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ARTICLE

Antifouling activity of marine bacterial extracts: A non-toxic alternative as biocide

Actividad anti-bioincrustante de extractos de bacterias marinas: Una alternativa de biocidas no tóxicos

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Resumen.- El bioincrustamiento es generado por un acondicionamiento bioquímico del sustrato para formar comunidades complejas; esto podría tener un impacto negativo a nivel ecológico e industrial. Para inhibir este proceso se utilizan recubrimientos a base de metales pesados. Los microorganismos son fuentes prometedoras de compuestos antiincrustantes que no son tóxicos o son menos tóxicos que los productos químicos comerciales que se utilizan en la actualidad. Para evaluar la eficacia antiincrustante de los extractos bacterianos, se obtuvieron siete extractos crudos de cultivos de bacterias aisladas de organismos marinos. Se sometieron a una prueba de toxicidad con nauplios de *Artemia*, a diferentes concentraciones. Los extractos no tóxicos se incorporaron a un agente gelificante (Phytagel™) y se expusieron al mar durante 30 días para evaluar la inhibición en la adhesión de diatomeas y macroorganismos. Se identificaron 67 taxones de diatomeas, pertenecientes a 45 géneros, además se identificaron cuatro grupos de macroorganismos no fotosintéticos; estos son ascidias, balanos, briozoos y poliquetos. La prueba de toxicidad mostró que solo el extracto de *Bacillus safensis* (C2) era tóxico y se excluyó de las pruebas posteriores. Existen diferencias significativas entre los tratamientos, los extractos con mejor actividad son los de *Shewanella algae* (18) y *Staphylococcus aureus* (28). Estos extractos contienen diferentes compuestos orgánicos como saponinas, terpenos, esteroles y proteínas, los cuales podrían ser responsables de su actividad. Con estos resultados se concluye que los microorganismos son una alternativa eficaz para sustituir las sustancias químicas nocivas para la biota que actualmente se utilizan en los recubrimientos, ya que los extractos, además de ser menos tóxicos, son potenciales inhibidores o letárgicos del proceso de colonización epibiótica.

Palabras clave: Bacteria, anti-incrustante, epibiontes, diatomeas, extractos

Abstract.- Biofouling is generated by the biochemical condition of the substrate to form complex communities; this could be negative at an ecological and industrial level. To inhibit this process, coatings based on heavy metals are used. Microorganisms are promising sources of antifouling compounds that are non-toxic or less toxic than the commercial chemicals in use today. In order to evaluate the antifouling effectiveness of bacterial extracts, seven crude extracts from cultures of bacteria isolated from marine organisms were obtained. They were subjected to a toxicity test with *Artemia* nauplii, at different concentrations. Non-toxic extracts were incorporated into a gelling agent (Phytagel™) and exposed to the sea for 30 days to evaluate the inhibition of diatoms and macroorganisms adhesion. Sixty-seven diatoms' taxa were identified, belonging to 45 genera, besides four groups of non-photosynthetic macroorganisms were identified; these are sea squirts, barnacles, bryozoans, and polychaetes. Toxicity test showed that only *Bacillus safensis* (C2) extract was toxic, and it was excluded from subsequent tests. There are significant differences between treatments, the extracts with the best activity are *Shewanella algae* (18) and *Staphylococcus aureus* (28). These extracts contain different organic compounds such as saponins, terpenes, sterols, and proteins, which could be responsible for their activity. These results suggest that microorganisms are an effective alternative to replace harmful chemical substances currently used in antifouling coatings. The extracts, being less toxic, are potential inhibitors or retardants of the epibiotic colonization process.

Key words: Bacteria, antifouling, epibionts, diatoms, extracts

Introduction

Biofouling is a natural phenomenon that is common in aquatic ecosystems (Martínez-Díaz 2010), the process is generally divided into three phases: (1) conditioning, a primary biofilm composed of proteins, colloidal organic matter, and polysaccharides, (2) settlement of microorganisms, where bacteria, fungi, rotifers, and diatoms stand out, the latter being almost always the largest component of the community since they are notable for their abundance and speed as colonizers, (3) as a consequence of the formation of the previous phases, there is an establishment of macroorganisms, which can correspond to flora and/or fauna (Nurioglu & Esteves 2015).

The use of chemical substances to prevent the development of biofouling in industrial activities has been common (Cao *et al*. 2011). Antifouling paints based on tributyltin (TBT) were the recurrent strategy to counteract the formation of biofilms on surfaces in contact with water. Other antifouling substances that are used have been developed based on toxic compounds such as arsenic and mercury (Jones 2009). However, the prohibition of all these toxic substances was necessary because they are not specific biocides and they bioaccumulate, in some cases, in organisms for human consumption (Castelló-Orvay 1993). Currently, commercially available biocides contain copper and zinc, although there is still uncertainty about the possible environmental impact of these products (Ciriminna *et al*. 2015). Research has shown that copper is highly toxic, not only for the fouling organism community but also for a wide range of marine species (Trepos *et al*. 2014).

For this reason, in recent years the application of antifouling compounds of natural origin, particularly those from marine organisms, has aroused great interest in the industry for the prevention of adhesion and accumulation of encrusting organisms on the hulls of vessels, as well as in the protection of submerged artificial structures (*e.g.*, aquaculture equipment, docks), without causing serious impacts to wild species (Dahms & Dobretsov 2017, Doiron *et al*. 2018, Al-Lihaibi *et al*. 2019, Sánchez-Lozano *et al*. 2019).

