

# Antifouling Activity of Isonitrosoacetanilides Against Micro and Macrofouling

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## Research Article

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# Abstract

Biofouling is responsible by structural and economic damage on man-made surfaces. Antifouling paints with biocides had been applied in these structures to avoid organisms adhesion, however they show high toxicity and they are not able to avoid all biofouling process, necessitating the mechanical removal of organisms periodically and paint reapplication. Thus, there is an urgent demand for novel, effective, and environmentally friendly antifouling alternative. As isonitrosoacetanilide is the precursor for many compounds with antibacterial activity, we believe that it could show an antifouling activity potential against microfouling and per consequence also against macrofouling. The aim of this work was to investigate the antifouling potential of six isonitrosoacetanilides compounds and their toxicity. The compounds were employed in different concentrations (0.625–1.25–2.5–5–10  $\mu\text{g mL}^{-1}$ ) in this study. The biofilm and planktonic bacteria inhibition and biofilm eradication potential were evaluated through by crystal violet assay, while *Amphibalus amphitrite* barnacle was evaluated by cyprid settlement assay. Toxicity evaluation ( $\text{LC}_{50}$  and  $\text{EC}_{50}$ ) was performed with *A. amphitrite* nauplii II and cyprid larvae. 4-Br-INA, 4- $\text{CH}_3$ -INA, and 2-Br-INA compounds, at least one of the tested concentrations, showed non-toxic antifouling activity against microfouling (antibiofilm) and macrofouling (anti-settlement). However, only 4- $\text{CH}_3$ -INA and 2-Br-INA showed also biofilm eradication potential. These compounds with antibiofilm activity and non-toxic effects could be combined with acrylic base paint resin or added directly into commercial paints in place of toxicant biocides to cover artificial structures as friendly antifouling.

## Introduction

Biofouling is a natural phenomenon that often involves the adhesion of microfouling and macrofouling on hard substrates (Gu 2005). Microfouling refers to microorganisms (e.g., bacterial biofilm) and are the first colonizers; whereas, macrofouling refers to macrorganisms (e.g., invertebrates), and end the process (Agostini et al. 2018). According to researches (Gu 2005; Agostini et al. 2017, 2018) microfouling controls the ecological succession, as consequence the macrofouling settlement.

Macrofouling is the main responsible by structural and economic damage on man-made surfaces, as pipelines and ship hulls (Schultz et al. 2011, Agostini et al. 2019a), being the global losses around fifteen billion dollars per year (Azis et al., 2001). To avoid biofouling, antifouling paints with biocides derived from organic, metallic, and inorganic compounds have been applied on man-made structures immersed in aquatic environment (Telegdi et al. 2016). The traditional antifouling paints prevent the adhesion of organisms on surfaces by killing them (Agostini et al. 2019b), and because of this show high toxicity to both target and non-target organisms (Amara et al. 2018). Besides that, the nowadays antifouling paints (mainly copper oxide-based) are not able to avoid all biofouling process, necessitating the mechanical removal of organisms periodically and paint reapplication (Desai 2008, Agostini et al. 2019a). Thus, there is an urgent demand for novel, effective, and ecological safe antifouling compounds (Wang et al. 2017).

An environmentally friendly antifouling strategy has been explored in recent years, being focused on the characterization and synthesis of compounds that are based on the natural chemical defenses from

organisms (e.g., marine bacteria, fungi, cyanobacteria, aquatic plants, and terrestrial plants) (Dahms et al. 2006; Dobretsov et al. 2006; Qian et al. 2010).

Convolutamydine A, an oxindole alkaloid and a marine natural product was isolated from the Floridian marine bryozoan species *Amathia convolute* (Lamarck, 1816) (Zhang et al. 1994). Bryozoans produces this kind of chemical as antifouling and antipredation strategy (Lopanik et al. 2004). According to Figuerola et al. (2014, 2017) this product include defense against microfouling. Convolutamydine can be derivative from isatins, which are heterocyclic compounds with considerable synthetic versatility (Silva 2013). Isatin and its derivatives have been found to have antimicrobial activity (Silva et al. 2010; Majik et al. 2014), including against marine bacteria species (Majik et al. 2014). Isatin is found in nature as metabolic products of terrestrial plants (e.g., *Isatis*, *Calanthe discolor*, *Melochia tomentosa*, *Boronella koniamboensis* and *Coroupita guianenses*), in the secretion of the frog parotid gland (e.g., *Bufo*) and in eggs of mollusc (e.g., *Dicathais orbita*) (Baker and Sutherland 1968; Wei et al. 1982; Guo, Y.; Chen 1986; Grougnet et al. 2005). On the other hand, can also be synthesized from isonitrosoacetanilide, which is considered a synthetic intermediate to get isatin and consequently convolutamydine A (Silva 2013). Isonitrosoacetanilides presented oxime in its structure, group that have gained a considerable amount of attention due to their notable broad-spectrum biological activity (e.g., antibacterial and antifungal) (Raghav et al. 2018). As isonitrosoacetanilide is the precursor for many compounds with antibacterial activity, we believe that it could show an antifouling activity potential against microfouling and per consequence also macrofouling.

Hence, the objective of this work was to investigate the antifouling potential of six isonitrosoacetanilides compounds (Fig. 1) against micro (bacterial biofilm) and macrofouling (*Amphibalanus amphitrite*) adhesion, evaluating their toxicity.

