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Experimental Assessment of a Conducting Polymer (PEDOT) and Microbial Biofilms as Deterrents and Facilitators of Macro-Biofouling: Larval Settlement of the Barnacle *Notobalanus flosculus* (Darwin, 1854) from Central Chile

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Abstract: Maritime enterprises have long sought solutions to reduce the negative consequences of the settlement and growth of marine biofouling (micro- and macro-organisms) on virtually all surfaces and materials deployed at sea. The development of biofouling control strategies requires solutions that are cost-effective and environmentally friendly. Polymer-based coatings, such as the poly (3,4-ethylenedioxythiophene) (PEDOT) and its potential applications, have blossomed over the last decade thanks to their low cost, nontoxicity, and high versatility. Here, using multiple-choice larval settlement experiments, we assessed the efficacy of PEDOT against the balanoid barnacle *Notobalanus flosculus* one of the most common biofouling species in Southeastern Pacific shores, and compared results against a commercially available antifouling (AF) coating, and biofilms at different stages of succession (1, 2, 4 and 8 weeks). We show that larval settlement on PEDOT-coated surfaces was similar to the settlement on AF-coated surfaces, while larvae settled abundantly on roughened acrylic and on early-to-intermediate stages of biofilm (one to four weeks old). These results are promising and suggest that PEDOT is a good candidate for fouling-resistant coating for specific applications at sea. Further studies to improve our understanding of the mechanisms of barnacle larval deterrence, as well as exposure to field conditions, are encouraged.

Keywords: biofouling; environmental protection; coastal waters; larval settlement-biofilm interactions; *Notobalanus flosculus*; Chile

1. Introduction

Marine biofouling refers to the settlement and growth of organisms on artificial structures deployed in the ocean and is an age-old problem that has been a target for control since the beginning of human maritime enterprises [1]. In the marine environment biofouling includes the biofilms of microorganisms that rapidly settle on virtually all

materials as well as large bodied invertebrates and macroalgae and can attain large biomass per unit area [2]. Biofilms create diverse, extensive challenges for industries dedicated to the development of new technologies, such as marine renewable energy (MRE, [3,4]) and great economic losses to the navigation and aquaculture sectors [5,6]. The transformation and rapid expansion of the aquaculture and MRE sectors [7–9] must cope with the challenges imposed by marine biofouling [10].

Several factors act simultaneously to define biofouling risks: (a) the characteristics of local biofouling species [11], (b) the properties of the material, such as surface topography (e.g., roughness), wettability and colour [12], (c) the type of application of the material (small rigid sensor, large moving energy converter) (d) the local environmental conditions, such as temperature, local productivity, hydrodynamics and oxygen conditions [13] and (e) the biotic interactions among species during different phases of the ecological succession, such as the initial biofilm–larval interactions [4,14,15]. It is, therefore, fundamental to understand the complex interactions, often of inter-kingdom origins [16] arising among different organisms involved in the biofouling process and between those organisms and abiotic forces [14]. In fact, the existence of a wide range of fouling species demands a variety of antifouling coating strategies, which can be summarized as follows [17]: (i) fouling-resistant coatings that prevent adhesion of biofilm and/or algae, (ii) fouling-release coatings, which allow an initial weak foulant-surface adhesion, followed by an easy removal by the application of mechanical forces, and (iii) fouling-degrading coatings, which degrade adsorbed organic material and/or kill biofilm by the action of bactericides. While the application of fouling-degrading coatings, (e.g., copper and zinc-based anti-fouling coatings) and recurrent maintenance has been the standard approach in the industry, this is either impractical for many applications (e.g., sensors, soft flexible materials, etc.) or questioned for environmental consequences (e.g., the inevitable release of chemical pollutants to the environment), making this approach highly questionable and subject to increasing restrictions [4,18]. Thus, the race is on to find alternative, environmentally friendly coatings and strategies to reduce the "biofouling problem", improve sustainability of the industries and promote the development of new marine technologies and instrumentation [19].

Fouling-resistant and fouling-release strategies using polymer-based coatings [17] have blossomed over the last decade, thanks to advances in medical science [20,21] and nanotechnologies [22]. These coatings are low cost, nontoxic, biocompatible, highly versatile, and their functionalities and architectures can be easily modified, allowing interfacial adjustments of the antifouling properties [17]. Among polymer coatings, the conducting polymer poly (3,4-ethylenedioxythiophene) (PEDOT) and its composites have been used as antifouling and anticlotting coatings in medical applications [20,23,24] and biocorrosion in marine environments [25–27]. An important factor associated to its application are the experimental conditions necessary to obtain the PEDOT on different surfaces, which are related to its adherence, conductivity, and stability. Usually three electrochemical methods are used to obtain PEDOT, which are cyclic voltammetry (application of a linearly variable voltage), chronopotentiometry (constant current), and chronoamperometry (constant potential) [28]. In addition, the effects of other experimental variables have been observed (e.g., solvent, starting unit, monomer and supporting electrolyte kind and concentration, temperature effect) on the mechanism and process of electropolymerization [29,30]. It is therefore imperative to specifically study each system to optimize the electropolymerization conditions according to the intended use for the conducting polymer electrodeposited on the working electrode. Nonetheless, PEDOT seems to be particularly promising to prevent marine fouling because it prevents adsorption of non-specific proteins associated with a biofilm [31,32]. Recently, laboratory experiments showed that PEDOT can delay bacterial formations by about 35 days in submerged PEDOT-coated coupons compared to uncoated ones [27]. To our knowledge, there is no evidence of the efficiency of conducting polymers in general and PEDOT in particular, on preventing direct colonization by macrofouling, the most harmful components of biofouling for many applications. Here we provide an experimental assessment to start filling this important information gap for the development

