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Involvement of reactive oxygen species in the electrochemical inhibition of barnacle (*Amphibalanus amphitrite*) settlement

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The role of reactive oxygen species (ROS) in electrochemical biofouling inhibition was investigated using a series of abiotic tests and settlement experiments with larvae of the barnacle *Amphibalanus amphitrite*, a cosmopolitan fouler. Larval settlement, a measure of biofouling potential, was reduced from 43% ± 14% to 5% ± 6% upon the application of pulsed electric signals. The application of ROS scavengers such as glutathione and catalase counteracted the inhibitory effects of the electric signals, allowing settlement, and thus indicating that ROS are antifouling agents. Based on the experimental evidence, the proposed mechanism for ROS-based fouling prevention with interdigitated electrodes involved the electrochemical generation of hydrogen peroxide by oxygen reduction, and its likely reduction to hydroxyl radicals. Either hydroxyl radicals or products of hydroxyl radical reactions appeared to be the main deterrents of larval settlement.

Keywords: reactive oxygen species; pulsed electric fields; settlement; barnacles; antifouling coatings; interdigitated electrodes

Introduction

Biofouling on surfaces exposed to marine environments is deleterious to a wide range of human activities (Flemming 2002; Railkin 2004). The most common solution to this problem is to apply biocides directly to the water or mix these into paint formulations, which induces environmental damages due to the release of long-lived toxic compounds into the water (Omae 2003). Thus, most of the research on antifouling (AF) coatings is devoted to the development of paints of low or no toxicity that are easy to clean (Yebra et al. 2004). An alternative recently reported as a successful AF system against marine bacteria and invertebrates is the use of electric signals (Matsunaga et al. 1998; Wake et al. 2006). It has been hypothesized that the mechanism by which electrochemical AF operates is the generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and hydroxyl ions, but direct evidence of ROS formation and involvement on AF has not been provided (Wake et al. 2006). Support for this hypothesis comes from the demonstrated relevance of ROS on electrochemical bacterial inactivation (Jeong et al. 2006; Chang et al. 2007; Hong et al. 2008), although inactivation may not be a necessary requirement for electrochemical biofouling prevention. As barnacles are known for their ubiquity, abundance in fouling communities, and adhesion strength to marine structures (Aldred and Clare 2008), this study aimed to (i) provide direct evidence for ROS formation during electrochemical fouling prevention experiments, and (ii) determine whether ROS influence barnacle settlement. The study employed abiotic tests with surfaces receiving pulsed electric fields and biotic experiments with larvae of the species *Amphibalanus* (= *Balanus*) *amphitrite* (Pitombo 2004), an inhabitant of tropical and subtropical waters extensively used in biofouling studies (Aldred and Clare 2008).

Materials and methods

Experiments on surfaces subjected to electric fields were performed by slightly modifying the procedure described by Pérez-Roa et al. (2008). Planar, interdigitated electrodes (IDEs) made of sputtered titanium over borosilicate glass (Intelligent MEMS Design, Toronto, Canada) were used in most of the experiments. The IDE architecture consisted in an array of two intercalated titanium combs facing each other; each comb finger was 25 μm wide, and the spacing between opposite fingers was also 25 μm (Figure 1a). The active area (ie the surface covered by the electrode strips and the space between them) was 361 mm^2.

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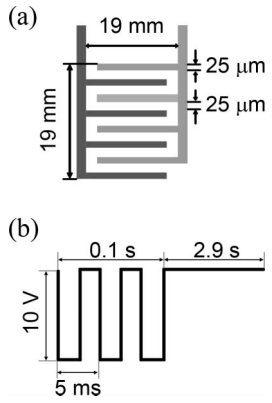

Figure 1. Schematics of (a) IDE and (b) electric signals used to prevent barnacle larval settlement. (Figures are not at scale to enhance relevant features.)

