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RESEARCH ARTICLE

Posidonia oceanica (L.) (Delile, 1813) extracts as a potential booster biocide in fouling-release coatings

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Abstract

Since the banning of tributyltin, the addition of inorganic (metal oxides) and organic (pesticides, herbicides) biocides in antifouling paint has represented an unavoidable step to counteract biofouling and the resulting biodeterioration of submerged surfaces. Therefore, the development of new methods that balance antifouling efficacy with environmental impact has become a topic of great importance. Among several proposed strategies, natural extracts may represent one of the most suitable alternatives to the widely used toxic biocides. *Posidonia oceanica* is one of the most representative organisms of the Mediterranean Sea and contains hundreds of bioactive compounds. In this study, we prepared, characterized, and assessed a hydroalcoholic extract of *P. oceanica* and then compared it to three model species. Together, these four species belong to relevant groups of biofoulers: bacteria (*Aliivibrio fischeri*), diatoms (*Phaeodactylum tricornutum*), and serpulid polychaetes (*Ficopomatus enigmaticus*). We also added the same *P. oceanica* extract to a PDMS-based coating formula. We tested this coating agent with *Navicula salinicola* and *Ficopomatus enigmaticus* to evaluate both its biocidal performance and its antifouling properties. Our results indicate that our *P. oceanica* extract provides suitable levels of protection against all the tested organisms and significantly reduces adhesion of *N. salinicola* cells and facilitates their release in low-intensity waterflows.

Keywords Model species · Biofouling · Natural extract · PDMS-based coating · Ecotoxicology · Bioactivity

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Introduction

Biofouling is considered one of the main unresolved issues in the aquatic environment. Biofouling is commonly defined as the unwanted growth of organisms, usually associated with the deposition of organic and inorganic matter on surfaces that are in contact with freshwater, brackish water, or seawater (Callow and Callow 2011). This has substantial adverse effects on anthropic coastal and offshore activities. In particular, biofouling reduces the efficiency of submerged structures, including heat exchangers, pipelines and flow channels, turbines, reverse osmosis membranes, and ship hulls (Gu 2005; Schultz 2007; Schultz et al. 2011).

Biocides (such as copper, zinc, herbicides and pesticides) are the most commonly used method to prevent biofouling globally (Ytreberg et al. 2010). However, the release of these chemicals in large quantities into the aquatic environment, combined with their significant impact on the biota and habitat quality, was underestimated until the mid-1990s when the concept of "Green Chemistry" was first introduced (Anastas and Williamson 1996). Since then, the release of hazardous substances, whether accidental or intentional, into the environment has received much attention, resulting in the banning of certain compounds. An example of this is tributyltin (Champ 2000; *Ban on organotin compounds (TBT)*; IMO 2001; Regulation 11153/2002 n.d.), which caused well-documented adverse environmental effects on organisms (e.g. the imposex phenomenon on gastropods and other invertebrates (Gibbs and Bryan 1986)). Therefore, it became crucial to find environmentally sustainable alternatives. Two strategies to prevent biofouling have since been proposed. These include (a) the preparation of specific surfaces that are selected based on their fouling-release properties that promote surface cleaning and (b) the use of "less toxic biocides" due to their antifouling properties that aim to prevent surface colonization.

Fouling-release surfaces are surfaces that prevent strong adhesion of proteins, cells, algae, and other micro-/macro-organisms without any leaching of biocides (Maan et al. 2020). According to the available literature, the most successful coatings are those based on poly(dimethylsiloxane) (PDMS) elastomers (Pretti et al. 2013; Martinelli et al. 2019). Moreover, several strategies have been tested to produce novel fouling-release coatings that have different polymer chemistries, including amphiphilic and hydrolyzable copolymers (Guazzelli et al. 2020a, b; Hu et al. 2020), zwitterionic polymers (Jiang and Cao 2010), phase-segregated polysiloxane-urethanes (Majumdar et al. 2007), polymer nanocomposites (Beigbeder et al. 2008; Carl et al. 2012; Guazzelli et al. 2020c), and polyphosphonate polymers (Masotti et al. 2020; Guazzelli et al. 2021). Despite this, the search for biocidal compounds to add to antifouling compounds is ongoing, with the main goal of finding substances that are able to affect biofouling organisms without emitting organic and/or toxic solvent-based contaminants into the environment (Benito-González et al. 2019). Currently, new strategies have been studied to improve silicone-based matrix properties, including chemical modification with epoxy and urethane moieties, functionalization, and immobilization of bioactive agents (Gu et al. 2020; Hu et al. 2020), which, after being approved, may represent a potentially viable integration of both antifouling and fouling-release coatings. One of the most promising technologies for biofouling-formation control is the use of active compounds that are naturally produced by living organisms (Qian et al. 2009; Qian et al. 2015), including bacteria (Ma et al. 2017; Pan et al. 2019), sponges (Hellio et al. 2005; De Marchi et al. 2022), macroalgae (Saha et al. 2018), and plants (Feng et al. 2018). These solutions rely on the prevention of settlement/adhesion of micro- and macro-organisms on submersed surfaces, lowering the chemical-based environmental impact. Overall, the present study is part of a continuous program of research that seeks to discover natural marine products, capable of inhibiting marine biofouling that can be used as additives for fouling-release formulations. These compounds, after being approved and inserted in EU and other countries' regulatory plans, may act as a valid alternative to antifouling paints without losing their effectiveness when it comes to the protection of submerged structures from biofouling-linked deterioration.