## Material And Methods

### Compounds

### Synthesis of isonitrosoacetanilides and stock-solution preparation

To a 500 mL round-bottom flask were added 0.915 mol of sodium sulfate, 120 mL of distilled water, 0.109 mol of chloral hydrate, 0.079 mol of hydroxylamine sulfate dissolved in 50 mL of distilled water, 0.1 mol of the respective aniline in 60 mL of distilled water and 8.6 mL of concentrated hydrochloric acid. The reaction mixture was gradually heated to 70° C. After adding 100mL of ethyl alcohol, the mixture was heated under reflux until TLC indicated that formation of isonitrosoacetanilide was complete. The mixture was poured onto ice/H<sub>2</sub>O and the solid isonitrosoacetanilide formed was collected by filtration and washed with water (Garden et al 1997, Silva et al 2011).

Assays were performed using 48-well polystyrene plates (Barloworld Scientific Ltd., Sandton, South Africa). Each compound was first dissolved in a small amount of dimethyl sulfoxide (DMSO) (final concentration = 0.1%) that was first diluted with filtered 0.22 µm and autoclaved seawater 25 PSU (FSW) to achieve stock-solution (2.4 mg mL<sup>-1</sup>). The active compounds were diluted with FSW to get 0.625–1.25–2.5–5 – 10 µg mL<sup>-1</sup> (based on Chen et al. 2014). This range was chosen followed Kitano et al. (2011). In all assays 0.1% DMSO served as the solvent control (°C), while FSW as negative control (°C).

## Microfouling Assays

### Bacteria inoculum

The bacterial biofilm consortium were represented by Proteobacteria and Bacteroidetes phyla (98%): *Vibrio* (24%), *Neptuniibacter* (16%), *Phaeobacter* (13%), *Alteromonas* (11%), *Oceanospirillum* (10%), *Pseudoalteromonas* (7%), *Methylophaga* (7%), *Pseudomonas* (7%), *Oleibacter* (5%), and *Marinomonas* (3%) (Agostini et al., 2019, 2020).

### Planktonic and Biofilm Bacteria assays

Crystal violet assay for the estimation of bacterial biomass inhibition and eradication. The ability of the compounds to prevent bacterial adhesion and EPS formation on a virgin surface (inhibition of planktonic and biofilm biomass) and to destroy an established marine bacterial biofilm (eradication of biofilm biomass) were assessed using four replicates per treatment using the crystal violet assay (Agostini et al., 2019b, 2020).

For the biomass inhibition assay, 100 µL of bacterial inoculum, 100 µL of FSW, and/or compounds stock-solution were mixed in 96-well plates (Barloworld

Scientific Ltd., Sandton, South Africa) and incubated at 25°C for 48 h in the dark (OD<sub>570</sub> = 0.002). For the biomass eradication assay, 200 µL of bacterial inoculum

(OD<sub>570</sub> = 0.002) was incubated at 25°C for 24 h in the dark in 96-well plates. The supernatant in the wells was removed and the compounds were added to these wells and incubated at 25°C for 24 h in the dark.

The planktonic bacterial biomass (free-living bacteria) was estimated by the difference in the absorbance at the beginning (OD<sub>600</sub> = 0.002) and end of the incubation. For biofilm assays, the content of the wells was removed, and washed three times with sterile saline solution. The attached bacteria were heat-fixed at 60°C for 1 h. The biofilm layer formed was stained with 0.4% w/v crystal violet for 15 min at 20°C and the plates were washed four times with tap water. The stain bound to the cells was solubilized with 99.5% ethanol (Sigma-Aldrich Co., St. Louis, MO, USA) for 30 min, and the absorbance was measured (Malafaia et al., 2017) using the Spectramax M2e Multimode Microplate Reader (Molecular Devices San Jose, CA, USA).

# Macrofouling Assays

Adult barnacles from Istituto per lo studio degli impatti Antropici e Sostenibilità in ambiente marino (IAS) - CNR were maintained in aerated, filtered (0.45  $\mu\text{m}$ ) natural seawater at 20°C, on a 16-h:8-h light-dark cycle. They were fed every two days with *Artemia salina* (50 to 100 mL; 200 larvae mL<sup>-1</sup>) and *Tetraselmis suecica* (100 to 200 mL;  $2 \times 10^6$  cells mL<sup>-1</sup>). The seawater was changed three times per week, and the barnacles were periodically rinsed with fresh water to remove epibionts or debris.

To obtain the nauplii for cyprid cultures, the adults were left to dry for 30 min to 40 min and then immersed in fresh seawater. The hatched nauplii were attracted to a light source and collected using a Pasteur pipette. They were reared to the cyprid stage as described in Faimali et al. (2003), by keeping them at 28°C in natural filtered (0.22  $\mu\text{m}$ ) seawater and feeding them three times a week with *Tetraselmis suecica* ( $2 \times 10^6$  cells mL<sup>-1</sup>). In these conditions nauplii reach the cyprids stage in 5–6 days. The cyprids were harvested by filtration and aged for 4 days prior to use, in filtered (0.45  $\mu\text{m}$ ) natural seawater at 4°C in the dark (Rittschof et al. 1992).