Marine microorganisms are promising sources of antifouling compounds which, in addition to prevent the settlement and/or growth of other microorganisms, also inhibit the fixation of macroalgae, larvae, and invertebrates spores (Viju *et al*. 2019). The application of these products in the formulation of antifouling paints can be an environmentally friendly alternative to replace coatings based on components that are harmful to marine species (Holmström & Kjelleberg 1999, Armstrong *et al*. 2000, García *et al*. 2015).

Within the great diversity of marine microorganisms, bacteria have exhibited a wide variety of compounds with biological activity such as antibacterial, antifouling, algaecide, antifungal and antiviral (Kelecom 2002, Blunt *et al*. 2007, León *et al*. 2010). In addition, marine bacteria can grow under controlled conditions, which facilitates the constant production of metabolites in large quantities at a reasonable cost (Anderson & Williams 2000). Therefore, the objective of this study was to evaluate the inhibition of diatoms and microorganism settlement in the biofouling process on inert gels.

Materials and methods

Bacterial extracts

Seven extracts from six strains and a co-culture of marine bacteria, with potential antifouling activity, previously tested by Sánchez-Rodríguez *et al.* (2018), were used (Table 1).

Each strain was grown in 250 mL of marine broth and incubated on a rotary shaker at 150 rpm for 72 h at 35 °C. In the case of co-culture (Mix), the three strains (Table 1) were grown together in the culture medium. The obtained

Table 1. Isolation source and identification number of evaluated bacteria / Fuente de aislamiento y número de identificación de las bacterias evaluadas

ID bacterial extract	Isolation source	Strains
1	*Ulva lactuca* (Chlorophyceae)	*Bacillus licheniformis*
18	*Hippocampus ingens* (Syngnathidae)	*Shewanella algae*
28	*Rhizophora mangle* (leaf) (Rhizophoraceae)	*Staphylococcus aureus*
38	*Aplysina gerardogreeni* (Demospongiae)	*Bacillus subtilis*
88	*Rhizophora mangle* (root) (Rhizophoraceae)	*Pseudoalteromonas ruthenica*
94	*Hippocampus ingens* (Syngnathidae)	*Marinobacter* sp.
Mix	*Aplysina gerardogreeni* (Demospongiae)	Co-culture *Bacillus subtilis/B. pumilus/B. licheniformis*
C2	*Ulva lactuca* (Chlorophyceae)	*Bacillus safensis*

fermentation broth was extracted using Diaion™ HP-20 (Sigma-Aldrich) resin. The adsorbent resin was soaked in 100% methanol for 12 h to remove impurities, the solvent was completely removed by rinsing with distilled water. The wet resin was weighed at 5% level and added to the fermented broth. This mix was agitated on a shaker for 12 h at 150 rpm. The fermented broth with adsorbent resin was filtered and the supernatant was discarded. Two hundred milliliters of methanol were added to the pellet containing Diaion™ HP-20 and cell mass, this was agitated on a shaker for 1 h at 150 rpm, and metabolites were extracted. The pellet was re-extracted with ethyl acetate following the same procedure. The methanol and ethyl acetate fractions were aggregated and concentrated in a vacuum by rotary evaporator at 38 °C and 200 rpm. The extract was dissolved in the minimum amount possible of methanol, collected in pre-weighed vials, and evaporated to dryness. The extract weight was estimated and stored at 4 °C (Pinzón-Espinoza 2012).

Toxicity bioassay

To determine the toxicity of the extracts, *Artemia franciscana* nauplii were used, testing each extract at different concentrations in triplicate (1500, 2000, 2500, 3000, µg mL^{-1}), in 96-well microplates. A 100 µL solution of the final concentration of the corresponding extract dissolved in sterile seawater, together with 100 µL of seawater were added to 10-15 nauplii of *Artemia franciscana* Kellogg, 1906, in addition to a negative control (sterile seawater only) and a positive control (copper sulfate was used because copper oxide is not soluble in water, 25 µg mL^{-1}). After 24 h, the plates were examined under stereoscopic microscope and dead nauplii (inert organisms at the bottom of the well) were counted. Then, 100 µL of methanol were added; 15 min later the total nauplii were counted in the microwell, obtaining the percentage of mortality and the mean lethal concentration was calculated by Probit analysis (LC_{50}) (Mekapogu 2021)[1].

Phytagel™ assay

Non-toxic extracts were added by triplicate in Phytagel™ plates using Petri dishes of 100 x 15 mm as containers, as Henrikson & Pawlik (1995) and Arias *et al.* (2006) proposed. A plate with Phytagel™ without any extract was used as negative control (CN), and copper sulfate as a positive control (CP).

Field trials were carried out during the warm weather season (July-August) in La Paz, Baja California Sur, in the dock area known as La Costa (24°9'22.91"N; 110°19'40.61"W). For the bioassay, containers were fixed in polyvinyl chloride (PVC) structures suspended horizontally at a depth of approximately 1 m and 90 cm from the seabed, in triplicate. To avoid stagnation of water inside Petri dishes, these were oriented towards the sediment. Containers were removed from the PVC structure after 30 days and were transferred to laboratory with seawater from the sampling site (Fig. 1).