Posidonia oceanica, besides being studied as an ecological key species for Mediterranean Sea biodiversity maintenance (Gobert et al. 2006), oxygen balance (Campagne et al. 2015), and coastal dynamics (Boudouresque et al. 2006), is actually recognized as a rich source of different compounds, such as phenolic compounds and derivatives (Agostini et al. 1998), chicoric acid (Haznedaroglu and Zeybek 2007), long-chain fatty acids (Viso et al. 1993), terpenes (Hammami et al. 2013), and flavonoids (Heglmeier and Zidorn 2010). It undertakes a wide range of bioactivities, which are potentially relevant for human activities, such as medicine (Vasarri et al. 2021a), industry, agriculture, and food maintenance (Lorenzo et al. 2018).

In this study, a hydroalcoholic extract was prepared from the leaves of *P. oceanica*, and its potential biological properties were evaluated, both as a raw form and as an additive in a PDMS-based fouling-release coating. In particular, we evaluated if the *P. oceanica* extract:

I) Exerts biological effects on three model species that are partially or fully comparable to known biofoulers

II) Produces comparable biological results to commonly used biocides
III) Interferes with the preparation of PDMS-based fouling-release coatings
IV) Maintains its biological effects even after being added to a PDMS formula
V) Can be considered a potential booster biocide for PDMS-based coatings by enhancing the protection of submerged surfaces from biodeterioration

Materials and methods

Posidonia oceanica extract preparation and characterization

Leaves of *P. oceanica* ((L.) Delile, 1813) from naturally available cuttings (left behind from mooring activities) were collected in July 2020 in the "Secche della Meloria" marine protected area (Livorno, Italy) by authorized personnel of CIBM (Livorno, Italy). A water-ethanol extraction method (30:70 v/v) was used in order to obtain hydrophilic compounds, including polyphenols and carbohydrates, from the *P. oceanica* leaves (Barletta et al. 2015). The leaves were washed, cleared of epiphytes, dried, chopped, and then incubated in 10 mL of water-ethanol solution per gram of leaves overnight at 37 °C while being stirred, and then left at 65 °C for 3h. Two centrifugations (4000×*g*) of 25 min each were performed to remove the leaf residue and recover the supernatant. The hydroalcoholic extract was then added to a separating funnel along with *n*-hexane in a 1:1 ratio. After repeated agitations, the hydroalcoholic extract was allowed to stand for the separation of two phases. The lower hydrophilic phase was then recovered and brought to dryness. Upon use, the dry extract was resuspended in water-ethanol (30:70 v/v).

The total polyphenol and carbohydrate content of the hydroalcoholic extract was analyzed by in vitro colorimetric assays using the Folin-Ciocalteu method and the phenol-sulfuric method, respectively, as described in Barletta et al. (2015) without modifications. The total polyphenol content in *P. oceanica* extract was reported as milligrams of gallic acid equivalents per milligrams of dry extract, while the total carbohydrate content was reported as milligrams of glucose equivalents per milligrams of dry extract. Each in vitro test was repeated at least three times.

Water media preparation

Natural seawater (pH 8.11, S 39 psu) was manually collected in Quercianella (LI), stored in opaque HDPE tanks, and maintained at 5±2 °C until use. Before use, seawater was filtered twice, at 41 µm and then 0.45 µm. The salinity necessary for bioassay media preparation (30 psu) was obtained by diluting with ultrapure water. Seawater used for all tests was identified as filtered natural sea water (FNSW).