## Amphibalanus amphitrite cyprid settlement and mortality assay

To evaluate the macrofouling settlement inhibition was used barnacle cyprids of *A. amphitrite*. In the assays, the treatments were conducted in 24-well microplates (Barloworld Scientific Ltd., Sandton, South Africa) by adding 20 to 25 cyprids per well, with each well containing 2 mL of medium, representing the treatments. The test plates were sealed to prevent evaporation and incubated at 28°C in the dark. The settlement and the mortality were evaluated after 72 h of incubation. The larvae were examined under a dissecting microscope, to record the number of dead and permanently attached individuals. The experiments were terminated by the addition of three droplets of 40% formaldehyde into each test well and the counting of the settled and non-settled larvae. The results were expressed as the percentages ( $\pm$  standard error) of the settlement of the total number of larvae incubated (Piazza et al. 2014).

## Amphibalanus amphitrite nauplii II swimming alteration and mortality assay

Acute (mortality) and sub-acute (immobility) toxicities were assessed using II stage nauplii *A. amphitrite*. All of these tests were performed in 48-well microplates (Barloworld Scientific Ltd., Sandton, South Africa) with 10–15 nauplii II per well, each well containing 1 mL of medium. The organisms were evaluated after 24 h and 48 h of incubation at 20°C in the dark. After the exposure the larvae were examined under a dissecting microscope, and the number of dead and not swimming larvae was recorded (Piazza et al. 2014). Larvae that were completely motionless (unable to change their barycenter position or move any appendages in 10 s) were counted as dead, while the larvae that could not swim (unable to change their barycenter position but move appendages) were counted as having a swimming alteration (Agostini et al.

2019b, 2020). The results were expressed as the percentages ( $\pm$  standard error) of the settlement of the total number of larvae incubated (Piazza et al. 2014).

## Data Analyses

For the bacterial biofilm data, the plant extract that killed planktonic bacteria and consequently prevented bacterial adhesion and biofilm formation was called antibiotic, while the plant extract that prevented bacterial adhesion without killing planktonic bacteria was called antibiofilm (Agostini et al., 2019, 2020). Variance analysis (one-way ANOVA) was used to evaluate possible differences in the bacterial density and biomass (response variables) according to different treatments (predictive variables) after confirming adherence to all the statistical assumptions required by ANOVA (continuous data; normal distribution of residuals, assumption of homogeneity of variances/covariances and mean and variances independence) (Gotelli & Ellison, 2013). Generalized Linear Models (GLM) were used to evaluate settlement and toxicological effects (response variables) of the treatments (predictive variables). Binomial distribution was applied to the logit link function, where  $Y = n =$  number of mortalities/swimming alteration/settlement of the total of "n individuals" in the experimental units (Lopes et al., 2018). For toxicity, the tests were considered acceptable when the mortality in the control was  $< 10\%$  (Garaventa et al., 2010). Tukey's post hoc test was used when the alternative hypotheses from ANOVA and GLM were acceptable through the multcomp package (Hothorn et al., 2008). Differences were considered significant when the calculated p value was less than 0.05. Analyses were performed on R (version 3.5.1) (R Core Team, 2019). For toxicological assays, the median endpoint values ( $LC_{50}$  and  $EC_{50}$ ) and related 95% Confidence Intervals were calculated using Spearman and Karber analysis (Finney 1978). The median values were expressed as  $LC_{50}$  for mortality (the concentration able to cause the mortality of the 50% of the tested population) and  $EC_{50}$  for mobility alteration (the concentration able to cause an alteration of larvae mobility for 50% of tested population). We consider immobility (EC) the sum between mortality and swimming alteration.

## Results

The antifouling and toxic effects of six isonitrosoacetanilides compounds were investigate. About microfouling biomass, five compounds (H-INA, 4-Br-INA, 4-CH<sub>3</sub>-INA, 2-Br-INA and 3,4-Cl-INA) at least two of the concentrations tested inhibited the growth of marine bacterial biofilm when compared to controls ( $p < 0.05$ ). The compound 4-Cl-INA was the only one that did not show effect against microfouling. From the five compounds that showed biofilm inhibition, part of concentrations also inhibited planktonic bacteria (antibiotic activity) ( $p < 0.05$ ) and part did not (antibiofilm activity) ( $p > 0.05$ ) when compared to controls. The antibiofilm activity was observed to: H-INA ( $1.25-10 \mu\text{g mL}^{-1}$ ), 4-Br-INA ( $0.625-1.25 \mu\text{g mL}^{-1}$ ), 4-CH<sub>3</sub>-INA ( $2.5 \mu\text{g mL}^{-1}$ ), 2-Br-INA ( $5-10 \mu\text{g mL}^{-1}$ ) and 3,4-Cl-INA ( $2.5-10 \mu\text{g mL}^{-1}$ ) (Fig. 2).

About microfouling eradication biomass, four compounds at least three of the concentrations tested eliminated the biofilm already formed when compared to controls ( $p < 0.05$ ). The biofilm eradication was

observed to: 4-CH<sub>3</sub>-INA (0.625-2.5 µg mL<sup>-1</sup>), 2-Br-INA (0.625-2.5 µg mL<sup>-1</sup>), 4-Cl-INA (1.25-5 µg mL<sup>-1</sup>) and 3,4-Cl-INA (0.625-5 µg mL<sup>-1</sup>) (Fig. 3)

Regarding to macrofouling, five compounds (4-Br-INA, 4-CH<sub>3</sub>-INA, 2-Br-INA, 4-Cl-INA and 3,4-Cl-INA) at least two of the concentrations tested inhibited the *A. amphitrite* cyprid settlement when compared to controls ( $p < 0.05$ ). While, only four compounds avoid the settlement without cause acute toxicity to barnacles (stage cyprid) when compared to controls ( $p > 0.05$ ). The non-toxic anti-settlement activity was observed to: 4-Br-INA (0.625-5 µg mL<sup>-1</sup>), 4-CH<sub>3</sub>-INA (1.25-10 µg mL<sup>-1</sup>), 2-Br-INA (0.625-10 µg mL<sup>-1</sup>) and 3,4-Cl-INA (1.25 µg mL<sup>-1</sup>) (Fig. 4).