Some experiments were performed using a flat titanium foil (Alfa-Aesar, Ward Hill, MA) instead of the IDE. Prior to each experiment, surfaces were cleaned by sonication with Terg-a-zime enzymatic detergent (Alconox, White Plains, NY) for 1 h, followed by a soaking step in 10% v/v HNO_3, for another hour. The cleaned surfaces were then placed in a convection oven at 250°C for 2 h. For reusing the test surfaces, attached barnacles were removed with a spatula and rinsed off with water before starting the cleaning procedure.

For each experiment, a test surface (either an IDE or a piece of titanium foil) was placed in a Petri dish, and if required, the IDE was wired to a computer-based multichannel pulse generator (model PCI 6723, National Instruments, Austin, TX). In the Petri dish, the test surface was surrounded with wet paper towels in order to maintain a moist microenvironment throughout the duration of the test. Electric signals consisted of pulse trains (Figure 1b) 10 V in magnitude, a frequency of 200 Hz and 100 ms in duration, applied in cycles of 3 s (ie 0.1 s on and 2.9 s off). This specific electric pulse configuration has been shown to produce significant inhibition of settlement of barnacle larvae (Pérez-Roa et al. 2008).

Abiotic tests

To evaluate the formation of ROS on surfaces exposed to electric fields, a set of 18 IDEs (9 wired and 9 unwired) were used. A 0.2 ml drop of natural seawater (1 μm-filtered and aged seawater collected at Duke University Marine Laboratory (Beaufort, NC)) was placed on each IDE. Then, every 24 h, six IDEs (three wired, three unwired) were removed from the Petri dishes, and the seawater was collected for quantitative measurements of H_2O_2 using a colorimetric method based on copper reduction by H_2O_2 in the presence of 2,9-dimethyl-1,10-phenanthroline (DMP) (Baga et al. 1988). Free chlorine was measured using Standard Method 4500-Cl-G, with *n*,*n*′-diethyl-*p*-phenylenediamine (DPD) (American Public Health Association et al. 1995).

To assess the decomposition of H_2O_2 on IDE surfaces not subjected to electric fields, nine unwired IDE were set up as described above, except that at the beginning of the experiment they received 60 μM H_2O_2 dissolved in seawater. The H_2O_2 concentration was measured on three of these non-wired IDEs every 24 h. Furthermore, to indirectly detect any formation of hydroxyl radicals (OH•), separate experiments were performed using *n*,*n*-dimethyl-*p*-nitrosoaniline (RNO) as a scavenger added to the seawater. RNO reacts with OH• and its degradation can be easily detected by absorbance at 440 nm (Comninellis 1994). In these experiments, a 20 μM solution of RNO in artificial seawater was placed directly on the electrodes, using a similar set up to that described above.

To further investigate the activity of free radicals on surfaces subjected to electric fields, 3-μm porous silica beads coated with boron dipyrromethene difluoride-based dye BODIPY$^{581/591}$(Molecular Probes/Invitrogen, Carlsbad, CA) were employed. The BODIPY$^{581/591}$ dye was covalently attached to the beads to reach a target concentration of 4 nanomoles of dye mg^{-1} of beads, then suspended in isopropanol at a concentration of 25 mg of beads ml^{-1} and stored frozen at −80°C. With fluorescent excitation at 488 nm, the reduced form of the dye emits fluorescence at 595 nm (red), a single oxidation cleaves the molecule resulting in fluorescence emission at 515 nm (green), while multiple oxidations result in total loss of fluorescence (Drummen et al. 2002). Thus, the ratio of red to green fluorescence (R/G ratio) is a surrogate measure of oxidation (Drummen et al. 2002; Hunt 2006).

