
Antifouling on Artificial Substrates of *Bacillus pumilus* Bacteria Against Diatom Biofilms *Nitzschia ovalis arnott* and *Navicula incerta*

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Abstract: Microalgae and bacteria are the primary colonists of marine surfaces, forming the initial biofilm layer needed for posterior macro organism fixation. This phenomenon, termed biofouling, greatly deteriorates ships, piers, and aquaculture systems. Biofouling is currently treated with toxic and poorly degradable chemicals, resulting in significant environmental concerns. Consequently, increasing research efforts are focused on antifouling microorganisms as a measure to take care of the environment and avoid the use of harmful chemicals for marine flora and fauna. The objective of the present study was to evaluate the ability of the marine bacterium *Bacillus pumilus* (C32-MESO) in inhibiting to the substrate fixation of the benthic microalgae *Nitzschia ovalis arnott* and *Navicula incerta* known for forming biofilms on substrates. A total of 26 bacterial morphotypes were isolated that grow associated with microalgae cultures *N. ovalis arnott* and *N. incerta*. The results showed that the bacteria *B. pumilus* (C32-MESO) inhibited 96.4% of these isolated morphotypes and significantly decreased microalgae density when inoculated with the cultures. Substrate adherence assessments revealed that *B. pumilus* (C32-MESO) decreased the adherence of both microalgae to shell and PVC substrates. In conclusion, this study lays the foundation for future research into characterizing the active metabolites derived from *B. pumilus* (C32-MESO) and evaluate the biotechnological applications of this bacterium in naturally inhibiting the early stages of biofilms such as fixing to substrates of microalgae.

Keywords: Adherence, Antifouling, Bacteria, Bacillus, Microalgae, *Navicula incerta*, *Nitzschia ovalis arnott*

1. Introduction

Biofouling is a phenomenon in which diverse marine microorganisms, algae, and macroinvertebrates adhere to submerged surfaces [1]. The initial surface fixation of bacteria is the first, and perhaps most important, step for the posterior adherence of macroorganisms [2]. In particular, macroorganisms use bacteria-produced metabolites [3, 4]. Bacterial communities adhered to substrates are 1,000 times more resistant to antibacterial agents than planktonic communities [5], making the eradication of marine surface bacteria a highly complex process.

The biofouling on the surfaces of intertidal structures results in biological shape contact barrier with surrounding

environment. This so-termed microbiological corrosion occurs on a surface with highly particular and conditioned physicochemical and biological modifications, meaning that interpretations cannot be based on the same parameters used for inorganic corrosion [6]. Preventing or reverting biofouling is of particular relevance considering that this phenomenon causes significant economic loses, such as through costs related to cleaning work crews, additional fuel consumption, the use of antifouling chemicals, and structural damage [7, 8].

Biofouling can be inhibited through the use of chemical compounds that cover submerged structures. These compounds protect structures against the accumulation of algae, bacteria, and calcium carbonate sediments [9, 10]. However, these chemical agents are toxic. For example, antifouling paints contain tributyltin, which is not easily

degraded and can detach from submerged surfaces, thereby affecting non-targeted marine organisms and the environment [11]. Considering this toxicity, the International Maritime Organization banned the use of tributyltin in 2008 [12]. Notable interest also exists in substituting toxic chemical agents for environmentally friendly alternatives [13].

Marine microorganisms have potential antifouling applications. Indeed, some microorganisms associated with corals, sponges, sea squirts, and algae have known antimicrobial and antifouling properties [12, 14]. The antifouling properties of bacteria that adhere to substrates have also been studied. Bacteria and microalgae compete for inorganic nutrients in seawater. At the same time, the liberation of organic material from phytoplankton stimulates the incorporation of carbonated compounds and nutrients in heterotrophic bacterial communities [15]. In turn, bacteria release vital growth factors for microalgae, thereby promoting the recirculation of nutrients [16]. Therefore, inhibiting bacteria associated with microalgae might inhibit microalgae growth. The primary microalgae acting in biofouling are diatoms, the most known of which include species within the *Navicula* and *Nitzschia* gender [17].