About the toxicity assay with nauplii II stage of *A. amphitrite*, the six compounds showed immobility (mortality + swimming alteration) to barnacles at least two of the concentrations tested when compared to controls ( $p < 0.05$ ). In the same way, that the non-toxic effect was observed for the same six compounds in different concentrations ( $p > 0.05$ ). The treatments that did not caused mortality and/or swimming alteration at 24 and 48 h were: H-INA (0.625-2.5 µg mL<sup>-1</sup>), 4-Br-INA (0.625-10 µg mL<sup>-1</sup>), 4-CH<sub>3</sub>-INA (0.625-2.5 µg mL<sup>-1</sup>), 2-Br-INA (0.625-2.5 µg mL<sup>-1</sup>) and 3,4-Cl-INA (0.625 µg mL<sup>-1</sup>) (Fig. 5). The Table 1 shows the LC<sub>50</sub> and EC<sub>50</sub> values.

Table 1

Toxicity of five isonitrosoacetanilides compounds against cyprid and nauplii II larvae of *Amphibalanus Amphitrite*. LC<sub>50</sub> for mortality and EC<sub>50</sub> for mortality more swimming alteration.

Compounds	72 h - LC <sub>50</sub> (cyprid) µg mL <sup>-1</sup> (CL 95%)	48 h - LC <sub>50</sub> (nauplii) µg mL <sup>-1</sup> (CL 95%)	48 h - EC <sub>50</sub> (nauplii) µg mL <sup>-1</sup> (CL 95%)
H-INA	> 10	> 10	> 10
4-Br-INA	> 10	> 10	> 10
4-CH <sub>3</sub> -INA	> 10	> 10	> 10
2-Br-INA	> 10	> 10	> 10
4-Cl-INA	7.48 (6.73–8.32)	> 10	> 10
3,4-Cl-INA	5.69 (5.30–6.11)	> 10	4.64 (4.08–5.27)

## Discussion

This study describes the first antifouling screening using isonitrosoacetanilides compounds against natural marine biofilm bacteria consortium (microfouling) and barnacles (macrofouling).

The isonitrosoacetanilides compounds assays showed significant bacterial biofilm biomass inhibition. The bacterium consortium used in this study is composed by Gram-negative bacteria. In seawater, Gram-

negative is the most representative (95% of bacteria) (Zobell 1946) and usually they present higher resistance than Gram-positive bacteria (Biswas et al. 2013; Boulekbache-Makhlouf et al. 2013), because comprise a hydrophilic lipopolysaccharide outer layer and enzymes that avoid the chemical penetration (Djihane et al. 2017), being more difficult to inhibit. In the same way that biofilm-associated is more difficult to inhibit than planktonic bacteria (Famuyide et al. 2019). Majik et al. (2014) suggest that the presence of NH group is necessary for marine bacteria inhibitory activity.

Only 12 from 30 treatments showed antibiofilm activity, represented by five isonitrosoacetanilides compounds. Although the antibiofilm and antibiotic activities reflect a marine bacterial inhibition (Agostini et al. 2020), isonitrosoacetanilides compounds with antibiofilm action is considered promisor, because did not kill bacteria, only repeal the planktonic cell adhesion (pelagic-benthic coupling), avoiding resistance (Salta et al. 2013; Antunes et al. 2019). The motive associated to non-adhesion of planktonic bacteria were not investigated in the current study. However, could be attributed to an interference in electrostatic interaction forces between the cells and the surface (Tiwari et al. 2018) or the capacity of the compounds in to interfere in the availability of nutrients (Sandasi et al. 2010).

Only 13 from 30 treatments showed biofilm biomass eradication, represented by four isonitrosoacetanilides compounds. H-INA and 4-Br-INA compounds that presented biofilm inhibition did not present the capacity of biofilm eradication. While 4-Cl-INA only got the capacity to eradicate the biofilm. Eradication is more difficult to achieve than inhibition of biofilms, since the polymeric matrix that surrounds the bacteria acts as a protective barrier against physical and chemical stress, preventing the contact of the compound with the cells (Stewart & Costerton, 2001). The motive that 4-Cl-INA compound did not inhibit but eradicated the biofilm could be associated to a specific effect disrupting and dissolving the matrix associated to 4-Cl group, diminishing the biomass.

The majority of the studies with the effect of isatin derivatives are associated with clinic pathogenic antibacterial potential (Patel et al. 2006; Pakravan et al. 2013; Lian et al. 2016). Although the aquatic antifungal activity on shrimp embryos had been observed in medium with isatin (Gil-Turnes et al. 1989). About marine antibacterial activity, Majik et al. (2014) evaluated the initial antifouling potential of synthetic analogues of isatin and few of them exhibited potent inhibitory activity against different ecologically relevant marine. On the other hand, in the current study, we find that the majority of the isonitrosoacetanilides compounds tested showed promisor results against microfouling. Maybe the different potential observed be related to isatin as synthetic derivative from isonitrosoacetanilides showing lower antibacterial effect than its precursor. The author did not evaluate the type of the antibacterial activity (antibiotic or antibiofilm) or the effect against macrofouling.