Ecotoxicological characterization

Before testing *P. oceanica* extract as a potential booster biocide in antifouling compositions, an ecotoxicological screening, characterized by three different endpoints, was performed in order to investigate the extract's bioactivities. The selected assays were the inhibition of bioluminescence in the marine bacterium *Aliivibrio fischeri*, the inhibition of growth in the marine diatom *Phaeodactylum tricornutum*, and the inhibition of the larval development in the brackish water serpulid *Ficopomatus enigmaticus* (Fauvel, 1923). Species selection was based on the organism's relevance as a biofouler. *F. enigmaticus* is an example of an effective hard fouler, whereas *P. tricornutum* and *A. fischeri* are comparable to microalgal and bacterial biofilm-forming organisms. Moreover, each model species was tested against a specific reference biocide, selected from those that are used in existing antifouling paints. Selected biocides were Zn^{2+} for *A. fischeri*, Diuron® for *P. tricornutum*, and Cu^{2+} for *F. enigmaticus*. Biocides were assessed following the same protocol as for the extract effect evaluation.

Sample preparation

Frozen lyophilized *P. oceanica* extract (9.6 mg) was resuspended in 1.5 mL of ethanol:water (70:30, v/v) in order to obtain a 6.4 g/L stock solution.

Stock solution was diluted with algal growth medium (for *P. tricornutum* bioassay) and with FNSW (for the other 2 assays) to obtain working concentrations (total range 90–0.09 mg/L). The highest concentration was selected to obtain a final ethanol concentration lower than 1 %. All tests run together with a negative control and a 1 % ethanol control.

Inhibition of bioluminescence in *Aliivibrio fischeri*

The acute luminescent bacteria test was performed in accordance with the ISO 11348 2007 methodology. Bacteria were purchased as freeze-dried bacterial cells (strain n. 19A4002A, Ecotox LDS, Pregnana Milanese, MI, Italy).

Growth inhibition in *Phaeodactylum tricornutum*

The assessment of growth inhibition in *P. tricornutum* was performed following ISO procedures (ISO 10253 2016). *P. tricornutum* Bholin strain (CCAP 1052/1A) was purchased from the reference center CCAP (Culture Collection of Alga and Protozoa Scottish Association for Marine Science/SAMS Research Services Ltd).

To carry out the test, the algal culture was grown for 72 h under continuous light (6000–8000 lux), obtaining a logarithmic-phase algal culture. After 72 h, the culture was diluted to obtain a concentration of 1×10^6 cell/mL. Twenty microliters of diluted algal culture and 2 mL of samples (three replicates) or controls (ASTM-ESM) were pipetted into 24-well plates. The sample preparation was reported in the "*Posidonia oceanica* extract preparation and characterization" section. Plates were left at 20 ± 2 °C under continuous *illumination* (6000–8000 lx) and slow shaking (80 rpm) for 72 h. To evaluate *P. tricornutum* growth, absorbance was read spectrophotometrically at 670 nm (Abs 670) and algal concentration (cells/mL) was calculated using the following equation: cells/mL = Abs $670/10^{-7}$.

The Linear Interpolation Method for Sublethal Toxicity software (U.S.EPA, 1993) was used to calculate the values of $EC_{10}/_{50}$ for all *P. oceanica* extracts. The reference toxicant was potassium dichromate (ISO 10253 2016).

Larval development in *Ficopomatus enigmaticus*

The brackish water serpulid *F. enigmaticus* was collected in S. Rossore-Migliarino Regional Park (Pisa, Italy) and transported to the laboratory covered with a wet towel. The reef was kept in tanks under controlled conditions: temperature 22 ± 1 °C, oxygen saturation > 90 %, pH 8.1 ± 0.1, photoperiod 10-h light:14-h darkness, and salinity was increased up to a maximum of 5 points/day, from salinity registered at sampling site to 30 psu (Oliva et al. 2018). Organisms were fed daily with an *Isochrysis galbana* algal suspension (1×10^4 cells/mL).

As reported by Oliva et al. (2018), the serpulid's calcareous tube was broken with tweezers, and *F. enigmaticus* individuals were gently removed from their tubes and then individually placed in wells of a 24-well plate filled up with 1 mL of FNSW. This destructive method induced the emission of the gamete within 10 min. The larval development assay was performed according to Oliva et al. (2019). The number of normal and abnormal larvae, observed under the microscope, allowed us to calculate the percentage of poorly developed larvae. The acceptability threshold of the assay was set at 20 % of poorly developed larvae in controls. $EC_{10}/_{50}$ values and their 95 % confidence intervals were calculated via PROBIT analysis.

Antifouling/fouling-release assays

Testing slide preparation and characterization

Disilanol-terminated polydimethylsiloxane (HO-PDMS-OH, M_n = 26000 g/mol) matrix and poly(diethoxysiloxane) crosslinker (ES40) were purchased from ABCR and used as received. Tetrabutylammonium fluoride trihydrate (TBAF, Merk), ethyl acetate, and ethanol (Carlo Erba reagents) were used without further purification.

A stock dispersion (213 mg/L) of *P. oceanica* extract was prepared by adding 3.2 mg of the extract to 1 mL of ethanol. After vortex mixing, the obtained dispersion was diluted with 14 mL of ethyl acetate and sonicated in an ultrasonic bath for 30 min.