of environmentally friendly antifouling strategies in the maritime industry. Moreover, little information is available on strategies that prevent macrofouling colonization of submerged materials in general, in regions characterised by high productivity, such as the Humboldt Upwelling Ecosystem, in the Southern Pacific [33].

Several studies have shown that the settlement and fast growth of fouling species in the Humboldt Upwelling Ecosystem can reach very high biomass accumulation rates [34–36], among the highest reported in the world. Understanding biofouling dynamics and mechanisms to prevent it in this productive region is a major concern for the success of the MRE and aquaculture industries [36]. The ecological succession at wave exposed sites was characterised by initial settlement of microbial biofilms that were rapidly colonized by fast growing invertebrate larvae, leading to deterministic final stages of succession dominated by barnacles and tunicates of large biomass [36]. Further experiments found that diverse materials, deployed above and below the thermocline, were colonized indistinctively by late successional species (barnacles and tunicates of large biomass) and that standard, copper-based antifouling paint was an effective deterrent after seven months of exposure [36]. Here we advance our understanding on biofouling dynamics by testing the antifouling efficiency of PEDOT with settling larvae of barnacle *Notobalanus flosculus*, an intertidal/shallow subtidal barnacle [37] found in abundance in biofouling communities [36,38]. Using multiple choice experiments, we tested PEDOT efficiency as a fouling-resistant coating against barnacle settlement using both a positive control substratum (roughened acrylic plates) and a negative control (self-polishing Cu_2O-based antifouling paint). Furthermore, since larval-biofilm interactions are complex and some barnacle species actively select substrate with different biofilm composition [14,15] (or even without bacterial deposition, Roberts et al. 1991), we evaluated settlement preferences for different stages of biofilm (i.e., biofilms with different age of deposition).

2. Materials and Methods

2.1. Study Species and Sampling Area

We chose the common balanoid barnacle *N. flosculus* [39] as test model for our experiments because the species is one of the most frequent barnacle present in the biofouling of wave-exposed habitats of central Chile [36]. The species is found along the entire coast of central-northern Chile, and it can attain high abundances from the low intertidal rocky shore to shallow subtidal environments [37]. Although it can be displaced in late stages of ecological succession by the larger barnacle *Austromegabalanus psittacus* and the tunicate *Pyura chilensis* [36], larval culture and availability make it an ideal laboratory test model species for south-eastern Pacific waters. Moreover, understanding the interaction between anti-fouling materials and barnacles can shed light on other important barnacle fouling species. Specimens of *N. flosculus* were collected at Las Cruces, Central Chile (S33.5. W71.6) from the rocky intertidal shores during Summer 2019. This site was chosen for three reasons: (i) availability and proximity to the facilities of the larval laboratory of ECIM (Pontificia Universidad Católica de Chile), (ii) existence of biofouling pilot experiments on *N. flosculus* at the same location, and (iii) the existence of Wave Energy Converter projects in the same area (https://lascrucesem.cl).

2.2. Settlement Substrates

In the laboratory experiments described below, we tested five different settlement substrates, including biofilms. First, since many field studies have used roughened acrylic plates to monitor barnacle settlement [34–36,40], we used this material as a positive reference for larval settlement to contrast settlement on PEDOT. We used an AF paint to assess whether it inhibits barnacle settlement. Most commercially available antifouling paints use copper (cuprous oxide, Cu_2O) as biocide. To prevent excessive Cu_2O leaching within the experimental aquaria, we opted for a coating with Self-Polishing Copolymer technology (SPC), which has a controlled and stable hydrolysis release over time (SeaVoyage CDP100, Sherwin Williams; https://www.sherwin.cl/industrial/marino/). The paint was applied

on roughened acrylic plates following manufacturer instructions. Although the product labeling does not specify the presence of copper, the coating does have copper as active compound and the manufacturer ensures that encapsulation of Cu_2O guarantees long-term retention in the matrix with negligible leaching (below detection; see manufacturer instructions attached as supplementary material). Considering the short duration of our experiments and water movement, leaching could not be detected. In any case, AF paint plates were deployed in the numbers in all aquaria, thus preventing any potentially confounding effects. Both roughened acrylic plates and AF paint on which larvae are known to settle and not settle, respectively, can be considered as the positive and negative references for our broad hypothesis (Ho: Larval settlement is similar among the different materials). As organic coating, we used PEDOT, deposited on stainless steel (SS) plates (see details below). Finally, two different biofilm stages were used, (i) early and (ii) late biofilm stages (see details below), grown on roughened acrylic plates.