Coverage determination

In a sterile environment, the containers were rinsed with distilled water to eliminate any organisms not adhered to the Phytagel™ and, thus, obtain the fixed diatoms and other macroorganisms in the gel. Epibiont macroorganisms adhered to the gel were carefully removed with sterile forceps and conserved (alcohol, 70º) for treatment and replicates for their identification, using keys and taxonomic guides corresponding to the region (Solís-Marín *et al.* 1997, Medina-López 1999, García-Madrigal 2007, Kerstitch & Bertsch 2007, Brusca 2010, Prado-Navarro *et al.* 2016).

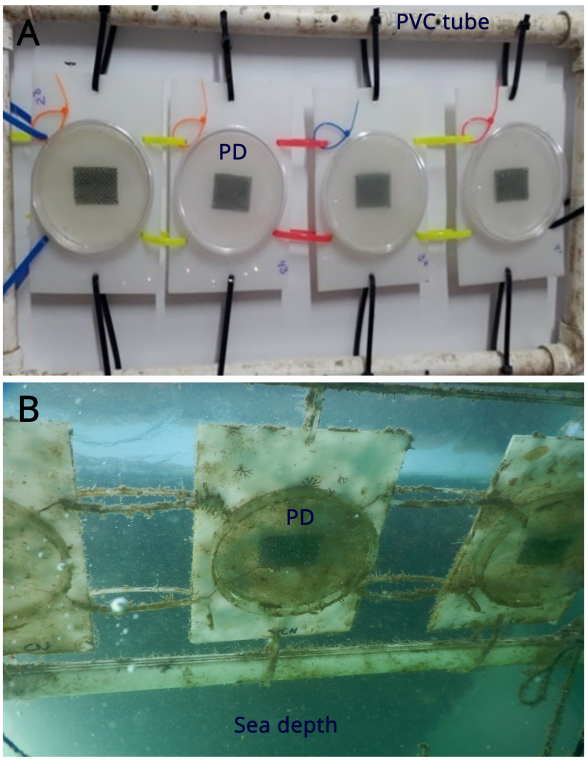

Figure 1. Field trails for Phytagel™ assay. A) Cointainers in PVC structures, B) Structures suspended horizontally in the depth of the sea. PD: Petri dish / Ensayos de campo para las pruebas con Phytagel™. A) Contenedores en estructuras de PVC, B) Estructuras suspendidas horizontalmente en la profundidad del mar. PD: Placa Petri

[1]Mekapogu AR. 2021. Finney's probit analysis spreadsheet calculator (Version 2021). <https://probitanalysis.wordpress.com/>

Photographs of each of the plates were taken to define the coverage of each group adhered through the Coral Point Count with Excel extensions (CPCe) program (Kohler & Gill 2006). Also, an analysis of variance was carried out to determine significant differences between the coverage of the different treatments, with a probability of $\alpha = 0.05$.

Diatoms identification

Scrapings of each replicate were made with a sterile swab which was immersed in 15 mL conical tubes containing 10 mL of f/2+silicates medium in duplicate; the tubes were incubated for seven days under a constant temperature of 22 °C and incident irradiance of 140 µM $m^{-2}s^{-1}$. At the end of the culture period, 3 mL of each repetition were taken for taxonomic identification of diatoms.

In order to remove organic matter, which would preclude the observation of diatom frustules, 3 mL samples were oxidized by adding 3 mL of nitric acid and 2 mL of ethanol while heating with a burner. Oxidized sample were repeatedly rinsed with purified water until reaching a circumneutral pH (> 6). A total of 54 permanent slides were mounted using Zyrax® (RI= 1.7) (made and distributed by Prof. Bill Daily, University of Pennsylvania). Mounted slides were observed under a Zeiss® Axio Lab A1 compound microscope (Zeiss, Germany) with phase-contrast optics.

Diatom identification was in accordance with Péragallo & Péragallo (1908), Hustedt (1930, 1955, 1959, 1961-1966), Foged (1975, 1984), Navarro (1982), Navarro & Torres (1987), Moreno *et al*. (1996), Witkowski *et al*. (2000), Siqueiros-Beltrones (2002) and López-Fuerte *et al*. (2010). Nomenclatural updates were made according to AlgaeBase (Guiry & Guiry 2023), Catalogue of Diatom Names (California Academy of Sciences)[2] , and WoRMS (2022)

Preliminary chemical profile of the extract by thin-layer chromatography

In order to identify the main groups of metabolites present in the extracts, detection of substances was carried out through the analysis of chromatographic plates (Fried & Sherma 1996), using Dragendorff reagent to determine alkaloids; Lieberman-Burchard reagent for unsaturated sterols, unsaturated and saturated triterpenes; solution of iron chloride ($FeCl_3$) in n-butanol ($C_4H_{10}O$) and hydrochloric acid (HCl) at 5% for phenols, pyrogallic tannins and catecholic tannins; 1% solution of aluminum chloride ($AlCl_3$) in ethanol for flavonoids; 10% potassium hydroxide (KOH) solution in ethanol for coumarins, anthraquinones and anthrones; 10% sulfuric acid ($H2SO_4$) solution in ethanol for saponins and a 5:3 isopropan-water mixture developed with ninhydrin solution for amino acids and protein compounds. Subsequently, plates were analyzed with ultraviolet light at a wavelength of 250 and 360 nm, where the colorimetric reactions were interpreted as the presence or absence of the mentioned chemical groups.