PDMS-based films (P90) were prepared by solvent casting a condensation cure reactive formulation. The latter was composed of HO-PDMS-OH as matrix (1 g), ethyl acetate as solvent (3 mL), ES40 as crosslinker (25 mg), and an aliquot of the stock dispersion of the *P. oceanica* extract. The amount of the aliquot was selected so that the final concentration of *P. oceanica* extract was 90 mg/L with respect to HO-PDMS-OH. The formulation was vortex mixed for a minute. Finally, TBAF (0.15 wt% with respect to HO-PDMS-OH) was added as an activator and the mixture was vortex mixed for an additional minute. The formulation was cast on cleaned glass slides and the crosslinking reaction was allowed to proceed at room temperature for 24 h (dry film thickness ≈ 300 μm). Films of PDMS alone (PDMS) without the extract were also prepared as controls following the same experimental conditions.

Prior to use, both P90- and PDMS-coated glass slides were maintained in FNSW for 24 h, rinsed in ultrapure water, and dried at room temperature to remove eventual surface impurities.

Navicula salinicola settlement and detachment assays

The settlement of the diatom *N. salinicola* (CCAP 1050/10) was assessed by exposing the prepared slides to a 10^4 cells/mL algal suspension in static conditions (temperature 20±2 °C; photoperiod 14:10 h light:darkness; light intensity 3000 lux), for 24 h. After this period, all films were gently rinsed with clean FNSW to remove un-adhered cells. Slides were exposed for 5 min to a 5 Pa shear stress (corresponding to a 50 L/min water stream), using turbulent channel flow apparatus. Adherent algae on the slides were evaluated before and after waterflow exposure by measuring the autofluorescence of chlorophyll (a+b) with a microplate reader (Synergy-HTX, Biotek, Winooski, VT, USA), as reported in Guazzelli et al. (2020c) without modifications. Background noise of each slide was measured before the test following the same procedure, and removed from the relative chlorophyll autofluorescence measures.

Ficopomatus enigmaticus settlement assay

Adults of *F. enigmaticus* were collected in S. Rossore-Migliarino-Massaciuccoli Regional Park (Pisa, Italy) and acclimated, prior to use, in a laboratory to test conditions (temperature 20 ± 2 °C, water salinity 30 psu, pH

8.12, photoperiod 14:10 h light:darkness). Larval culture for test setup and testing procedures were performed as reported in Guazzelli et al. (2020c), without modifications. The larvae were reared until they reached "competent larva" stage. Rearing conditions were the same as for the adult maintenance. Twenty competent larvae were pipetted on each sample replicate. All replicates were incubated in the darkness at 21 ± 2 °C, in wet conditions. Incubation was carried on for a total time of 5 days, counting the amount of settled larvae at the end of the exposure and calculating the settlement percentage on each sample replica.

Statistical analysis

For each ecotoxicological bioassay, EC_{10} and EC_{50} parameters were calculated depending on the test. INTERPOLATION v2.0 (USEPA, 1993) software was used for *P. tricornutum* inhibition of growth. For *A. fischeri* inhibition of bioluminescence, the software MicrotoxOmni® was used for both test performance and automatic ecotoxicological parameter calculation. For *F. enigmaticus* larval development assay, a PROBIT model was adopted (Finney, 1952). For all three assays, differences among mean effects at each tested concentration were compared with an ANOVA analysis of variance, followed by a Tukey post-test for multiple comparisons, performed with GraphPad Prism® 5. Moreover, differences of mean effect at each tested concentration and the relative control were compared with a Student's *t* test, performed with the same software for all bioassays.

For all adhesion/settlement and detachment assay, differences between samples were compared with a Student's *t* test, performed with GraphPad Prism® 5.

Results

Extraction yield from *P. oceanica* leaves

From the hydroalcoholic extraction carried out in this work, 4 mg of dry extract were recovered from 1 g of dry leaves. A preliminary ultra-performance liquid chromatography (UPLC) characterization analysis reported that the *P. oceanica* extract consisted mainly of polyphenols. The phenolic composition of the hydroalcoholic fraction of *P. oceanica* extract is reported in Table 1. This extraction method allowed us to obtain the hydrophilic component from the leaves of *P. oceanica*. Specifically, the dry extract obtained from this preparation was found to be composed for 28 ± 2 % of polyphenols equivalent to gallic acid, and for 50 ± 10 % of carbohydrates equivalent to glucose.

Furthermore, because the harvesting period of *P. oceanica* leaves occurred in the same seasonal period as for previous harvests, the extraction yield and composition are in agreement with our previous work (Leri et al. 2018; Vasarri et al. 2020a, b; Vasarri et al. 2021b), supporting the reproducibility of the extraction method.