2.3. Preparation of PEDOT Coated Stainless Steel

The electrochemical synthesis of PEDOT was conducted in a conventional three-electrode cell following the procedures described by Aguirre and colleagues [27], using a cylindrical mesh of AISI 316L SS (surface area 42 cm^2) as counter electrode, Ag/AgCl (KCl saturated) as reference electrode, and the AISI 304 SS coupons as working electrodes, exposing a surface area of $\approx$ 20 cm^2. The conducting polymer was electrodeposited from an organic solution containing 0.1 mol L^{-1} monomers of 3,4-ethylenedioxythiophene (EDOT) and 0.10 mol L^{-1} $LiClO_4$ in acetonitrile (CH_3CN). To prevent the oxidation of the monomers prior to electropolymerization, the solution was de-aerated by purging with nitrogen gas. Deposition of PEDOT on the working electrode was achieved by cyclic voltammetry, applying 10 potentiodynamic cycles at a scan rate of 0.05 $V \cdot s^{-1}$ in a potential window between -0.7 to 1.3 V. These experiments were carried out using an OrigaLys potentiostat (OrigaFlex OGF500).

2.4. Surface Roughness and Wettability of PEDOT, Acrylic and AF Coating

We measured surface roughness (SR) and wettability (W) for the PEDOT, acrylic and AF plates. The SR was measured using a BioLogic Optical Surface Profiler (OSP470) along two perpendicular transects within a 4 $\times$ 2 cm area on a randomly chosen plate of each substrate. All measurements were then expressed as RMS (mean square root) and given in μm^2. Surface wettability was measured by determining the water contact angle (WCA) following ASTM D7334-08 (ASTM International, 2013), using water-type II reagent (distilled) after ASTM D1193-06 (ASTM International, 2011). The W value was used to define hydrophobic and hydrophilic properties of the substrate from critical surface tension theory, assigning hydrophilic property to WCA < 45°, hydrophobic property to WCA > 90°, and intermediate property to 45° < WCA < 90°. Finally, the quality of polymer adhesion was tested by means of a tape-and-peel test, conducted according to ASTM D3359-09ε2 standard (method A, ASTM International).

2.5. Biofilm Deposition and Characterization

The biofilms were grown on 40 finely roughened acrylic plates using emery paper (grit 240) and exposed in aquaria (50 $\times$ 30 $\times$ 17 cm) to the running sea water system of ECIM, which pumps seawater from ca. 1.5 m depth and filtered at 1 mm. Prior to exposure to running seawater, plates were left in 95% ethanol overnight in the dark. Acrylic plates were then left undisturbed in the aquaria under natural sunlight, photoperiod, and seawater temperature, allowing biofilm formation. Different biofilm ages were used in the two trials described below, early, and late, since the exact age of the biofilm varied between experiments as mandated by the availability of competent barnacle larvae (see below).

At the end of the biofilm depositions and before deployment in experimental arenas, each plate was placed in a clean Petri dish and photographed with a digital camera (Nikon D80 set at f/2, 1/60 s exposure, ISO 400, 50 mm focal length, no flash, and recording in RAW

format). Lighting was controlled using overhead lights. The photo images (Figure 1A) were then analysed using the software Image-J2 [41] to calculate the % of biofilm cover per plate using the standard technique described by Otsu [42]. Briefly, this technique establishes a threshold to delineate foreground (biofilm) from background (clean acrylic surface) by reducing a grayscale image (8-bit image) to a binary image and assuming pixel values form a bimodal histogram. Threshold value were chosen algorithmically using the Otsu's method of thresholding, which is one of the most referenced algorithmic techniques. Once photographed, biofilms were kept in aquaria with still FSSW in the dark avoiding successive biomass growth for a maximum of three days.

