** showed a broad spectrum of activity against diatoms however with higher EC_{50} values (EC_{50} = 10–20 μM). Cholic acid (**3**) did not show any AF effects. Based on these results, the microfouling spectrum of activity of the natural deoxycholic acid **1** could be further investigated to combat the early stages of marine biofouling formation.

Methyl ester derivatives (**5** and **8–10**) were the most potent compounds against *Mytilus galloprovincialis* larvae presenting EC_{50} values lower than 10 μM. Lengthening the side chain did not lead to an increase in the antisettlement activity, as indicated by the lower potency of butyl deoxycholate (**6**) compared with methyl deoxycholate (**5**). Considering effectiveness *vs* toxicity, the methyl ester derivatives **9** and **10** were as effective as the commercial biocide ECONEA® (EC_{50} around 4 μM), while showing lower toxicity to *Mytilus galloprovincialis* larvae. The slight toxicity found in mussel larvae exposed only to compounds **9** and **10** seems to indicate that at least these two compounds might not act on a specific AF molecular target (directly related with settlement or adhesion metabolism); their mode of action might consist in a broad-spectrum effect target leading to lethal consequences at high concentrations in mussel larvae. Mode of action of these compounds is probably distinct from the other bile acid derivatives in study.

No effect was observed in the activity of AChE indicating that these compounds may have no impact in the cholinergic system, as described for some biocides.

Esterification of bile acids (**5**, **6**, and **9**) and the introduction of a primary amine (**7**) led to compounds with increased inhibitory activity against the growth of marine biofilm-forming bacteria. These data are in accordance with previous reports in which these molecular modifications improved the AF activity of other scaffolds, such as bromotyramines (Schoenfeld et al., 2002; Andjouh and Blache, 2016), piperamides (Huang et al., 2014), and butenolides (Li et al., 2012).

Again, esterification (**5**, **6**, and **8–10**) and the introduction of a primary amine (**7**) seemed to influence the AF activity against the growth of diatoms. Some compounds, namely derivatives **8** and **10**, were more potent inhibitors of the growth of *Halamphora* sp., and

Table 3
Bacterial growth inhibition by compounds **1** and **5–9**.

Compound	EC_{50}*; μM (95% CI)				
	C. marina	*V. harveyi*	*P. atlantica*	*H. aquamarina*	*R. litoralis*
1	–	–	–	–	16.81 (6.23–178.99)
5	–	26.49 (19.37–44.29)	21.97 (13.93–50.55)	–	9.94 (1.23–27.85)
6	–	–	–	–	28.52 (17.13–156.72)
7	28.92 (21.73–46.07)	–	21.55 (13.70–48.48)	18.92 (12.89–32.01)	9.85 (3.94–20.09)
8	67.23 (23.80–733.35)	–	35.38 (24.34–54.23)	–	34.06 (21.13–64.00)
9	–	24.32 (14.54–93.42)	18.12 (10.97–37.80)	14.83 (11.04–20.80)	–

*EC_{50} - concentration that inhibited 50% of bacterial growth; ECONEA®: EC_{30} (*H. aquamarina*) = 15.31 (95% CI: 7.3–70.4); CI – confidence interval.

Table 4
Diatoms growth inhibition by compounds **1**, **2**, and **5–10**.

Compound	EC_{50}*; μM (95% CI)			
	Cylindrotheca sp.	*Halamphora* sp.	*Nitzschia* sp.	*Navicula* sp.
1	7.07 (6.14–8.16)	6.91 (4.67–10.60)	3.84 (3.23–4.53)	11.55 (9.58–14.30)
2	13.68 (11.41–16.91)	18.89 (15.10–24.99)	15.09 (12.15–19.40)	17.13 (14.02–21.86)
5	6.57 (4.60–9.52)	5.93 (4.93–7.14)	5.18 (3.45–7.61)	10.60 (8.47–13.79)
6	–	16.49 (9.99–39.65)	–	–
7	6.85 (5.90–7.98)	8.67 (7.29–10.46)	–	–
8	9.52 (4.46–28.23)	4.80 (3.09–6.60)	10.32 (8.03–12.49)	17.20 (6.98–400.33)
9	5.06 (4.38–5.90)	6.62 (5.74–7.65)	6.50 (4.36–9.34)	13.76 (11.75–16.45)
10	4.84 (4.17–5.60)	14.11 (10.20–21.77)	–	17.15 (13.99–21.97)
ECONEA®*	5.22 (2.92–10.84)	6.04 (5.13–7.22)	4.97 (2.58–11.03)	5.65 (2.82–15.97)

*ECONEA® values according to Almeida et al. (2017) *CI – confidence interval; EC_{50} – concentration that inhibited 50% of bacterial growth.

Cylindrotheca sp., respectively, than ECONEA®.

Overall, compounds showing the most promising AF profile were methyl ester derivatives **5** and **8–10** once they were found to have a broad profile, inhibiting both antimicrofouling and antimacrofouling organisms. The butyl ester derivative **6** have only inhibited the bacteria *R. litoralis* and diatom *Halamphora* sp. showing that lengthening the side chain might change a broad spectrum to a species-specific profile. The fact that compound **4**, a persulfated derivative with no free hydroxyl groups, did not show any AF activity, allowed to hypothesize that the presence of hydroxyl groups in the bile acid scaffold may be important for the AF activity.