Preliminary research supports that *Bacillus pumilus* has inhibitory properties against *Vibrio parahaemolyticus* [18, 19] and *Vibrio cholerae* [20], in addition to presenting antifouling traits [21]. Based on this background, the aim of the present study was to evaluate *B. pumilus* as a potential natural antifouling agent. In particular, the ability of this marine bacterium to inhibit the substrate fixation of *Nitzschia ovalis arnott* and *Navicula incerta* was evaluated.

2. Methods

2.1. Obtaining Microalgae and *B. pumilus* (C32-MESO)

The benthic microalgae *Nitzschia ovalis arnott* was isolated previously from a commercial abalone (*Haliotis rufescens*) hatchery in Caldera, Chile (27°03'24"S-70°51'30"W) [22] and *N. incerta* were obtained from the strain collection of the Unit of Applied Microbiology (UMA acronyms in Spanish), Universidad de Antofagasta, Antofagasta (Chile). Each microalgae was maintained in the F/2 medium [23] with a continuous photoperiod (72 $\mu\text{E m}^{-2}\text{s}^{-1}$) and a constant temperature of 20°C \pm 1°C. The C32-MESO *B. pumilus* strain was obtained from the strain collection of the Mesocosmos Marino Laboratory, Universidad de Antofagasta. This strain was previously isolated by [24]. *B. pumilus* was maintained in a tryptone soya agar (TSA Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with 2% NaCl the plates were incubated a constant temperature of 20°C \pm 1.

2.2. Isolation of Bacteria Associated with the Cultive of the Microalgae *N. ovalis arnott* and *N. incerta*

The microalgae (*N. ovalis arnott* and *N. incerta*) were

collected during the exponential phase and sonicated (15 s), 100 μL was diluted (10^{-4}) and were extensively seeded onto tryptone soya agar (TSA) plates. Then the plates were incubated at 20 \pm 1°C for 5 days, and colony-forming units (CFU) developed were isolated and maintained in batch cultures. The criterion used to isolate the strains of bacteria was the appearance of the colonies, according to shape, pigmentation, elevation, surface, and rim [25] and stored at -20°C in cryoinstant beads (Scharlab, S. L., Barcelona, Spain).

2.3. *B. pumilus* Inhibitor of Bacteria Associated with the Cultivation of *N. incerta* and *N. ovalis arnott*

Bacteria-bacteria inhibition was evaluated using the double layer method [26]. For this, *B. pumilus* (10 μL) was cultured overnight in the Mueller Hinton medium (Difco, Leeuwarden, Netherlands) and incubated at 20°C \pm 1°C for 48 hours. The macro-colony was exposed to chloroform vapors for 45 min. Then, a second layer of semisolid tryptone soya agar (Oxoid Ltd.) was added; the applied tryptone soya agar contained 0.9% bacteriological agar (Oxoid Ltd.) and was supplemented with 2% NaCl previously inoculated with bacteria (1×10^6 cells/mL) isolated from the microalgae cultures. Each dish was incubated for 48 hours at 20°C \pm 1°C. The presence of an inhibition halo around the macro-colonies was used to define positive antibacterial activity. Experiments were performed in triplicate, and the degrees of inhibition were determined by measuring the diameters of each inhibition halo. Diameters greater than 5 mm were used to establish inhibition, as described by [24].

2.4. Inhibition of Fixation of Microalgae to Substrate

Inhibition of the formation of microalgae biofilms in substrates previously bio-encrusted with bacteria was evaluated. For this purpose, 4 cm² test coupons of shells (Rough surface texture) and PVC (Polyvinylchloride, smooth surface texture) were used substrates, which were washed and sterilized at 121°C for 15 min. The substrates of shell and PVC were hung with nylon (0.35 mm) previously autoclaved and were submerged without touching the bottom separately in transparent containers with 300 ml of M9 medium (100 mL; 30 g/L Na₂HPO₄, 15 g/L KH₂PO₄, 5 g/L NH₄Cl, 100 g/L NaCl, 50 mL/L MgSO₄, 50 mL/L CaCl, 100 mL/L glucose, 1 mL/L vitamin B, and 5 g/L casamino acid) and was inoculate *B. pumilus* bacteria (1×10^6 cells/mL), for 72 hours at 20°C \pm 1°C. Control substrates were maintained under the same conditions, but not exposed to the bacteria *B. pumilus*. The entire system (treatments and controls) was incubated with continuous photoperiod for 72 hours at 20°C \pm 1°C for biofouling of bacteria. After 72 hours, the culture medium was removed from each vessel and the substrates were washed 2 times with sterile seawater to remove bacteria that were not fixed. Each type of substrate was transferred and hung with its nylon to the glass bolons