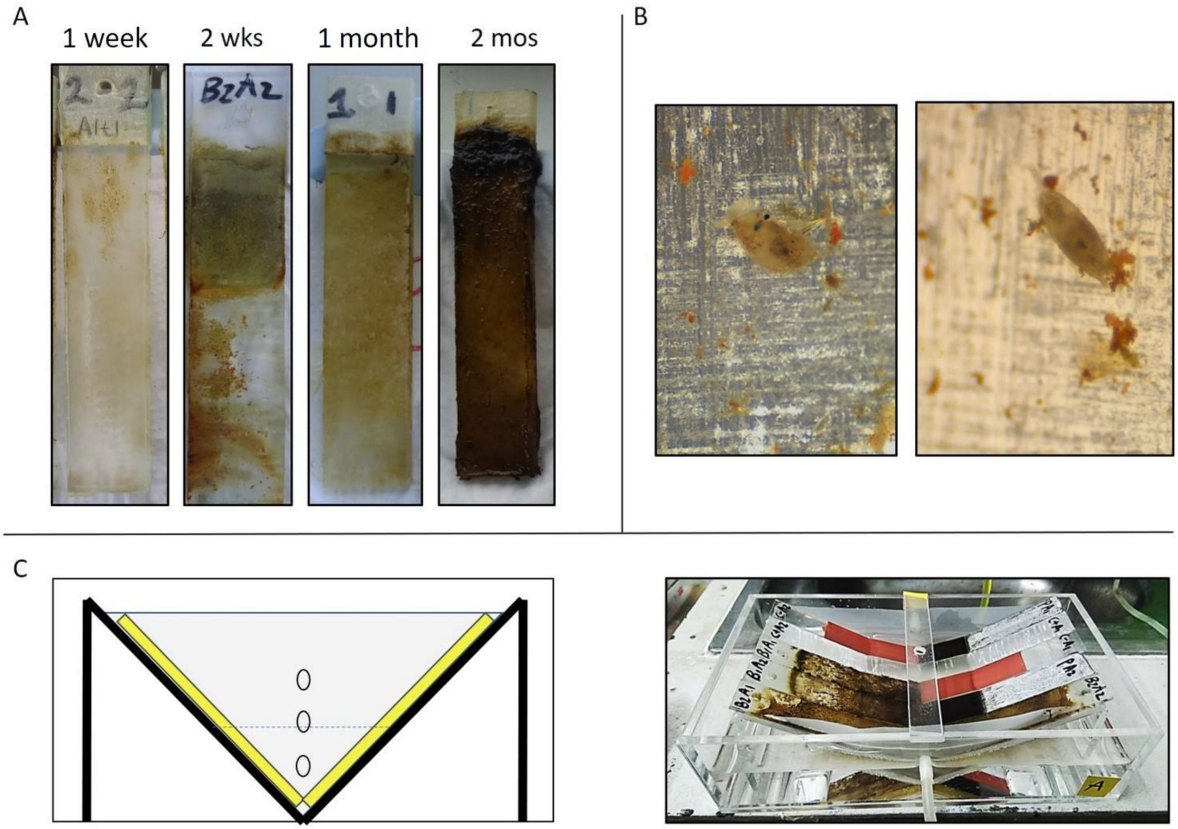

Figure 1. Upper-left panel (**A**): photographs of some of the plates with different biofilm age (from 1-week to 2-months) used for settlement experiments and image analysis (see material and methods for details). Upper-right panel (**B**): two photographs of cyprids of *Notobalanus flosculus* settling in the biofilm plates (**C**) schematic drawing of a lateral view of the V-shaped stand placed in the outer aquarium used in the settlement experiments and a photograph of the stand with the experimental plates mounted. In the drawing, the grey-shaded triangle represents the 90 µm nylon mesh used to constrain larvae during the trial, the yellow rectangle represents the experimental plates, and the dashed blue line represents the level of water within the aquarium (see material and methods for details).

2.6. Barnacle Collection, Spawning and Larval Rearing

Animals were collected manually by removing approximately 10 small boulders colonized by adult *N. flosculus* from the low intertidal zone. In the laboratory rocks were inspected under a stereoscope and other species were carefully removed with tweezers and scalpels. External shells of *N. flosculus* and naked rocks were gently brushed cleaned using a diluted solution (1:50) of ethanol to reduce the microbial load (i.e., biofilm) and then rinsed carefully using pre-filtered (1 µm), UV sterilized seawater (FSSW). Rocks were cut into units of 10 cm long max with 20 to 30 individual barnacles and randomly assigned to five different aquaria (20 L each) connected to a flow-through system provided with serial polyethylene filtering—(from 20 to 1µm) and UV light sterilizing apparatus (FSSW). The water leaving

each aquarium was collected in a 15 × 15 × 20 cm (W × L × H) plastic boxes equipped with 100 µm mesh for larval retention. The entire system (aquaria with adult individuals and larval retention filters) was placed inside a temperature-controlled chamber (hereafter incubation chamber) which allowed full control of light intensity, photoperiod (14:10 h dark:light) and temperature (15 °C, simulating the temperature at the study site when animals were collected) to stimulate spawning (see below). Barnacles were fed ad libitum with a daily dose of the microalgae *Skeletonema* sp. of approximately 2×10^6 cells mL^{-1} and recently hatched nauplius of *Artemia salina*. Spawning in the laboratory facilities was achieved using a light-stress technique. After a week of acclimation to laboratory conditions in the incubation chamber, animals were kept completely in the dark and starved for 48 h. After this period, light was applied at full intensity (6 led bulbs of 2520 lumens each) for the following 24 h, which led to the release of larvae in delayed batches over a period of about a week. Hatched larvae were collected daily and placed in pre-autoclaved 10 × 10 × 2.5 cm glass containers half-filled with 150 mL of FSSW. The number of total larvae per container depended on the number of larvae released per batch, with a maximum concentration of 100 larvae per container. Larvae were kept separated by day of collection to rear similar cohorts within each container. Following Jonsson and collaborators [43] we identified 6 nauplius stages over the larval developmental time starting from nauplius 2–3 until cypris stage which is competent to settle (Figure 1B). Larvae were fed with a microalgal mix (*Skeletonema* sp. and *Isochrysis* sp.) given at a concentration of 1 to 2×10^6 cells mL^{-1}. The FSSW of the glass containers was changed daily.