Since only compounds with low toxicity to non-target organisms and low bioaccumulative potential will be able to become an approved product, the marine ecotoxicity of methyl ester derivatives and their bioaccumulative potential were evaluated. Compounds **5** and **8–10** were not toxic to the marine brine shrimp *Artemia salina*, in contrast to ECONEA® which caused 100% of mortality at the same concentration range. From the $LogK_{ow}$ values, compound **8** showed the lowest bioaccumulation potential. Although sulfation at position 3 did not significantly improve biofouling effects, this molecular modification increased the water solubility of compound **5**, decreasing its $LogK_{ow}$. However, between these two methyl ester derivatives, compound **5** was selected for incorporation into polymeric coatings due to a more potent AF activity against all the tested organisms (mussels, bacteria, and diatoms) and a feasible synthesis with higher yields. This bile acid derivative revealed to be compatible with both PU and PDMS coating systems and the AF activity was maintained even after its incorporation. These results indicate that compound **5** is a good candidate for further *in situ* testing.

5. Conclusions and future directions

In this work, methyl esters of bile acids were disclosed as good starting point to develop broad spectrum AF agents with both anti-macrofouling and antimicrofouling activity (Fig. 3). The development of these compounds as AF agents is of great interest for the following reasons: the starting materials are readily available from commercial sources at a reasonable cost, their syntheses are moderate to high yielding, and the purification processes are relatively simple and not time consuming, particularly for compound **5**.

Moreover, the high compatibility and preliminary antimacrofouling effects demonstrated by the representative bile acid derivative **5** after incorporation into polymeric marine coating matrices support the high potential of these bile acid derivatives as AF agents in real protective systems (Fig. 3).

In the future, it would be important to study the biodegradability of these bile acids in various environmental conditions (temperatures, pH, salinity) and the toxicity of the transformation products to assure low impact to the environment. Although no toxicity was found for bile acid and their derivatives to *Artemia salina*, other non-target marine organisms should be tested to confirm the safety of these compounds.

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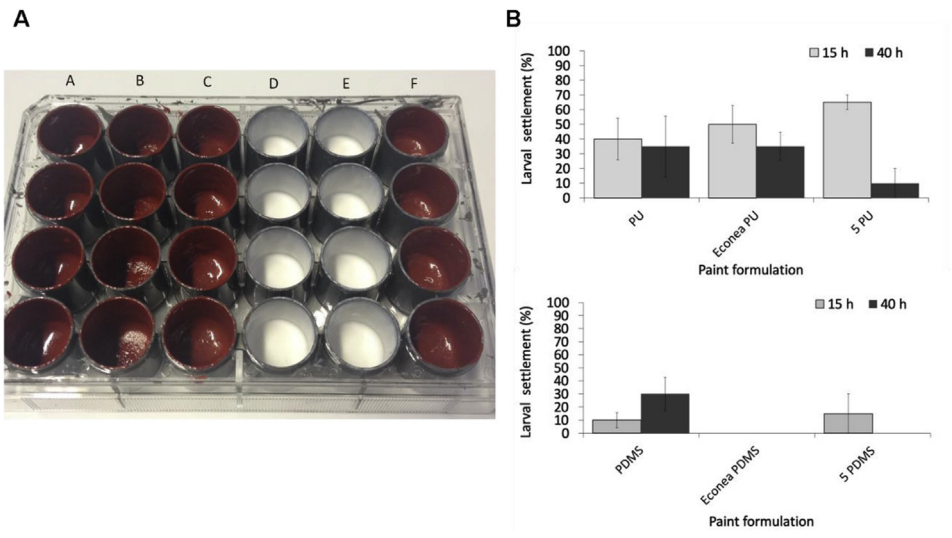

Fig. 2. Bile acid-based coatings and assessment of antimicrofouling activity. **A** - Image of a 24-well microplate coated with bile acid-based polyurethane (red coating, A-C and F) and polydimethylsiloxane (PDMS) coatings (white coating, D and E): columns A and D - free of bioactive agents, column B – other matrix for comparative purposes (data not shown), columns C and E – containing 0.57 ± 0.01 wt% of bile acid derivative **5**, column F – containing 0.56 wt% of ECONEA® biocide; **B** - Antisettlement activity towards plantigrade larvae of the mussel *Mytilus galloprovincialis* by polyurethane (PU) and polydimethylsiloxane (PDMS) coatings containing compound **5** and ECONEA® as reference. Negative controls: PU and PDMS, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

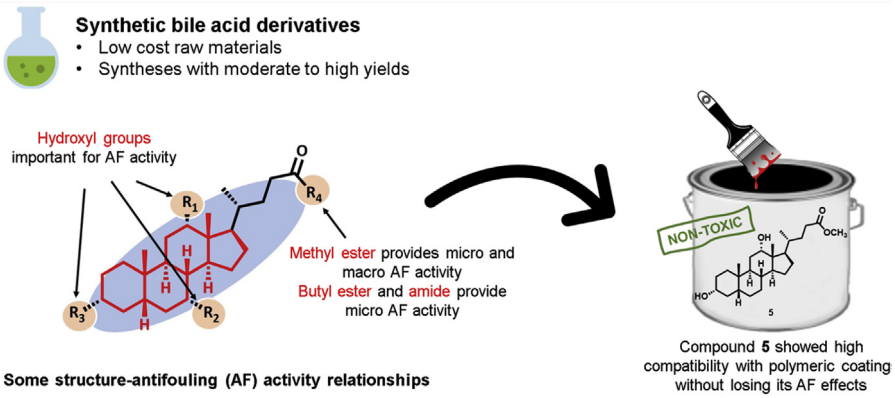

Fig. 3. Main findings of this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.109812.

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