Effects on model species

The effects of the *P. oceanica* extract on the three tested organisms are reported in Fig. 1. The bioluminescence of *A. fischeri* was evaluated in the presence of *P. oceanica* extract at concentrations ranging from 90 to 0.70 mg/L. Specifically, in Fig. 1A, the recorded bioluminescence of *A. fischeri* was reported, indicating a significant concentration-dependent trend with the exception of the two lower concentrations after 30 min of incubation. All assessed concentrations, except for the lowest two, showed a significantly lower bioluminescence compared to the control. EC_{10} and EC_{50}, expressed as mg/L of extract, were 1.73 and 26.87, respectively.

The effects of different concentrations (ranging from 90 to 5.62 mg/mL) of *P. oceanica* extract in terms of growth inhibition of *P. tricornutum* are shown in Fig. 1B. A concentration-dependent effect was observed after 72 h. Despite the difference in algal growth at each concentration and the fact that all concentrations were statistically different from the control, the algal cell concentration measured at 90, 45, and 22.5 mg/L was lower than the control, while at 11.25 and 5.62 mg/L cellular concentrations, it was significantly higher than the control. EC_{10} and EC_{50} values were 15.56 and 51.62 mg/L, respectively.

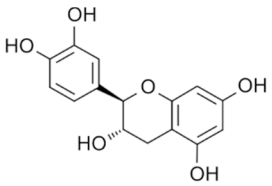
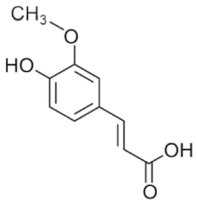
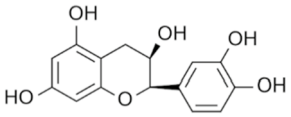
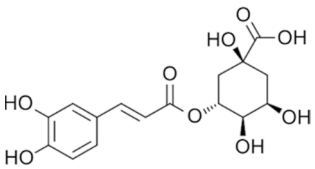
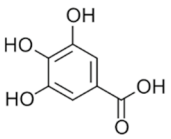
F. enigmaticus larval development impairment was evaluated in the presence of *P. oceanica* extract at concentrations in the range 18–0.09 mg/L. Figure 1C shows that the extract concentrations of 18, 9, and 4.5 mg/L significantly reduced the percentage of correctly developed larvae compared to the control. A concentration-dependent effect was also seen for *P. tricornutum*. EC_{10} and E_{50} values (2.41 and 24.97 mg/L, respectively) were similar to those observed for *A. fischeri*. Comparing with the other two tested organisms, fertilized eggs of *F. enigmaticus* needed to be exposed at 18 mg/L as the highest extract concentration as concentrations higher than 30 mg/L resulted in a complete interruption of zygote divisions (data not shown).

Biological effects, in terms of EC_{50}, of all reference biocides on the relative model organism are reported in Table 2.

Antifouling and fouling-release simulations

In the assay with *Navicula salinicola* (Fig. 2), a significant decrease of cellular adhesion was observed for P90 after 48 h of incubation compared with PDMS. Figure 3

Table 1 Polyphenolic profile characterization of *P. oceanica* leaf extract by UPLC analysis performed by Barletta et al. (2015)

Phenolic compound	Structure	Molecular Formula	Composition (%)
(+)-Catechin		$C_{15}H_{14}O_6$	84.762
Ferulic acid		$C_{10}H_{10}O_4$	1.729
Epicatechin		$C_{15}H_{14}O_6$	1.383
Chlorogenic acid		$C_{16}H_{18}O_9$	0.639
Gallic acid		$C_7H_6O_5$	0.374
Other	-	-	11.113

shows the percentage of removed algae from the same slides when it was submitted to a turbulent channel flow system, under different flow rates (10-50-150 L/min), after 2 min of exposure to the selected flow. The number of detached cells significantly increased by increasing the flow rate from 10 to 50 L/min, but not from 50 to 150 L/min. No significant differences were observed between PDMS and P90 at the same flow rate.

Figure 4 shows the adhesion percentage of *F. enigmaticus* competent larvae on both P90 and PDMS, which acted as the controls, after 5 days of incubation. In contrast to diatoms, no statistically significant differences were observed between the two coatings. However, in both cases, a percentage of adhesion lower than 50% was registered.

Discussion

In the present study, natural constituents of the Mediterranean seagrass *Posidonia oceanica* were extracted using the hydroalcoholic extraction method previously described (Barletta et al. 2015) to evaluate their biological effects against biofouling organisms. For this reason, three model organisms were selected to identify the biocidal potential of the extracts. Test organisms were selected among the commonly used model species in ecotoxicological bioassays, choosing biofoulers, or biofouler-like species. In particular, *Ficopomatus enigmaticus* is described as a worldwide diffused invasive brackish water biofouler (Charles et al. 2018; Peria and Pernet 2019), recently described as a potential model organism in both ecotoxicological bioassays (Oliva et al. 2018, 2019, 2020; De Marchi et al.