2.7. Settlement Choice Experiments

2.7.1. Experimental Set-Up

Settlement choice experiments consisted in offering larvae the five selected materials and biofilms conditions. Experiments were performed using a flow-through, acrylic aquarium modified from [44]. Briefly, aquarium consisted of an inner V-shaped stand in which experimental plates could be placed in a 120° angle facing each other (Figure 1C). To the sides of each stands a 90 µm nylon mesh, which was glued allowing FSSW to flow through while retaining larvae. The design of the experimental chambers ensured that the experimental plates were the only substrate available to settle (settlement on nylon mesh was zero; see also [43]). The stand was placed inside an aquarium fed with FSSW from a header tank. Water temperature during the experiments was kept constant at 15 °C by placing the header tank and the experimental aquarium within an incubation chamber (same used above for the nauplius rearing).

Each of the five substrates (treatment) was offered on two 10 × 2 cm plates that covered the entire available surface the V-shape stands. A total of 10 plates (five substrates) were therefore available in each aquarium. Using a table of random numbers, where each number represented a plate, we placed one plate of each substrate on the left and the other on the right-hand side of the V-shape stand, in this manner preventing potential bias due to unforeseen gradients. Before analyses, the number of settled larvae on the two plates was added within each replicate aquarium.

2.7.2. Laboratory Trials

As larvae developed and turned into the cyprid stage, they were placed inside separate glass containers (same as above) filled with 150 mL of FSSW and left in the dark at 6 °C for a maximum of five days [44] until a total 360 individual cyprids were obtained (60 from a first batch and 300 from a second batch).

Two separated settlement experimental trials were performed, using the two separate batches of cyprids, so the only difference between the two trials was the age of the biofilms, which cannot be controlled with precision. In the first trial (named experiment 1), we had 2-month old biofilm and 2-week old biofilm, besides the PEDOT, AF coating and acrylic plates. A total of 60 cyprids were used in experiment 1. In the second experiment (named experiment 2), we had 1-month old biofilm and 1-week old biofilm, besides the other three

materials. A total of 300 cyprids were used in the experiment 2. In both experiments, cyprids were carefully placed in three (experiment 1) and five (experiment 2) replicated aquaria with the settlement plates and left undisturbed with flowing FFSW seawater, in the dark at 15 °C, for one week. After this incubation time, aquaria were inspected under a stereoscope for the evidence/confirmation of larval settlement. Those larvae found attached to each substrate were gently stimulated using a micropipette to ensure that settlement had occurred. After this preliminary inspection, and if the majority (>80%) of larvae had settled, plates were individually transferred to a large Petri dish filled with FSSW and settlers were carefully counted, and their general condition assessed. The number of larvae settled was registered for each plate and aquarium. Larvae that did not settle within the experimental time were recorded but not considered in analyses. Assessment of statistical significance of larval settlement among plates was conducted through separate Pearson chi-square tests for each experiment. The test compared observed numbers of larvae settled on the different substrates against the expectation of equal frequencies on all substrates. Larvae which had settled within each replicated plate on the aquaria were averaged before the analysis.

3. Results

3.1. Physical Properties of Materials and Biofilm Coverage

The cyclic voltammetry profiles recorded during EDOT electro-polymerization at the AISI 304 SS surface in ACN+LiClO4 supporting electrolyte are shown in Figure 2, as well as a proposed electrochemical polymerization mechanism. In the first cycle (red line) at 1.0 V onset the oxidation of the monomer and a nucleation current loop can be observed. This is the expected result for a 2D nucleation followed by 3D growth [27]. Later, in the consecutive cycles, it was possible to observe the redox processes of the polymer. There is a current increase with the number of scans until a stable cyclic voltammogram (blue line) is reached, which can be correlated with the doping–undoping processes of the polymer. This behavior indicates the gradual formation of a conductive film on the AISI 304 SS coupon and has been previously reported for PEDOT and other conducting polymers [29]. In addition, under the same conditions for obtaining the conducting polymer, a thickness of ~2 μm has been reported [27].