Regarding macrofouling assays, the *A. amphitrite* settlement was avoided by all compounds with exception to H-INA. However, the anti-settlement and non-toxic effect to cyprid larvae was recorded to 4-Br-INA, 4-CH<sub>3</sub>-INA, 2-Br-INA and 3,4-Cl-INA with concentration from 0.625 to 10 µg mL<sup>-1</sup>. The LC<sub>50</sub> was not found to 4-Br-INA, 4-CH<sub>3</sub>-INA, 2-Br-INA, being higher than the values tested (> 10 µg mL<sup>-1</sup>). For 3,4-Cl-INA the non-toxic effect was recorded in the lower concentration, while the LC<sub>50</sub> was 5.69 µg mL<sup>-1</sup>.

Usually nauplii II stage of *A. amphitrite* is more sensitive than cyprid stage (Piazza et al. 2014). However, in the current work the  $L_{50}$  for cyprid was lower than for nauplii II. For all isonitrosoacetanilides compounds the  $L_{50}$  was not found ( $> 10 \mu\text{g mL}^{-1}$ ). While only 3,4-Cl-INA showed an  $EC_{50}$  inside the tested values ( $4.64 \mu\text{g mL}^{-1}$ ). The differences in the toxicity observed between cyprid and nauplii II could be associated to the time of evaluation, higher to cyprid (72h) than for nauplii (48h), keep the larvae more time exposed to the chemical. According to U.S. Navy program (Chen et al. 2014), a novel antifouling may be a barnacle larval anti-settlement effected using concentration lower than  $25.0 \mu\text{g mL}^{-1}$ , corroborating the antifouling potential of the isonitrosoacetanilides compounds tested.

4-Br-INA ( $0.625\text{--}1.25 \mu\text{g mL}^{-1}$ ), 4- $\text{CH}_3$ -INA ( $2.5 \mu\text{g mL}^{-1}$ ) and 2-Br-INA ( $5\text{--}10 \mu\text{g mL}^{-1}$ ) isonitrosoacetanilides compounds showed non-toxic antifouling activity against microfouling (antibiofilm) and macrofouling (anti-settlement). However, only 4- $\text{CH}_3$ -INA and 2-Br-INA showed also biofilm eradication potential. These compounds with antibiofilm activity and non-toxic effects could be combined with acrylic base paint resin or added directly into commercial paints in place of toxicant biocides to cover artificial structures as friendly antifouling.

## Declarations

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### Author contribution

All authors contributed to the study conception and design, as well as, material preparation, data collection and analysis. VOA performed the assays. VOA and STM wrote the original draft of the manuscript. GLP, AJM and EM reviewed and edited the manuscript. All authors read and approved the final manuscript.

**Ethics approval and Consent to participate** – Not applicable

**Consent for publication** - All authors agree with the publication

**Competing interests** - The authors declare that they have no competing interests.

## References

1. Agostini VO, Ritter MdoN, Macedo AJ, Muxagata E, Erthal F (2017) What determines sclerobiont colonization on marine mollusk shells? PLoS ONE 12(9):e0184745. <https://doi.org/10.1371/journal.pone.0184745>
2. Agostini VO, Macedo AJ, Muxagata E (2018a) O papel do biofilme bacteriano no acoplamento bento-pelágico, durante o processo de bioincrustação. Revista Liberato 19(31):1e134. <https://doi.org/10.31514/rliberato.2018v19n31.p23>
3. Agostini VO, Macedo AJ, Muxagata E, Pinho GLL (2019a) Surface coatings select their micro and macrofouling communities differently on steel. Environ Pollut 254:113086. <https://doi.org/10.1016/j.envpol.2019.113086>
4. Agostini VO, Macedo AJ, Muxagata E, Silva MV, Pinho GLL (2019b) Natural and non-toxic products from Fabaceae Brazilian plants as a replacement for traditional antifouling biocides: an inhibition potential against initial biofouling. Environ Sci Pollut Res Int 26(26):27112–27127. <https://doi.org/10.1007/s11356-019-05744-4>
5. Agostini VO, Macedo AJ, Muxagata E, Silva MV, Pinho GLL (2020) Non-toxic antifouling potential of Caatinga plant extracts: effective inhibition of marine initial biofouling. Hydrobiologia 847:45–60. <https://doi.org/10.1007/s10750-019-04071-6>
6. Almeida JR, Silva MC, Sousa E, Antunes J, Pinto M, Vasconcelos V, Cunha I (2017) Antifouling potential of Natureinspired sulfated compounds. Sci Rep 7:42424. <https://doi.org/10.1038/srep42424>
7. Antunes AL, Trentin DS, Bonfanti JW, Pinto CC, Perez LR, Macedo AJ, Barth A (2010) Application of a feasible method for determination of biofilm antimicrobial susceptibility in staphylococci. Acta Pathologica Microbiologica et Immunologica Scandinavica 118:873–877. <https://doi.org/10.1111/j.1600-0463.2010.02681.x>
8. Amara I, Miled W, Slama RB, Ladhari N (2018) Antifouling processes and toxicity effects of antifouling paints on marine environment. A review. Environ Toxicol Pharmacol 57:115–130. <https://doi.org/10.1016/j.etap.2017.12.001>
9. Azis PKA, Al-Tisan I, Sasikumar N (2001) Biofouling potential and environmental factors of seawater at a desalination plant intake. Desalination 135:69–82. [https://doi.org/10.1016/S0011-9164\(01\)00140-0](https://doi.org/10.1016/S0011-9164(01)00140-0)
10. Baker JT, Sutherland MD (1968) Pigments of marine animals VIII. Precursors of 6,6'-dibromoindigotin (Tyrian Purple) from the marine mollusc *Dicathais orbita* Gmelin. Tetrahedron Lett 1:43–46 4
11. Biswas B, Rogers K, Mclaughlin F, Daniels D, Yadav A (2013) Antimicrobial activities of leaf extracts of guava (*Psidium Guajava* L.) on two gram-negative and gram-positive Bacteria. Int J Microbiol. 2013;2013. 52. <https://doi.org/10.1155/2013/746165>
12. Boulekbache-Makhlouf L, Slimani S, Madani K (2013) Total phenolic content, antioxidant and antibacterial activities of fruits of *Eucalyptus Globulus* cultivated in Algeria. Ind Crop Prod 41:85–89.