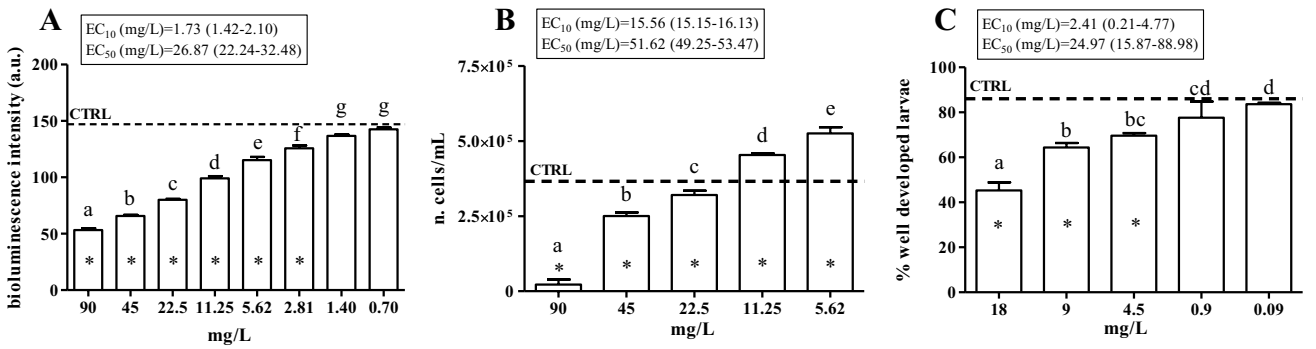


Fig. 1 **A** Concentration response of *A. fischeri* inhibition of bioluminescence assay. Dotted line represents the mean bioluminescence (I%) in controls. **B** Concentration response of *P. tricornutum* inhibition of growth assay. Dotted line represents the mean algal concentration in controls. **C** Concentration response graph *F. enigmaticus* larval development assay. Dotted line represents the mean well-developed larvae in controls. An ANOVA test was performed among all sample concentration, followed by a Tukey post-test for multiple comparison; different letters mean statistically significant differences, $p<0.05$. A Student's *t* test was performed for each sample concentration for comparison with control, *statistically significant difference, $p<0.05$. Each box shows mean EC_{10} and EC_{50} values, in brackets 95% confidence limits

Table 2 Results, in terms of EC_{50} and the relative confidence limits, of reference biocides selected for each bioassayed species

Species	Tested biocide	EC50	C.L. 95%
Aliivibrio fischeri	Zn^{2+}	8.92 mg/L	7.61–10.46
Phaeodactylum tricornutum	Diuron®	4.33 µg/L	3.20–5.21
Ficopomatus enigmaticus	Cu^{2+}	55.55 µg/L	40.05–71.02

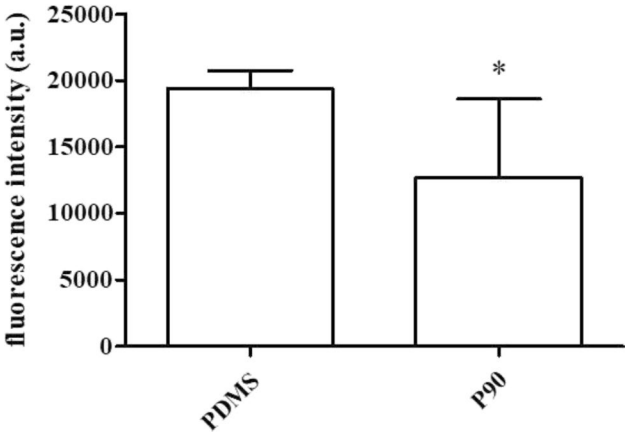


Fig. 2 Fluorescence intensity of *N. salinicola* adhered on PDMS and P90 films after 24h of exposure. A Student's *t* test was performed, *statistically significant difference, $p<0.05$

2019; Vieira Sanches et al. 2020; Cuccaro et al. 2021) and fouling-release biotests (Martinelli et al. 2015, 2018; Guazzelli et al. 2020b, c). Given that *F. enigmaticus* was selected as a representative of hard-fouling organisms, the other two assays, *P. tricornutum* and *A. fischeri*, were selected as representatives