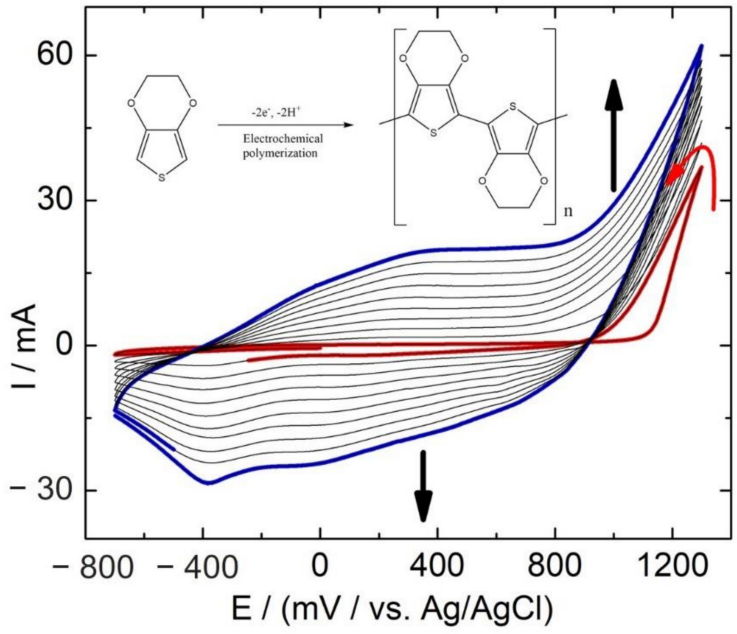

Figure 2. Cyclic voltammograms recorded during 10 cycles of EDOT electropolymerization in the potential range from −0.7 to 1.3 V on 304 SS at 0.05 $V \cdot s^{-1}$ from a 0.1 $mol \cdot L^{-1}$ EDOT + 0.1 $mol \cdot L^{-1}$ LiClO4 solution, in CH3CN. Inset: Proposed electrochemical polymerization mechanism of EDOT.

The physical properties (SR and W) of the experimental substrates are summarized in Table 1. Surface roughness was highest for the acrylic plate (0.332 μm^2) and lowest for the PEDOT (0.118 μm^2) with intermediate roughness observed for AF painting (0.243 μm^2). Wettability was lowest for PEDOT, which was categorized as the most hydrophobic substrate, while acrylic and AF painting had similar intermediate hydrophobicity values (see Table 1).

Table 1. Results of the analysis of surface roughness and wettability of the two materials (Acrylic, PEDOT) and antifouling (AF) paint. WCA = Water Contact Angle; High = WCA > 90°; Intermediate = 45 ° < WCA < 90°.

Plate	Surface Roughness (μm^2)	Wettability (WCA)	Hydrophobicity
Acrylic	0.332	81.9	Intermediate
PEDOT	0.118	103.4	Hydrophobic
AF	0.243	81.13	Intermediate

The mean cover (%) of biofilm, measured with image analysis, increased rapidly with biofilm age, from slightly over 10% in 1-week-old biofilm, to less than 50% in 2- to 4-week-old biofilms, to 100% cover in 2-month-old biofilm (Figure 3A).

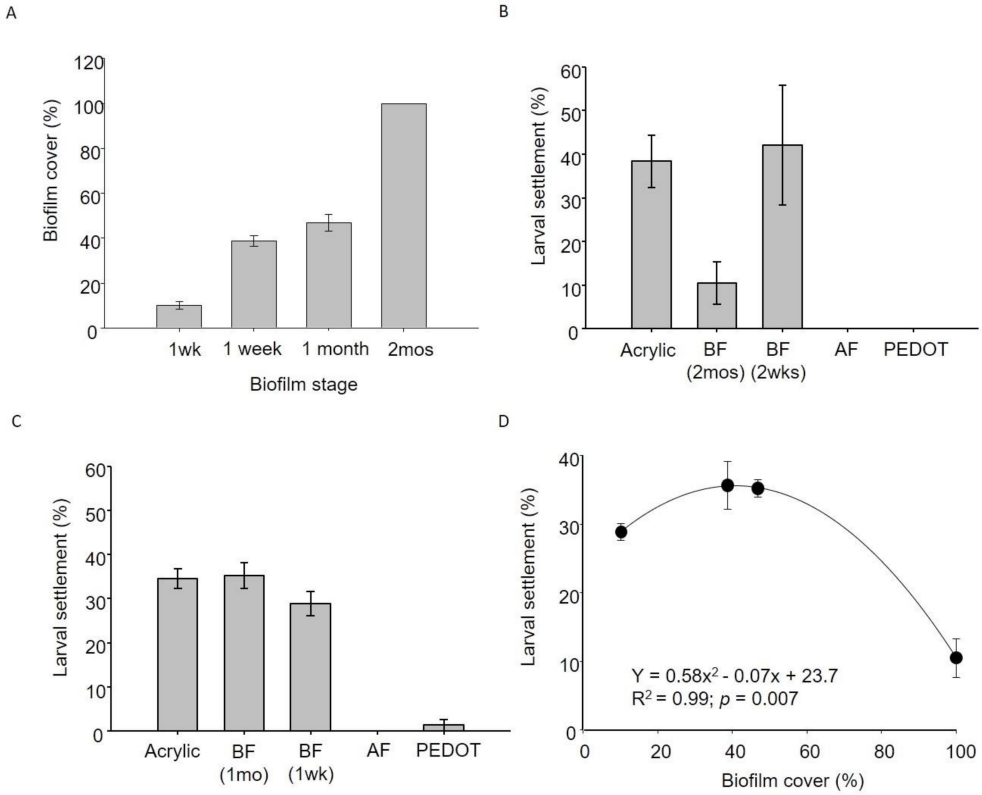

Figure 3. (**A**) Biofilm deposition cover (%) on acrylic plates calculated from the image analysis of the four different stages of biofilm, (**B**) proportion of barnacle settlement in the 5 experimental plates for the experiment 1, (**C**) proportion of barnacle settlement in the 5 experimental plates for the experiment 2, (**D**) results from the polynomial quadratic OLS regression between larval settlement (mean ± SE) and biofilm cover. BF = biofilm; AF = antifouling paint. Experimental time: 1 week.