<https://doi.org/10.1016/j.indcrop.2012.04.019>

13. Chen M, Shao C-L, Wang K-L, Xu Y, She Z-G, Wang C-Y (2014) Dihydroisocoumarin derivatives with antifouling activities from a gorgonian-derived *Eurotium* sp. fungus. *Tetrahedron* 70:9132e9138. <http://dx.doi.org/10.1016/j.tet.2014.08.055>
14. Dahms HU, Xu Y, Pfeiffer C (2006) Antifouling potential of cyanobacteria [mini-review]. *Biofouling* 22:317–327. <https://doi.org/10.1080/08927010600967261>
15. Desai DV (2008) Impact of Irgarol 1051 on the larval development and metamorphosis of *Balanus amphitrite* Darwin, the diatom *Amphora coffeaformis* and natural biofilm. *Biofouling* 24(5):393–403. <https://doi.org/10.1080/08927010802339764>
16. Djihane B, Wafa N, Elkhamssa S, Maria AE, Mihoub ZM (2017) Chemical constituents of *Helichrysum italicum* (Roth) G. Don essential oil and their antimicrobial activity against gram-positive and gram-negative Bacteria, filamentous Fungi and *Candida Albicans*. *Saudi Pharm J* 25(5):780–787. <https://doi.org/10.1016/j.jsps.2016.11.001>
17. Dobretsov S, Dahms HU, Qian PY (2006) Inhibition of biofouling by marine microorganisms and their metabolites. *Biofouling* 22:43–54. <https://doi.org/10.1080/08927010500504784>
18. Faimali M, Falugi C, Gallus L, Piazza V, Tagliaferro G (2003) Involvement of acetyl choline in settlement of *Balanus amphitrite*. *Biofouling* S19:213–220. <http://dx.doi.org/10.1080/0892701021000044228>
19. Figueiredo GSM, Zardo RS, Silva BV, Violante FA, Pinto AC, Fernandes PD (2013) Convolutamydine A and synthetic analogues have antinociceptive properties in mice. *Pharmacol Biochem Behav* 103:431–439. <https://doi.org/10.1016/j.jep.2009.10.025>
20. Figuerola B, Sala-Comorera L, Angulo-Preckler C, Vázquez J, Blanch AR, Montes MJ, García-Aljaro C, Mercadé E, Avila C (2014) Antimicrobial activity of Antarctic bryozoans: An ecological perspective with potential for clinical applications. *Mar Environ Res* 101:52–59. <https://doi.org/10.1016/j.marenvres.2014.09.001>
21. Figuerola B, Angulo-Preckler C, Núñez-Pons L, Moles J, Sala-Comorera L, García-Aljaro C, Blanch AR, Avila C (2017) Experimental evidence of chemical defence mechanisms in Antarctic bryozoans. *Mar Environ Res* 129:68–75. <https://doi.org/10.1016/j.marenvres.2017.04.014>
22. Finney DJ (1978) *Statistical Method in Biological Assay*, third edn. Charles Griffin & Co. Ltd, London, England
23. Finney DJ (1971) *Probit Analysis*, third edn. Cambridge University Press, London, England
24. Garden SJ, Torres JC, Ferreira AA, Silva RB, Pinto AC (1997) A Modified Sandmeyer Methodology and the Synthesis of (d:)-Convolutamydine A. *Tetrahedron Lett* 38:1501–1504. [https://doi.org/10.1016/S0040-4039\(97\)00140-8](https://doi.org/10.1016/S0040-4039(97)00140-8)
25. Gil-Turnes MS, Hay ME, Fenical W (1989) Symbiotic marine bacteria chemically defend crustacean embryos from a pathogenic fungus. *Science* 246:116–118. <https://doi.org/10.1126/science.2781297>