Results

Toxicity bioassay

The extract of *Bacillus safensis* (C2) was the only one that showed high toxicity with 88.5% mortality of *Artemia* nauplii (LC_{50} 795.45 µg mL^{-1}); hence, it was excluded from subsequent experiments. With the extract of *Pseudoalteromonas ruthenica* (88), the mortality of artemia was 6.8% (LC_{50} 4782.87 µg mL^{-1}). *Marinobacter* sp. (94) and co-culture (Mix) extracts showed low toxicity (1.38 and 1.45%, respectively). The remaining extracts had no negative effect on artemia, *i.e.*, they showed no toxicity (LC_{50} > 3000 µg mL^{-1}) (Table 2).

Table 2. Probit analysis and mortality percentages in bioassays with *Artemia franciscana* for extract toxicity evaluation / Análisis Probit y porcentajes de mortalidad en los bioensayos con *Artemia franciscana* para la evaluación de la toxicidad de los extractos

Extract	Mortality (%)	LC_{50}	95% Fiducial confidence intervals	
			Lower confidence limit	Upper confidence limit
1	0.689	>3000	ND	ND
18	0	>3000	ND	ND
28	0.704	>3000	ND	ND
38	0.735	>3000	ND	ND
88	6.897	4782.87	3562.69	6420.95
94	1.379	7315.76	4455.87	12011.19
Mix	1.449	6853.25	4025.21	10125.32
C2	88.513	795.45	538.85	1174.25
C+	100	<25	ND	ND
C-	0	>3000	ND	ND

ND: not determined; LC_{50}: Lethal Concentration for 50 percent of the exposed *A. franciscana*

[2]Catalogue of Diatom Names, California Academy of Sciences, On-line Version. Compiled by Elisabeth Fourtanier & J. Patrick Kociolek. <http://researcharchive.calacademy.org/research/diatoms/names/index.asp>

Diatoms identification

According to the isolation of the diatoms adhered to Phytagel™ plates, it was found that the negative control had the highest number of diatoms with 43 species, followed by the extracts of *Pseudoalteromonas ruthenica* (88) and *Marinobacter* sp. (94), for which 34 and 32 species were recorded, respectively. On the other hand, the extracts that presented lower richness were those obtained from *Bacillus* co-culture (Mix) with 22 species and the extract from *Bacillus licheniformis* (1) with 26 species (Table 3).

In the extracts and control plates, 67 diatoms taxa were identified, belonging to 45 genera, (Table 3); the highest percentage of taxa corresponded to diatoms with raphe. The genera with the highest number of species were *Nitzschia* with eight species which were present in all treatments, followed by *Navicula* with three species present in seven treatments. At a specific level, only six species were present in all treatments: *Amphora* sp., *Nitzschia longissima*, *N. incurva* var. *lorenziana*, *Pleurosigma* sp. 2, *Tryblionella coarctata* and *Thalassiosira decipiens*, while 14 species were present in only one treatment.

Table 3. Presence or absence of diatoms species in different Phytagel™ treatments with extracts and controls (C+) or (C-) / Presencia o ausencia de las especies de diatomeas en los diferentes tratamientos del ensayo en Phytagel™ con extractos y controles (C+) o (C-)