3.2. Settlement Experiments

In the experiment 1, from a total of 60 larvae (20 larvae per aquarium), 52 (87%) settled within the one-week experimental time. Of these, 42% settled on the 2-week-old biofilm, 38% on the acrylic plates, and 10% settled on the 2-month-old biofilm. No larvae settled on PEDOT or AF-painted plates (Figure 3B).

In the experiment 2, from a total of 300 larvae (60 per aquarium), 215 (72%) settled within the experimental time. Of these, 35.2% settled on the 1-month old biofilm, 34.5% on acrylic plates, 28.9% on 1-week-old biofilm while 1.4% settled on the PEDOT (Figure 3C), corresponding to three larvae observed in three plates. No larvae settled on the AF paint. Since no larvae settled on AF paint on any of the experiments and only three larvae settled on PEDOT (pooling results from experiment 1 and 2), these two substrates were not included in the statistical comparisons and must be considered significantly different to those in which positive settlement was observed [45]. In both experiments, the frequency of settlement in the three substrates compared in the analyses (acrylic plate, early and late biofilms) was significantly different from the equal settlement expectation. In experiment 1, larval settlement on 2-month-old biofilm was lower than expected ($\chi^2_{(2, 52}$ = 11.2311: p = 0.0034) and lower than on acrylic or 2-week-old biofilm (Figure 3). In experiment 2, settlement across treatment was more even and was not significantly different than expected ($\chi^2_{(2, 212)}$ = 1.623; p = 0.443).

Since we observed differences in settlement among different stages of biofilm, which were probably related to the differences in biofilm age and total cover (Figure 3A), we examined whether total larvae settled on biofilm (%) was associated to biofilm cover (Figure 3D). We used percentage of larvae settled as the response variable because the total number of larvae available to settle was different between the two experiments. The relationship was significant and non-linear (significant quadratic polynomial fit), with increased larval settlement at intermediate ages of the biofilm (between 2 weeks and 1 month) than when biofilm was only 1 week old or 2 months old.

4. Discussion

Our experiments showed that (i) the PEDOT is a good candidate for a fouling-resistant coating, as it showed extremely low to nil barnacle settlement, similar to the commercially available AF painting, (ii) *N. floscolus* is a good model species for biofouling experiments and to investigate larval settlement-biofilm interactions, as well as for implementation of environmentally friendly AF strategies, (iii) the cypris of *N. floscolus* prefer biofilms of intermediate age (between 2 weeks and 1 month), suggesting that larvae of this species perceive differences and actively choose among microbial communities of different age and composition.

A central issue of this study is the use of an AF paint as negative reference that contain copper (CU_2O), although it has a delayed releasing technology (see supplementary material). Copper could have affected larval settlement in the plates within each aquaria, especially those closer to the AF treatment. We recognize this as a limitation to the discussion of our results, although we found that randomizing the plates within each aquarium should have homogenized this effect. In fact, even assuming that copper was circulating in the assays, we have still showed differential settlement on the different plates, with low-to-zero settlement on PEDOT and higher settlement on acrylic and biofilms.

An important result that extends previous results, was the high efficiency of the conducting polymer coating PEDOT to inhibit *N. flosculus* larval settlement. A potential mechanism for this deterrent activity may be associated with the previously documented prevention of microbial biofilms by a PEDOT coating [27]. The previous authors showed that PEDOT can delay biofilm formation of at least 35 days, suggesting therefore that PEDOT should be a candidate for further experimentation, including field testing. The hydrophobic characteristic of PEDOT possibly also played an important role in settlement inhibition. Indeed, hydrophobicity of the material has been shown to inhibit larval settlement in *Balanus amphitrite* ([12,46], but see also [47] for the opposite pattern) and other important fouling organisms such as mussels [48] as well as spores of marine macroalgae [49]. The efficiency of PEDOT as antifouling coating against other taxa, which accounts for a large fraction of the final biofouling biomass in submerged structures, like the giant barnacle *Austromegabalanus psittacus* and the tunicate *Pyura chilensis* [36], should also be a priority if PEDOT is to be developed as a marine, environmentally friendly application.

The conducting polymers are interdisciplinary materials with cohesive aggregation of various areas, viz., electrochemistry, microbiology, environmental engineering, material sciences, biochemistry, and many other related areas, and are therefore a prime subject for research at the interface with marine biology.