Two types of experiments were conducted with the BODIPY-coated beads. First, a real-time fluorescence decay experiment was conducted by dissolving 5 μl of the bead/methanol suspension in 20 μl of seawater and placing this solution on a wired IDE that was set up directly on the stage of an inverted confocal laser scanning microscope (model LSM 510, Carl Zeiss North America, Thornwood, NY). In order to enhance the electrochemical process and improve visualization, continuous electric signals also were employed. In the second experiment, four IDE surfaces were placed in Petri dish humidity chambers and each received a 200 μl seawater drop containing 4 μl of bead suspension. The humidity chambers were covered with aluminum foil to prevent light penetration and photo-bleaching of the fluorescent beads. Two of the IDEs were wired and subjected to pulsed electric signals of 0.1 s in duration over 3-s cycles, while the other two served as unwired controls. After incubation

for 3 days, the extent of oxidation of the coated beads was assessed by confocal microscopy using the R/G ratio. The specific methodology for this quantification is described elsewhere (Hunt 2006).

Separate abiotic experiments were conducted to assess ozone formation in the IDEs. To enhance any potential ozone generation, continuous electric signals with the same voltage and frequency used in the pulsed signals were used. Ozone was detected colorimetrically using standard method 4500-O_3 (American Public Health Association et al. 1995). In addition, bromine-free artificial seawater (prepared according to Kester et al. (1967)) was used, because bromine species mask the colorimetric detection of ozone. In these experiments, small IDE devices (90 mm^2 of active area) were placed in a stirred electrochemical cell and immersed in 10 ml of bromine-free artificial seawater. The electric signals were applied for 24 h with an Agilent 33220A signal generator (Agilent Technologies, Santa Clara, CA). All experiments were performed at room temperature (23 $\pm$ 2°C).

Barnacle settlement experiments

Barnacle larvae were delivered to the test surfaces in the Petri dish humidity chambers by placing a 150 μl drop of seawater, containing approximately 25 larvae, directly on the plates. To evaluate the effect of ROS on barnacle settlement, two types of experiments were performed. First, ROS scavenger tests were conducted on wired IDEs by adding, every 12 h, 10 μl aliquots of seawater with a selected concentration of ROS scavenger. Second, to evaluate the effect of H_2O_2 when electric pulses were not present, barnacle settlement experiments were performed on titanium plates with seawater containing various initial H_2O_2 concentrations. In both cases, settlement experiments were terminated when control slides (ie unwired test surfaces not receiving H_2O_2 or ROS scavenger) had a settlement ratio of $\sim$40%, which usually occurred at day 3 (Pérez-Roa et al. 2008). As ROS scavengers, glutathione, an antioxidant ubiquitously present in living cells (Kosower and Kosower 1976), and the enzymatic scavengers catalase and superoxide dismutase (SOD), which catalyze the degradation of H_2O_2 and superoxide radical ($O_2^{-}\bullet$), respectively, were tested.

At the end of each biological experiment, attached and unattached barnacles were counted and the slides were photographed. The slides were then softly rinsed with deionized water to remove the unsettled barnacles and photographed again. Settlement ratios were compared using ANOVA and Kruskal–Wallis tests, the latter because the settlement data from wired IDEs not receiving ROS scavenger were skewed toward zero settlement values, thus invalidating the assumptions of normality and homoscedasticity required for ANOVA tests. At least four replicates were performed for each type of tested treatment.

Results

It was previously shown that the application of electric pulses in planar electrode surfaces significantly inhibits the settlement of marine barnacle larvae (Pérez-Roa et al. 2008). Likewise, Matsunaga et al. (1998) developed conductive paint electrodes and showed their effectiveness at preventing marine biofouling, and Wake et al. (2006) applied electric fields to create an AF system for applications in water intakes. In all cases, the generation of ROS was suggested as a possible AF mechanism, but none of the studies provided direct evidence of ROS formation.