Diatom species	Extracts							Control	
	1	18	28	38	88	94	Mix	C+	C-
Achnanthes brevipes var. *angustata*	0	1	1	0	0	0	0	0	1
Actinocyclus octonarius	0	1	0	0	0	1	0	0	0
Actinoptychus senarius	0	1	1	1	0	1	0	1	1
Amphora bigibba var. *interrupta*	1	1	1	1	0	1	1	1	1
Amphora sp.	1	1	1	1	1	1	1	1	1
Ardissonea formosa	0	0	0	0	0	0	0	1	1
Auliscus sculptus	0	0	0	0	1	0	0	0	0
Bacillaria socialis	0	0	0	1	0	0	0	1	1
Caloneis linearis	1	1	1	1	1	1	0	1	1
Campylodiscus fastuosus	1	1	1	0	1	0	1	1	1
Campylodiscus neofastuosus	0	0	0	0	1	0	0	0	1
Campylodiscus simulans	1	0	0	0	0	1	1	0	0
Cocconeis distants	0	0	0	0	0	0	1	0	0
Cocconeis sp.	0	1	1	0	0	0	0	1	1
Coronia ambigua	0	1	1	1	1	1	1	0	1
Cyclotella atomus	0	0	0	0	1	1	0	0	1
Cylindrotheca closterium	0	0	0	1	0	1	0	0	0
Delphineis australis	0	0	0	0	0	0	1	0	0
Diploneis suborbicularis	0	1	0	0	1	0	0	0	0
Entomoneis alata	1	0	1	0	1	0	1	0	1
Epithemia pacifica	1	0	1	0	0	0	0	1	0
Eupodiscus radiatus	0	1	0	0	0	0	0	0	0
Fallacia nummularia	0	0	0	0	1	0	0	0	0
Grammatophora marina	0	0	0	0	0	1	0	1	0
Hyalosynedra laevigata	1	1	0	0	1	1	0	1	1
Lyrella irrorata	0	0	1	1	1	0	0	1	1
Mastogloia sp.	0	1	1	0	1	1	0	1	1
Navicula arenaria	1	0	0	0	1	0	0	0	1
Navicula flagellifera	0	1	0	1	1	1	1	0	1
Navicula sp.	0	0	0	0	0	1	0	0	0
Neofragilaria burchardtiae	0	0	0	1	1	0	0	1	1
Nitzschia cf. *capitellata*	1	1	1	1	0	1	0	1	1
Nitzschia frustulum	0	0	0	0	0	0	0	0	1
Nitzschia hybrida	1	0	1	1	1	1	1	1	0
Nitzschia incerta	1	1	1	0	0	0	1	0	0
Nitzschia incurva var. *lorenziana*	1	1	1	1	1	1	1	1	1
Nitzschia lanceolate	0	0	0	1	1	0	0	0	1
Nitzschia longissima	1	1	1	1	1	1	1	1	1
Nitzschia sigma	0	0	0	1	1	1	1	0	1
Odontella turgida	0	0	0	0	1	0	0	0	0
Paralia sulcata	0	1	1	0	1	1	0	1	1
Parlibellus hagelsteinii	0	0	1	0	0	0	0	1	0
Perissonoë cruciata	1	0	0	0	0	1	0	1	0
Petrodictyon gemma	0	0	0	0	0	0	0	0	1
Petroneis granulata	0	0	0	0	0	0	1	0	0
Plagiogramma minus	1	1	1	0	1	1	0	1	1
Pleurosigma formosum	0	0	0	1	1	1	0	0	1
Pleurosigma sp. 1	0	1	0	0	0	1	0	0	0
Pleurosigma sp. 2	1	1	1	1	1	1	1	1	1
Podosira stelligera	0	1	0	1	0	0	0	0	0
Psammodictyon bombiforme	1	1	0	1	1	1	1	0	1
Psammodictyon cf. *panduriforme*	1	0	1	1	0	0	1	0	0
Pseudictyota dubia	0	1	1	0	1	0	1	1	1
Pseudictyota reticulata	1	0	0	0	0	0	0	1	1
Rhoicosigma oceanicum	0	0	0	0	0	1	0	0	0
Rhopalodia musculus	0	0	1	0	0	0	0	1	1
Seminavis cf. *eulensteinii*	0	0	0	0	1	0	0	0	0
Seminavis strigosa	0	0	0	1	0	0	0	1	1
Surirella sp.	1	1	0	1	1	1	0	0	1
Synedra gaillonii	0	0	0	0	0	0	0	0	1
Thalassiosira decipiens	1	1	1	1	1	1	1	1	1
Tropidoneis longa	0	0	0	0	0	0	0	0	1
Tryblionella coarctata	1	1	1	1	1	1	1	1	1
Tryblionella hungarica	1	1	0	1	1	0	1	0	1
Zygoceros rhombus	0	1	1	1	0	1	0	0	0
Total	24	30	27	27	33	31	22	29	41

Synedra gaillonii, *Nitzschia frustulum*, *N. sigma*, *Petrodictyon gemma*, and *Tropidoneis longa* were present exclusively in the negative control, while in extracts 88 and Mix the following exclusive species were found: *Auliscus sculptus*, *Fallacia nummularia*, *Seminavis strigosa*, and *Cocconeis distans*, *Delphineis australis* and *Petroneis granulata*, respectively.

Antifouling activity

In the plates with Phytagel™, four groups of non-photosynthetic macroorganisms were identified; these are sea squirts, barnacles, bryozoans, and polychaetes, in addition to other organisms that could not be identified. Table 4 shows the organisms belonging to the fouling community that was found on the surface of the experimental and control plates. When analyzing in the stereoscopic microscope the samples of the organisms that were removed from the surface of the plates, various non-sessile organisms were found and were classified as companion organisms, such as gammarids, crabs and sea spiders (Table 4).

Inhibition of settlement of marine organisms evaluated in the field, showed that there are significant differences between treatments ($F_{(7,16)}$= 6.6414, P = 0.00086) (Fig. 2); extracts 1, 18, 28, and 94 are statistically similar to the positive control and extracts 38 and Mix are similar to the negative control (Fig. 1); extract 88 was discarded for statistical analysis due to the variability observed within its replicates. The best activity was shown by the extract of *Staphylococcus aureus* (28) since it showed the smallest colonized area, the lowest net weight, and lowest average coverage (Figs. 3 and 4). The extract obtained from *Shewanella algae* (18) showed only 21.6% of coverage of fouling organisms, while the negative control presented a covered area of 55.2%, compared to the positive control, which inhibited the settlement of marine organisms in 97.8% of the surface (Fig. 4).