We showed that the surface roughness among PEDOT coated surfaces, AF and roughened acrylic largely differ (see Table 1), with PEDOT showing lower roughness than both positive and negative references. Given that rougher surfaces can generate higher settlement, this can be considered a cofounding effect limiting the interpretation of our results. Nevertheless, while it is true that the surface topography created by the polymer and adhered to the solid surface could have been one of the proximate mechanisms deterring establishment of competent settling larvae, it is also possible that changes in the hydrophobic nature of the polymer also played a role (as shown in other species). In previous work [50], we have shown that larvae of most macro-foulers (barnacles, tunicates) settle abundantly over a very wide range of surface roughness, bracketing the roughness range presented in these experiments. Simple comparison among materials of similar surface roughness will therefore not provide much insight into the mechanisms by which PEDOT deters larval settlement. The primary experiments presented here were not designed to test those mechanisms of larval inhibition, but only to determine whether a polymer-covered solid surface elicits deterrent activity for macrofouling. We consider that the PEDOT effect on larvae is rather indirect, though affecting the composition and attributes of the biofilms, as shown by Aguirre and collaborators [27]. We encourage future experiments to focus on disentangling the mechanisms of action and considering a wide range of roughness and hydrophobicity and, ideally, independent control of the biofilm.

Notobalanus floscolus demonstrated to be a good candidate for settlement studies because of its high abundance in the field, low mortality of adults, continuous larval production and low mortality rate of nauplius (data not shown), which led to a sufficient number cyprids available for the settlement experiments. These biological characteristics, its frequent presence in all man-made materials deployed at sea [35,36] and its extensive geographic distribution make it an ideal model species to compare experimental results across biofouling studies. While *N. flosculus* showed relatively high abundance among the biofouling species of central Chile [36] another barnacle species, such as the giant barnacle *Austromegabalanus psittacus*, can attain much larger biomass and create more serious problems to the maritime industry. The size of *A. psittacus*, however, make this species more difficult to rear under laboratory conditions, larval stages are harder to cultivate, and its availability along the shore is sparser. Settlement behaviour of barnacles, consisting in an elaborate sequence of exploratory activity, is well known among different species of balanoid barnacles, particularly among the family Balanidae of which *Balanus amphitrite* and *Semibalanus balanoides* (not present in the Southeastern Pacific) have been amply used as model experimental species. The native *N. flosculus* could fulfil a similar role in science exploration along the Pacific shores.

In both settlement experiments, cyprids showed settlement preferences for plates with free-space availability such as the acrylic plates and plates with biofilm covers between 40 and 50% (between 2 weeks and 1 month of biofilm stage), which correspond to mid-successional microbial biofilm communities [2,15]. It has been reported that larvae of *B. amphitrite* preferred biofilm-free surfaces ([51]), but observations with other barnacle species suggest larvae settle on the biofilm [15,16,52]. In our study, higher settlement found on the 2-week- and 1-month-old biofilms than on the 1-week-old biofilm that had higher bare surface (see Figure 3), suggested that *N. flosculus* larvae preferred to settle on the established biofilms probably with more favourable chemical and mechanical conditions than bare space. It is important to highlight that in our experiments, there was a little mixing of cyprids age during the assay, because not all nauplius turned in cyprids at the same time. Mixing of cyprids prior the start the experiments strongly reduced this effect, yet a possible confounding effect of cyprids age on settlement remains. Specifically, caution should be taken when drawing conclusions about the difference in settlement between early

and intermediate biofilm deposition ages. Nonetheless the results of low settlement on a more complete biofilm cover is clear, and suggest that later stages of microbial community succession are less suitable for larval settlement. While the association between biofilm age/density and larval settlement is generally positive [52–54], this is not always the case. For example, the settlement rate of the sea urchin *Tripneustes gratilla* larvae was not affected by the increasing age of a mixed consortium of bacteria [55].

Our experiments, as most others examining biofilm-larval interactions, could not separate between biofilm cover and biofilm age. As soon as the first microbial biofilm species get established on the surface, within hours of deployment in seawater [56], an ecological succession of highly diverse microbial species starts, changing diversity and composition over the course of weeks as the community advances towards late, more stable microbial composition [15]. Thus, differential *N. flosculus* settlement among different ages of biofilms are probably also or even mostly related to the changes in microbial community composition.

There is still much debate as to whether the microbial community can act as facilitator or inhibitor of macroscopic organisms (metazoans), such as barnacles [15], and clearly part of the answer is related to the state of the microbial succession with which larvae interact. This is today a very active and fascinating area of interdisciplinary research in which basic scientific breakthroughs could make major contributions to help solve the ages-old problem of marine biofouling.

5. Conclusions

In conclusion, the efficiency of PEDOT in reducing barnacle settlement was evident and promising and we showed that this organic coating is a good candidate as fouling-resistant strategy for specific application at sea. Further tests, however, are needed to confirm this potential deterrent efficacy against other fouling species, including other barnacles as the settlement behaviour of this taxonomic group highly depend on the substrate [15,16,36,52]. Future laboratory experiments should also be designed to examine in more detail the proximate mechanisms of deterrence of barnacle and other larvae by PEDOT coatings and its modulation by microbial biofilms, which will allow improvements in polymer designs. Beyond the laboratory testing, the final test of any antifouling strategy is the exposure of the material and technology to the diverse fouling community and environmental conditions encountered at sea where the application is to be deployed. This lies still ahead in the development of antifouling polymers.

Supplementary Materials: The following are available online at https://www.mdpi.com/2077-1312/9/1/82/s1,-Sea Voyage CDP 100 manufacturer instructions.

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