with flat bottom (1 L) containing 400 ml of sterile filtered water 0.2 μm . The microalgae *N. ovalis arnott* and *N. incerta* were inoculated at a concentration of 1×10^4 cells/mL. The treatment and control was performed separately and triplicate, the experimental bolons were incubated at 20°C with agitation, aeration and continuous photoperiod. To determine the density of the microalgae adhered to each surface (cells/cm²), in sterile conditions, three substrates were taken by treatment and control, were washed with sterile SSM (Marine saline solution) to remove any unfixed microorganisms. The samples were sonicated (Q125 Sonicator, Qsonica, LLC, Newtown, CT, USA) for 30 seconds in the medium M9 to ensure complete detachment of each microalga. The microalgal concentration was quantified through Neubauer chamber count using a microscope (Olympus BX51), every 24 hours over a period of seven days.

2.5. Statistical Analysis

Data normality and homoscedasticity it was verified, and also Tukey's Multiple Comparison Test to estimate the differences of adhesion between the means of the

microalgae and substrates. Analyzes were performed using the GraphPad Prism 5.0 statistical software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was established at $P < 0.05$.

3. Results and Discussion

A total of 26 bacterial morphotypes were isolated from the microalgae culture. From *N. ovalis arnott* was isolated 13 morphotypes and *N. incerta*. It was also isolated 13 morphotypes. Prior research has reported that bacterial growth on the surface of microalgae is an epiphytic behavior [27] and that microalgae development can benefit through associations with bacteria [26], where bacteria provide microalgae with vitamins and growth factors [15, 28]. In turn, the organic substances derived from microalgae are used by bacteria as growth substrates [16].

Bacillus pumilus (C32-MESO) presented inhibitory activity against the majority of the isolated bacterial morphotypes (88.5%). Total inhibition was achieved for 92.3% of morphotypes in the *N. incerta* culture and for 84.6% in the *N. ovalis arnott*, culture (Figure 1).

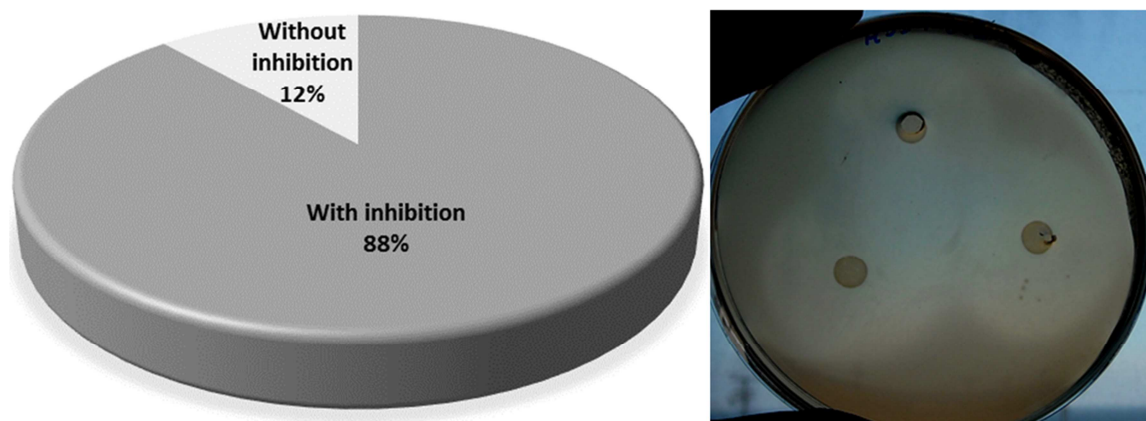


Figure 1. Left: Inhibition of the growth of bacterial strains (morphotypes) isolated from the cultures of the microalgae *Navicula incerta* and *Nitzschia ovalis Arnott* by the bacterium *Bacillus pumilus* (C32-MESO). Right: inhibitory activity of *B. pumillus* against the MNv1 morphotype isolated from the culture of the microalga *N. incerta*.