Table 4. Macrofauna identified in all Phytagel™ treatments for biofouling evaluation / Macrofauna identificada en todos los tratamientos del ensayo con Phytagel™ para la evaluación del bioincrustamiento

Group/species	Classification
Briozoaria	
Bugula neritina	Encrustan
Bugula californica	Encrustan
Polychaeta	
Dodecaceria concharum	Encrustan
Artropoda	
Eudevenopus sp.	Companion
Balanus amphitrite	Encrustan
Ammothella sp.	Companion
Gammarus sp.	Companion
Echinodermata	
Ophiocoma alexandri	Companion
Ophiocoma aethiops	Companion
Ascidiacea	
Aplidium coie	Encrustan
Distaplia stylifera	Encrustan
Lissoclinum fragile	Encrustan
Botryllus schlosseri	Encrustan

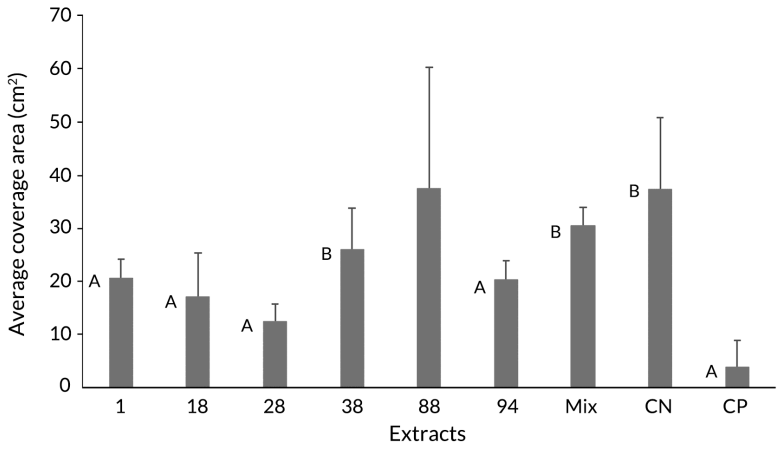

Figure 2. Average surface coverage of Phytagel™ with bacterial extracts and controls in antibiofouling activity assay/ Cobertura media de superficies de Phytagel™ con extractos bacterianos y controles en los ensayos de actividad anti-incrustante

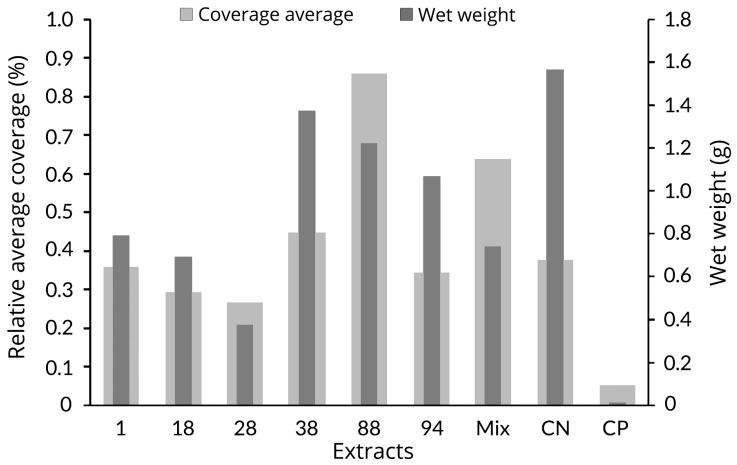

Figure 3. Evaluation of the antifouling effectiveness of bacterial extracts according to colonized surface area and final weight average in Phytagel™ assay / Evaluación de la efectividad anti-incrustante de los extractos bacterianos de acuerdo con la superficie colonizada y el promedio del peso final en el ensayo de Phytagel™