26. Grougnet R, Magiatis P, Fokialakis N, Mitaku S, Skaltsounis AL, Tillequin F, Sévenet T, Litaudon M (2005) Koniamborine, the first pyrano[3,2-b]indole alkaloid and other secondary metabolites from *Boronella koniambiensis*. *J. Nat. Prod.* 2005, 68, 1083. <https://doi.org/10.1021/np050013w>
27. Gu J-D (2005) Biofouling and prevention: corrosion, biodeterioration and Biodegradation of materials. In: Kutz, M. (Ed). *Handbook of Environmental Degradation of Materials*. 610 p. <https://doi.org/10.1016/B978-081551500-5.50011-2>
28. Guo Y, Chen F (1986) TLC-UV-spectrophotometric and TLC-scanning determination of *isatin* in leaf of *Isatis*. *Zhongcaoyao* 17:8–11
29. Hothorn T, Bretz F, Westfall P (2008) Simultaneous inference in general parametric models. *Biom J* 50:346–363. <https://doi.org/10.1002/bimj.200810425>
30. ICRAM (2001) *Metodologie analitiche di riferimento*. Ministero dell'Ambiente e della Tutela del Territorio. Servizio Difesa Mare, Roma
31. International Organization for Standardization 10253 (ISO 10253) (2006) Water Quality and Marine Algal Growth Inhibition Test with *Skeletonema costatum* and *Phaeodactylum tricornutum*. London
32. International Organization for Standardization 14669 (ISO 14669) (1999) Water quality – part 5: biological methods – section 5.24: determination of acute lethal toxicity to marine copepods. (Copepoda, Crustacea), London
33. Li Y, Zhang F, Xu Y, Matsumura K, Han Z, Liu L, Lin W, Jia Y, Qian P-Y (2012) Structural optimization and evaluation of butenolides as potent antifouling agents: modification of the side chain affects the biological activities of compounds. *Biofouling: The Journal of Bioadhesion and Biofilm Research* 28(8):857–864. <https://doi.org/10.1080/08927014.2012.717071>
34. Lian Z-M, Sun J, Zhu H-L (2016) Design, synthesis and antibacterial activity of isatin derivatives as FtsZ inhibitors. *J Mol Struct* 1117:8–16. <https://doi.org/10.1016/j.molstruc.2016.03.036>
35. Lopanik N, Lindquist N, Targett N (2004) Convolutamydine A and synthetic analogues have antinociceptive properties in mice *Oecologia*, 139, 131– 139. 15. <https://doi.org/10.1016/j.pbb.2012.09.023>
36. Lopes LFP, Agostini VO, Muxagata E (2018) Could some procedures commonly used in bioassays with the copepod *Acartia tonsa* Dana 1849 distort results? *Ecotox Environ Safe* 150:353–365. <http://dx.doi.org/10.1016/j.ecoenv.2017.12.004>
37. Majik MS, Rodrigues C, Mascarenhas S, D'Souza L (2014) Design and synthesis of marine natural product-based 1H-indole-2,3-dione scaffold as a new antifouling/ antibacterial agent against fouling bacteria. *Bioorg Chem* 54:89–95. <http://dx.doi.org/10.1016/j.bioorg.2014.05.001>
38. Pakravan P, Kashanian S, Khodaei MM, Harding FJ (2013) Biochemical and pharmacological characterization of isatin and its derivatives: from structure to activity. *Pharmacol Rep* 65(2):313–335. [https://doi.org/10.1016/s1734-1140\(13\)71007-7](https://doi.org/10.1016/s1734-1140(13)71007-7)
39. Patel A, Bari S, Talele G, Patel J, Sarangapani M (2006) Synthesis and Antimicrobial Activity of Some New Isatin Derivatives. *Iran J Pharm Res* 4:249–254. <https://doi.org/10.22037/IJPR.2010.685>

40. Qian P-Y, Xu Y, Fusetani N (2010) Natural products as antifouling compounds: recent progress and future perspectives. *Biofouling: The Journal of Bioadhesion and Biofilm Research* 26(2):223–234. <http://dx.doi.org/10.1080/08927010903470815>
41. R Core Team: R [Internet]. Auckland: A language and environment for statistical computing; [accessed 2019]. Available from: <https://www.R-project.org/>
42. Resgalla JRC, Laitano KS (2002) Sensibilidade dos organismos marinhos utilizados em testes de toxicidade no Brasil. *Notas Téc. FACIMAR* 6, 153–163. v6n1.p153-163
43. Rittschof D, Clare AS, Gerhart DJ, Mary SA (1992) Bonaventura, J. Barnacle *in vitro* assays for biologically active substances: Toxicity and settlement inhibition assays using mass cultured *Balanus amphitrite amphitrite* Darwin. *Biofouling* 6:115–122. <https://doi.org/10.1080/08927019209386217>
44. Sabatini ME (1990) The developmental stages (Copepodids I to VI) of *Acartia tonsa* Dana, 1849 (Copepoda Calanoida). *Crustaceana* 59:53–61
45. Sandasi M, Leonard C, Viljoen A (2010) The *in vitro* Antibiofilm activity of selected culinary herbs and medicinal plants against *listeria monocytogenes*. *Lett Appl Microbiol* 50(1):30–35. <http://dx.doi.org/10.1111/j.1472-765X.2009.02747.x>
46. Schultz MP, Bendick JA, Holm ER, Hertel WM (2011) Economic impact of biofouling on a naval surface ship. *Biofouling* 27(1):87–98. <http://dx.doi.org/10.1080/08927014.2010.542809>
47. Silva BV (2013) Isatin, a versatile molecule: studies in Brazil. *J Braz Chem Soc* 24(5):707–720. <https://dx.doi.org/10.5935/0103-5053.20130089>
48. Silva BNM, da, Bastos RS, Silva BV, Pinto AC (2010) Síntese de 5-nitro-isatina e 5-cloro-isatina a partir da isonitrosoacetanilida. *Quim Nova* 33(10):2279–2282. <http://dx.doi.org/10.1590/S0100-40422010001000043>
49. Silva BV, Violante FA, Pinto AC, Santos LS (2011) The mechanism of Sandmeyer's cyclization reaction by electroscopy ionization mass spectrometry. *Rapid Commun Mass Spectrom* 25:423–428. <https://www.ncbi.nlm.nih.gov/pubmed/21213361>
50. Teixeira PF, Kaminski SM, Avila TR, Cardozo AP, Bersano JGSilva, Garden JFM, Pinto SJ (2001) A. C. The chemistry of isatins: a review from 1975 to 1999. *J. Braz. Chem. Soc.* [online]. 2001, vol.12, n.3, pp.273–324. <http://dx.doi.org/10.1590/S0103-50532001000300002>
51. Bianchini A (2010) Diet influence on egg production of the copepod *Acartia tonsa* (Dana, 1896). *An. Acad Bras Cienc* 82:333–339. <http://dx.doi.org/10.1590/S0001-37652010000200009>
52. Telegdi J, Trif L, Románszki L (2016) Smart anti-biofouling composite coatings for naval applications. *Transport, structural environmental and energy applications. Woodhead Publishing Series in Composites Science and Engineering.* pp. 123–155. doi: <https://doi.org/10.1016/B978-1-78242-283-9.00005-1>
53. Throndsen J (1978) Preservation and storage. In: Sournia A (ed) *Phytoplankton Manual*. UNESCO, Paris, p 69e74