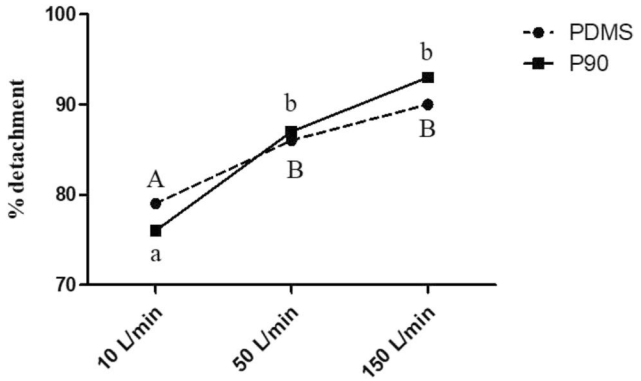


Fig. 3 *N. salinicola* percentage of detachment from PDMS and P90 films after 5 min of exposure to 5 Pa shear stress; A Student's *t* test was performed, *statistically significant difference, $p<0.05$

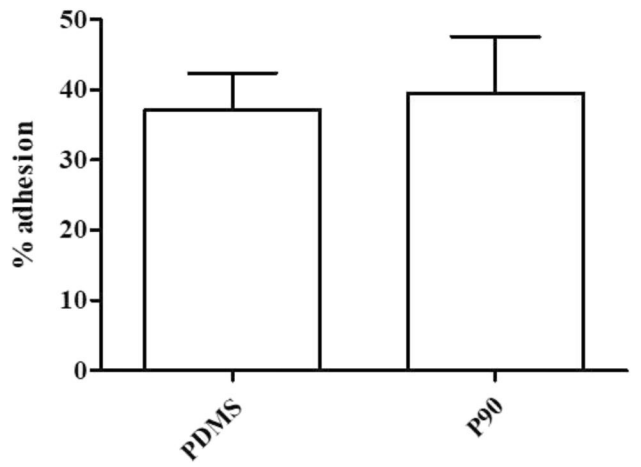


Fig. 4 Settlement percentage of *F. enigmaticus* competent larvae on PDMS and P90 films after 5 days of exposure. A Student's *t* test was performed, *statistically significant difference, $p<0.05$

for microalgal and bacterial biofilm-forming organisms, respectively. Although *P. tricornutum* is not an active biofouler, its assay was standardized by using an international testing protocol (ISO 10253:201), ensuring high scientific relevance and repeatability in compound effect biotesting. *P. tricornutum* belongs to the class Bacillariophyceae that are known as diatoms, which are common organisms in marine biofilms (Patil and Anil 2005).

Bioassay results indicated different trends of sensitivity to the *P. oceanica* extract across the test organisms. *A. fischeri* bioluminescence was significantly inhibited by the *P. oceanica* extract at a concentration of 2.813 mg/L compared to the control. *A. fischeri* and *F. enigmaticus* were the two most impacted species, with no effect concentration (EC_{10}) lower than *P. tricornutum*. This result, relative to the effect measured on *A. fischeri*, is in agreement with findings from the international scientific community, which have previously reported the antibacterial effect of *P. oceanica* constituents on both Gram-positive and Gram-negative bacteria (Berfad and Alnour 2014; Vasarri et al. 2021a). This has also been investigated for other seagrasses, such as *Zostera marina* (Papazian et al. 2019), *Cymodocea serrulata*, and *Syringodium isoetifolium* (Mayavu et al. 2009), which all induced similar effects on different bacteria strains.

Regarding the effect of the *P. oceanica* extract on diatoms, *P. tricornutum* growth was significantly inhibited at a sample concentration of 22.5 mg/L. Moreover, the two lowest tested concentrations significantly stimulated algal growth, probably indicating the presence of a relevant nutrient in the extract, which has also been reported by Alvarado et al. (2008) previously. In fact, the characterization analysis showed that the ethanol-water extraction method (70:30 v/v) allows the recovery of polyphenols and some carbohydrates in the hydrophilic phase (see Table 1). This nutrient load may be predominant under certain extract concentrations, enhancing algal growth, but becoming irrelevant when the natural toxic compound presence exceeds threshold values.

F. enigmaticus also showed a relevant sensitivity to *P. oceanica* extracts, with the percentage of well-developed larvae being significantly lower at the concentration of 4.5 mg/L compared to that of the control. While this value is far higher than the one observed with *A. fischeri*, the concentration response trend of *F. enigmaticus* was sharper if compared to both other assays, with the lowest calculated EC_{50} value. This result may indicate a strong chemical efficacy of *P. oceanica* in cutting down not only biofilm-forming organisms, but also the first developmental stages of macro-foulers.

The biological activity of natural extracts from marine seagrasses was recently investigated by Bel Mabrouk et al. (2020), which used methodologies similar to those reported in the present study. These authors indicated a strong biological effect against biofouling organisms, such as bacteria and mussel larvae, probably due to the bioactivity of phenolic compounds identified in *Halophila stipulacea* extracts.