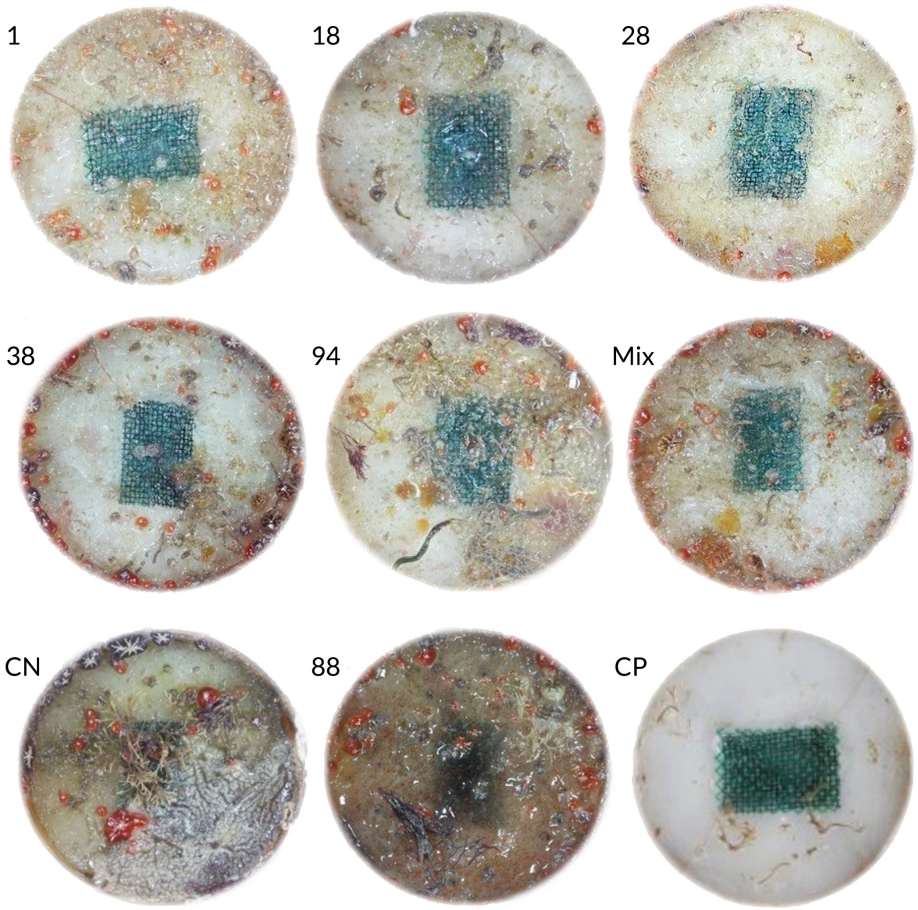

Figure 4. Experimental and control plates after 30 days of sea exposure, used for visual evaluation of antifouling effectiveness. In the upper left corner is the extract label. CN: Negative Control, CP: Positive control / Placas experimentales y control luego de 30 días de exposición en el mar, usadas para la evaluación visual de efectividad anti-incrustante. En la esquina superior izquierda se encuentra la clave de extracto. CN: Control negativo, CP: Control positivo

Preliminary chemical profile of the extract by thin-layer chromatography

The detection of the chemical components by thin-layer chromatography of the two extracts with the highest activity (18 and 28) reveal the presence of various bioactive secondary metabolites which might be responsible for their activity, such as saponins, flavonoids, coumarins, terpenes, protein compounds, and steroids (Table 5).

Discussion

The extracts have shown to be efficient inhibitors of the settlement of fouling species, evidencing their potential to be included in antifouling coatings in the future.

Toxicity bioassay

Bioassays of toxicity, used to recognize and evaluate the effects of pollutants on biota (*e.g.*, models with *Artemia franciscana*) have been considered one of the best indicators of the toxic capacity of a substance (Bartolomé-Camacho & Sánchez-Fortún 2007). The extracts of the strains used for this work did not show high toxicity, even more the most efficient extracts in inhibiting the settlement of epibiont organisms were the least toxic extracts against *Artemia franciscana*. The evaluation of the obtained bacterial extracts was essential to prevent the inclusion of toxic compounds in the environment, as well as to comply with the provisions of the regulations for said commercial products. According to the classification proposed by Nguta *et al.* (2012), toxicity of chemicals is divided into four categories based on their LC_{50}: (1) Strong cytotoxic activity between 0 and 100 µg mL^{-1}; (2) Moderate cytotoxic activity between 100 and 500 µg mL^{-1}; (3) Weak cytotoxic activity between 500 and 1000 µg mL^{-1} and, (4) No toxicity greater than 1000 µg mL^{-1}. Seven of the extracts showed low toxicity for the evaluated doses (> 1000 µg mL^{-1}), suggesting that they are ideal candidates for possible inclusions in safe and less toxic commercial coatings than those currently marketed that are made from heavy metals.

Diatoms identification

From the evaluation of the plates with the different extracts, it was found that pennate diatoms were the ones that presented the highest specific richness, of the six species that were recorded in all the samples, five were pennate and only one was central, coinciding with what was reported in previous investigations (Lee *et al*. 2000, Xu *et al*. 2012). Mitbavkar & Chandrashekar (2006) mention that pennate diatoms are usually the ones that predominate in phase two (settlement) of the biofouling process, since they have a raphe from which they secrete mucilage that they use both for their displacement and for their fixation to the substrate, a feature that is absent in most central forms. Regarding treatments with the extracts and controls, some differences were found in diatom species richness, considering six species that were present in all the treatments as species consistently involved in biofouling formation. On the other hand, species such as *Synedra gaillonii*, *Nitzschia frustulum*, *N. sigma*, *Petrodictyon gemma*, and *Tropidoneis longa*, which were exclusively present in the negative control, could be considered susceptible to treatments, both bacterial extracts and copper sulfate, since both inhibited their adhesion.

In several field studies, it has been determined that the main species of diatoms that adhere and grow on ship hulls (even if covered with a large variety of antifouling coatings) belong to the genera *Amphora*, *Navicula*, and *Nitzschia* (Patil & Chandrashekar 2005, Mitbavkar & Chandrashekar 2006, Cassè & Swain 2006, Satheesh & Wesley 2012, Zargiel & Swain 2014, Hunsucker *et al.* 2014). *Nitzschia* represented in this study the highest number of species and the greatest frequency of appearance, since at least one species was recorded in every treatment. However, in this study only two taxa of the genus *Amphora* were recorded.

Table 5. Phytochemistry of extracts with the highest antifouling activity in laboratory and field assays /
Fitoquímica de los extractos con mayor actividad antiincrustante en los ensayos de laboratorio y campo

Functional groups	*Shewanella algae*	*Staphylococcus aureus*	Observations
Saponins	+	+	Saponins in general
Triterpenes and/or Sterols	+	+	Sterols or triterpenes saturates
Phenols and Tannins	-	+	Phenols in general
Flavonoids	+	+	Flavonoids in general
Coumarins	+	+	Presence of anthrones in extract 18 and coumarins in general in extract 28
Alkaloids	-	-	