54. Tiwari M, Donelli G, Tiwari V (2018) Strategies for combating bacterial biofilms: a focus on anti-biofilm agents and their mechanisms of action au - Roy. *Ranita Virulence* 9(1):522–554. <https://doi.org/10.1080/21505594.2017.1313372>
55. Wang K-L, Wu Z-H, Wang Y, Wang C-Y, Xu Y (2017) Mini-review: antifouling natural products from marine microorganisms and their synthetic analogs. *Mar Drugs* 15:266. <https://doi.org/10.3390/md15090266>
56. Wei L, Wang Q, Liu X (1982) Application of thin-layer chromatography in quality control of Chinese medicinal preparations. II. Qualitative analysis of some Chinese medicinal preparations of Chansu. *Yaowu Fenxi Zazhi* 2(5):288–2913
57. Whale GF, Battersby N (2005) Case study: whole-effluent assessment using a combined biodegradation and toxicity approach. In: Thompson KC, Wadhia K, Loibner AP (eds) *Environmental Toxicity Testing*. Blackwell Publishing, Oxford, pp 310–336
58. Zagatto PA, Bertoletti E (2008) *Ecotoxicologia aquática: princípios e aplicações [Aquatic ecotoxicology: principles and applications]*. Rima, São Carlos
59. Zhang H, Shigemori H, Ishibashi M, Kosaka T, Pettit GR, Kamano Y, Kobayashi J (1994) Convolutamides A ~ F, novel  $\gamma$ -lactam alkaloids from the marine bryozoan *Amathia convoluta*. *Tetrahedron* 50:10201–10206. [https://doi.org/10.1016/S0040-4020\(01\)81752-X](https://doi.org/10.1016/S0040-4020(01)81752-X)
60. Raghav X, Ma C, Honrao JT, Wood O, Benchama H, Zhou S, Mallipeddi, Makriyannis A (2018) Financial burden of diabetic foot ulcers to world: a progressive topic to discuss always. *Bioorg Med Chem* 26:4963–4970. <https://doi.org/10.1177/2042018817744513>
61. Zobell CE (1946) *Marine microbiology*. Chronica Botanica, Waltham, Mass

## Figures

### Figure 1

Isonitrosoacetanilides compounds used to investigate antifouling activity against micro and macrofouling.

### Figure 2

Crystal violet assay showing biofilm bacteria and planktonic bacteria biomass inhibition at 48 h. The vertical lines denote 95% confidence intervals (standard error \* 1.96, n = 4). The letters above the columns indicate the statistical differences ( $p < 0.05$ ) or similarities ( $p > 0.05$ ) among the concentrations.  $^{\circ}\text{C} =$

solvent control;  $\bar{C}$  = negative control. Treatments with antibiofilm results (optimal) are delimited by the red box.

### Figure 3

Crystal violet assay showing biofilm bacteria biomass eradication at 24 h. The vertical lines denote 95% confidence intervals (standard error \* 1.96, n = 4). The letters above the columns indicate the statistical differences ( $p < 0.05$ ) or similarities ( $p > 0.05$ ) among the concentrations.  $^{\circ}C$  = solvent control;  $\bar{C}$  = negative control. Treatments with eradication action are delimited by the red box.

### Figure 4

*Amphibalanus amphitrite* assay showing cyprid settlement and mortality at 72 h. The vertical lines denote 95% confidence intervals (standard error \* 1.96, n = 3). The letters above the columns indicate the statistical differences ( $p < 0.05$ ) or similarities ( $p > 0.05$ ) among the concentrations.  $^{\circ}C$  = solvent control;  $\bar{C}$  = negative control. Treatments with anti-settlement and non-toxic results (optimal) are delimited by the red box.

### Figure 5

*Amphibalanus amphitrite* assay showing nauplii II mortality and swimming alteration at 24 and 48 h. The vertical lines denote 95% confidence intervals (standard error \* 1.96, n = 3). The letters above the columns indicate the statistical differences ( $p < 0.05$ ) or similarities ( $p > 0.05$ ) among the concentrations.  $^{\circ}C$  = solvent control;  $\bar{C}$  = negative control. Treatments with non-toxic results (optimal) are delimited by the red box.