The *P. oceanica* extract used in this study consisted of 28% of the dry extract of polyphenolic compounds. The rich variety of polyphenols of *P. oceanica* has been extensively described in the literature (Vasarri et al. 2021a; Messina et al. 2021). Many of the described biological activities of the hydroalcoholic extract of *P. oceanica* have been attributed to the synergistic action of its phenolic compounds (Vasarri et al. 2021a), and this reinforces the observations reported here on the anti-biofouling role of *P. oceanica* polyphenols. Compared to the reference biocides, raw *P. oceanica* extract generated lower biological effects on all model organisms. Therefore, once added to fouling-release coatings, it represents an alternative tool to combat the biodeterioration of submerged structures in a way that combines both fouling-release and antifouling properties.

Concerning antifouling and fouling-release assays performed on PDMS-based coatings that contained the *P. oceanica* extract, the effect on the larval settlement of *F. enigmaticus* was no longer present. This observation may be explained by the fact that competent larvae, generally known being less sensitive than zygotes or first-stage embryos (Mohammed 2013; Dean 2008), were not influenced by the extract because (i) the selected concentration was not sufficiently high to repel larval adhesion or, (ii) the effective concentration of *P. oceanica* extracts at the coating surface was too low to affect the larval adhesion. Although the EC_{50} value in *F. enigmaticus* bioassay was noticeably lower than the concentration used in coating composition (90 mg/L), it is necessary to underline that the *P. oceanica* extract was dispersed on a solid rubber-like matrix, so superficial concentration was probably comprised between the EC_{10} calculated for *P. tricornutum* and the EC_{50} for *F. enigmaticus*. To support the latter statement, it is necessary to consider the results obtained in the *Navicula salinicola* adhesion test. Indeed, there was a significant reduction in *N. salinicola* adhesion in P90 compared with PDMS. This finding supports the hypothesis of a certain presence of hydroalcoholic *P. oceanica* extract at the polymeric matrix surface level, enough to affect diatom biofilm formation. However, the presence of the extract in the coating matrix did not affect the fouling-release properties of PDMS, as observed by the detachment assay under all selected waterflow conditions. For this rationale, it is possible to identify the hydroalcoholic extract of *P. oceanica* leaves as a potential booster biocide, to add to both antifouling and fouling-coating formulations. Overall, the results obtained in this work underline the importance of tapping into marine resources to identify alternative marine/brackish water anti-biofouling compounds to safeguard

brackish and coastal system health. This undoubtedly innovative approach aims to limit anthropogenic human actions detrimental to the marine environment.

Conclusions

Posidonia oceanica leaf extract exerted a significant effect on all the three model organisms, selected ad hoc as biofouling-related species. Moreover, the extract did not negatively affect fouling-release coating preparation and efficacy, making it viable as an additive to fouling-release formulations. Although the measured effects were lower than reference biocides, they may still represent an alternative to antifouling paints to prevent the biodeterioration of submerged structures, while promoting the sustainable use of biocides. However, a massive production of *P. oceanica* extracts for commercial purposes is not even conceivable, due to its protected species status. Therefore, an in-depth chemical characterization of bioactive compounds of this species is a mandatory step in order to select those with best antifouling and environmentally friendly features for future approval, production, and in-field application.

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Author contribution Matteo Oliva, Donatella Degl'Innocenti, and Carlo Pretti conceived and planned the experiments. Carlo Pretti and Donatella Degl'Innocenti supervised the project. Matteo Oliva, Carlo Pretti, Donatella Degl'Innocenti, and Elisa Martinelli conceived of the presented idea. Matteo Oliva, Elisa Guazzelli, Elisa Martinelli, Marzia Vasarri, and Carlo Pretti verified the analytical methods. Matteo Oliva, Lucia De Marchi, Alessia Cuccaro, Giorgia Fumagalli, Gianfranca Monni, Elisa Guazzelli, and Marzia Vasarri carried out the experiment. Matteo Oliva, Lucia De Marchi, Alessia Cuccaro, Elisa Guazzelli, Marzia Vasarri, Elisa Martinelli, Donatella Degl'Innocenti, and Carlo Pretti contributed to the interpretation of results. Matteo Oliva wrote the original manuscript draft with support from Lucia De Marchi, Alessia Cuccaro, Giorgia Fumagalli, and Elisa Guazzelli. Matteo Oliva, Elisa Guazzelli, Elisa Martinelli, Marzia Vasarri, Donatella Degl'Innocenti, and Carlo Pretti reviewed and edited the final version of manuscript. All authors discussed results and contributed to the final manuscript.

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Declarations

Ethics approval and consent to participate All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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