Was evidenced inhibition halos between 93-213 mm in *N. incerta* and 67-213 mm in *N. ovalis arnott* (Table 1). These findings on the antibacterial properties of *B. pumilus* they agree with previously described activity against the pathogenic bacteria *V. parahaemolyticus* [24]. The *Bacillus*

genus evidences high potential for biomedical uses. In particular, *Bacillus* extracts are very active, presenting metabolites with antifungal and antibacterial properties that have been used to control phytopathogens [29, 30]. *Bacillus* species also have anti-biofilm potential against diatoms [8].

Table 1. Bacterial morphotypes isolated from microalgae *Navicula incerta* and *Nitzschia ovalis arnott* and growth inhibition register (mm) against *Bacillus pumillus* (C32-MESO).

Navicula incerta cultivation		Nitzschia sp. cultivation	
Morphotype v/s <i>B. pumillus</i>	Inhibition (mm)	Morphotype v/s <i>B. pumillus</i>	Inhibition (mm)
MNv1	213	MNc1	0
MNv2	147	MNc2	97
MNv3	213	MNc3	120
MNv4	196	MNc4	127
MNv5	133	MNc5	213
MNv6	133	MNc6	100
MNv7	113	MNc7	180
MNv8	173	MNc8	207
MNv9	106	MNc9	100

Navicula incerta cultivation		Nitzschia sp. cultivation	
Morphotype v/s <i>B. pumillus</i>	Inhibition (mm)	Morphotype v/s <i>B. pumillus</i>	Inhibition (mm)
MNv10	0	MNc10	0
MNv11	160	MNc11	100
MNv12	160	MNc12	67
MNv13	93	MNc13	133

The results indicate that the previous adhesion of *B. pumillus* in the Shell and PVC treatment substrates decreased the adhesion capacity of the microalgae of *N. incerta* and *N. ovalis arnott*. During the seven days culture period. The highest degree of inhibition is observed on the fourth day of culture with significant difference between the control and

treatment in the two microalgae and substrates types ($p < 0.05$) (Figure 2). When comparing the shell and PVC substrates, it can observe greater preference of both microalgae by the substrate Shell with respect to the PVC substrate ($p < 0.05$) (Figure 2).