Antifouling activity

Bacteria can play an important role in controlling the growth of micro and macroalgae. It has been reported that some bacteria and their excretion products can inhibit the growth of diatoms and other microalgae (Lee *et al*. 2000, Burgess *et al*. 2003). The most efficient extracts in terms of diatom inhibition were those of co-culture with *Bacillus subtilis*, *B. pumilus* and *B. licheniformis* (Mix), and extract 1 of the bacterium *Bacillus licheniformis* isolated from the green alga *Ulva lactuca* Linnaeus. *Bacillus* has been extensively studied to produce metabolites with antibacterial and non-stick properties, algicidal activity, and several pharmacological compounds (Ivanova *et al*. 1999, Jeong *et al*. 2003, Pabel *et al*. 2003) that have a high potential in the search for novel substances (Muscholl-Silberhorn *et al*. 2008). In addition, Valle *et al.* (2006) and Sayem *et al*. (2011) have suggested that polysaccharides produced by *Bacillus* spp. possess the ability to regulate and inhibit the formation of biofilms.

Adhered macroorganisms that were present in all the Phytagel™ plates, both with extract and controls, were barnacles, occupying a high percentage of coverage in addition to representing an important contribution on the weight of those plates that presented a small percentage of coverage. Therefore, they were classified as ineffective extracts, according to the criteria considered. Barnacles are one of the most conspicuous organisms and one of the most difficult to eliminate, considering that the secretion of carbonates increase weight of the structure and roughness of the matter. Because of this, they are considered one of the most important components of the group of fouling organisms (Rittschof 2001)

In the extract with the best activity against macroorganisms (*Staphylococcus aureus* 28), the decrease in the number of present barnacles was evident. According to the literature, other organisms that are part of the fouling community are mussels and polychaetes (Almeida & Vasconcelos 2015). Of these encrusting organisms, only polychaetes were found. It is likely that mussels and other larger invertebrates did not appear due to the period of exposure of the treatments and the size of the gel surface. Presence of *Botryllus schlosseri* (Pallas, 1766) and *Distaplia stylifera* (Kowalevsky, 1874), ascidians identified as invasive for the Mexican Pacific coast, were also observed (Tovar-Hernández & Yañes-Rivera 2012). They were two of the most abundant species in the experimental and control plates, suggesting that they are two of the most important components in the local fouling community.

The behavior of *Pseudoalteromonas ruthenica* (88) extract, which presented higher colonization than the negative control and surface coverage of 100%, could be a consequence of the stimuli or chemical signals released to the medium, which favored the fixation of larvae and other organisms. The opposite was the case with the *Staphylococcus aureus* extract (28), which showed to have a relevant antifouling activity against macroorganisms. Antimicrobial and antibiofilm activity have been attributed to this bacterial genus (Vandecandelaere *et al*. 2014, Ramírez-Granillo *et al*. 2015, Zhang *et al*. 2016, Camarillo-Márquez *et al.* 2018, Ebner *et al*. 2018).

Shewanella algae (18) was isolated from an adult male of *Hippocampus ingens* (Girard, 1858) (Sánchez-Rodríguez *et al*. 2018). Culture supernatant of this bacterial species presented inhibitory activity against biofilm-forming bacteria, identified as primary colonizers in the biofouling process: *Bacillus pumilus*, *B. altitudinis* and *B. subtilis* (Sánchez-Rodríguez *et al*. 2018). This strain (18) was also one of those that showed the best antifouling activity in the present study. In other research works, the antagonistic activity of bacteria isolated from hippocampi has been evaluated, showing activity against microorganisms that are harmful to fish, suggesting that they may be used as controllers of pathogenic bacteria in culture water, as well as probiotic organisms for *Hippocampus guttulatus* (Cuvier, 1829) (Balcázar *et al*. 2010).

Biotechnological potential of *Staphylococcus aureus* and *Shewanella algae* is evident, representing a source of alternatives for various industrial sectors, such as bacterial settlement and adherent macroorganisms inhibition on surfaces in contact with the sea. This offers a solution to mitigate the problems caused for shipping, aquaculture, and other industries whose work is directly related to the marine environment. An important aspect in the search for alternatives in implementing chemical compounds that counteract biofouling is to obtain extracts that do not represent a problem to species inhabiting the surroundings, since this compounds traditionally used as antifouling agents are potentially toxic to wild communities (Hellio *et al*. 2005). Therefore, considering what we observed in the toxicity test of the extracts obtained from *Staphylococcus aureus* and *Shewanella algae*, as well as the inhibition that they presented in the settlement of marine species in the field test, it is evident that these extracts are candidates to mitigate biofouling.

Preliminary chemical profile of the extract by thin-layer chromatography

The results observed in the preliminary chemical profile of these two extracts coincide with previous values mentioned in the literature, since the presence of chemical species belonging to saponins, terpenes, steroids, and protein compounds were confirmed. Some of these compounds have antifouling activity, such as glycosides (saponins), terpenoids, alkaloids, steroids and peptide derivatives (Fusetani 2004, Qi & Ma 2017). These chemical compounds can have different modes of action, either preventing the development of bacterial biofilms or inhibiting the settlement of macroorganisms, such as larvae (Faulkner 1994, Dobretsov *et al*. 2006).

Microorganisms as *Staphylococcus* and *Shewanella* offer effective alternatives to biota-harming chemicals currently used in commercial coatings, as extracts from these bacteria have been found to be potential inhibitors for the epibiotic colonization process.

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