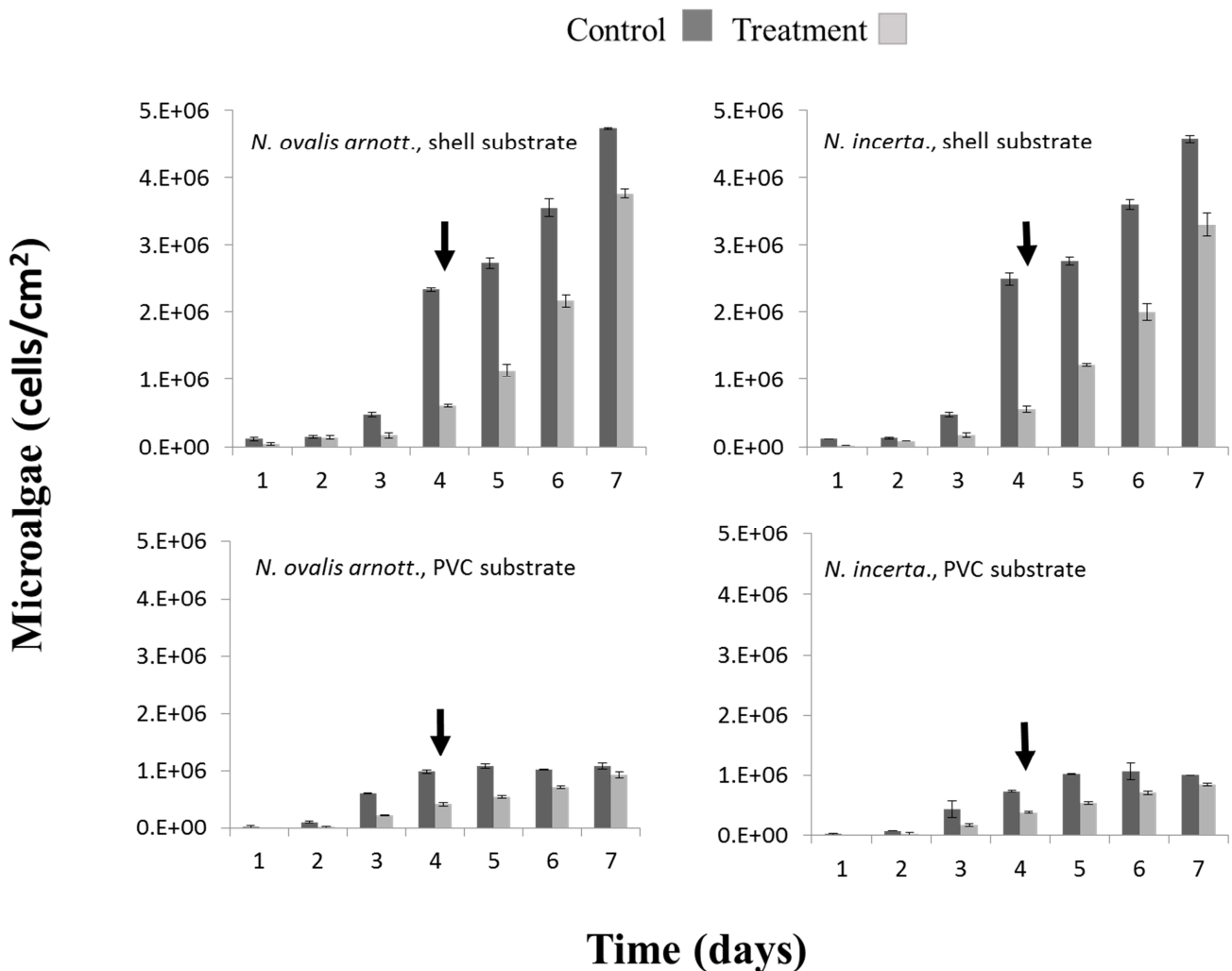


Figure 2. Inhibition of *Navicula incerta* and *Nitzschia ovalis Arnott* substrate (shell or PVC) fixation by *Bacillus pumilus* (C32-MESO). The arrow indicates the day in that observed the biggest inhibition with respect to control of microalgae fixed (cells/cm²) to substrates in both species. Bars indicate standard error.

In the microalgae *N. ovalis arnott* between PVC and Shell substrates, significant differences were observed in the adherence to substrate ($p < 0.05$). In the microalgae *N. incerta* we also observed significant differences ($p < 0.05$) in the adherence to the PVC and shell substrates. Only the treatment of *N. incerta* in shell substrate showed no significant differences with the treatment and control of PVC substrate (Figure 2).

The obtained results reinforce prior findings of a positive ecological relationship between microalgae and associated

bacteria. Furthermore, the presented data validate and widen the previously reported antibacterial potential of *B. pumilus* (C32-MESO) [18, 19, 20].

4. Conclusion

B. pumilus (C32-MESO) could have applications as an antifouling agent, particularly as the currently conducted experiments found this bacterium to decrease the growth of

bacteria naturally associated with the assessed microalgae, thus leading to a reduced microalgae density and, consequently, lowered substrate adherence. The decreased adherence of microalgae would ultimately mean reduced posterior fixation by macroorganisms. An important point to consider is that the degree of antifouling may depend on the concentration of inhibiting bacteria fixated to the substrate or the concentration of active bacterial products immobilized on the substrate. Overall, this study lays the foundation for future research into characterizing the active metabolites derived from *B. pumilus* (C32-MESO) and the uses of this bacterium in naturally inhibiting initial biofilm formation.

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Conflict of Interests

The authors declare that they have no competing interests

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