Thus, the first part of this study was aimed at detecting and measuring the potential formation of ROS upon the application of electric pulses to test surfaces. Figure 2 shows time series of H_2O_2 concentrations measured on unwired (controls) and wired (pulsed) IDEs, as well as H_2O_2 concentrations during a decay experiment on unwired IDEs. The pulsed experiment revealed the accumulation of H_2O_2 up to a concentration of 60 μM, although with high variability in the measurements. On the other hand, the unwired control experiment showed no detectable H_2O_2 formation, thus directly linking H_2O_2 accumulation to the application of the electric signals. Furthermore, the H_2O_2 decay experiment on unwired IDEs

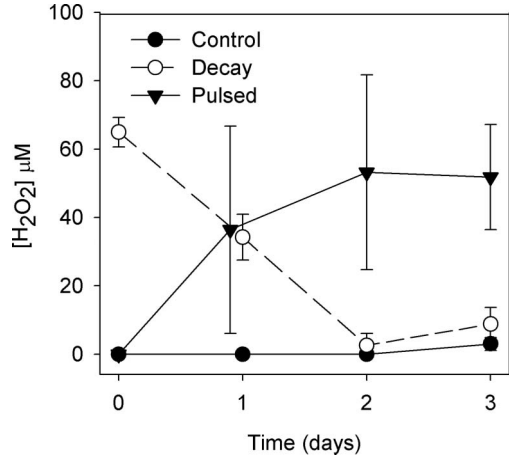

Figure 2. Evolution of H_2O_2 concentrations in seawater drops placed on IDEs for 3 days. The solid lines and filled symbol series show the time series of pulse-treated (▼) and control IDEs (●) without external H_2O_2 addition. The segmented line series with void circle symbols illustrates H_2O_2 decay in non-pulse-treated IDEs. Data points show average $\pm$ SD ($n \geq 2$ for each case).

showed the steady degradation of H_2O_2, which was likely catalyzed by a variety of chemical species found in natural seawater, such as metal and halide-containing compounds (Eul et al. 2001). Therefore, the leveling off of H_2O_2 concentrations in the pulsed IDEs suggested that equilibrium between H_2O_2 production and decay is established after incubation for 2 days.

To evaluate whether ROS other than H_2O_2 were formed during the application of electric pulses, the fluorescent dye BODIPY$^{581/591}$ was covalently attached to silica beads and the coated-beads were placed directly in contact with the IDE surface. Figure 3a shows microscopic images capturing fluorescence from the reduced form of the dye (ie fluorescence emission at 595 nm upon excitation at 488 nm) on an IDE exposed to a continuous electric signal. The striped pattern results from excitation light passing through the borosilicate glass in the IDE and being blocked by the titanium electrode strips, and therefore, the fluorescent beads are only visible on the glass portion of the slide placed on the inverted microscope stage. Anodic and cathodic strips are intercalated, with the images showing one anodic strip in the center and the two adjacent cathodic strips. As time progresses in the experiment, the loss of fluorescence signal is evident on the beads located right next to the cathode strips, while fluorescence from the beads located in close proximity to the anode strip was maintained, thus suggesting that the BODIPY$^{581/591}$ dye was being oxidized by ROS formed at the cathode. A quantification of this progressive loss of fluorescence is presented in Figure 3 and a microscopic image at a higher magnification is included as supplementary material [Supplementary material is available *via* a multimedia link on the online article webpage]. Using the fluorescence data after electric signal application for 300 s, a comparison of bead fluorescence as a function of the position of the beads, from the vicinity of the electrode strips toward the middle section between counter electrode strips, was performed. The gradient of loss of fluorescence of beads placed in the vicinity of the cathodic strips was significantly higher than those placed next to the anodes ($p < 10^{-5}$, ANOVA). As BODIPY$^{581/591}$ is not sensitive to H_2O_2, but it is oxidized by hydroxyl radicals (Drummen et al. 2004), these observations suggest the potential formation of OH• in the electrochemical process initiated by the application of electric signals to the IDEs.

As the microscopy experiments were conducted with a continuous signal rather than the pulsed electric signals (Figure 1b) that have been shown to have AF properties (Pérez-Roa et al. 2008), a confirmation experiment was conducted using the Petri dish humidity chambers with wired and unwired IDEs.

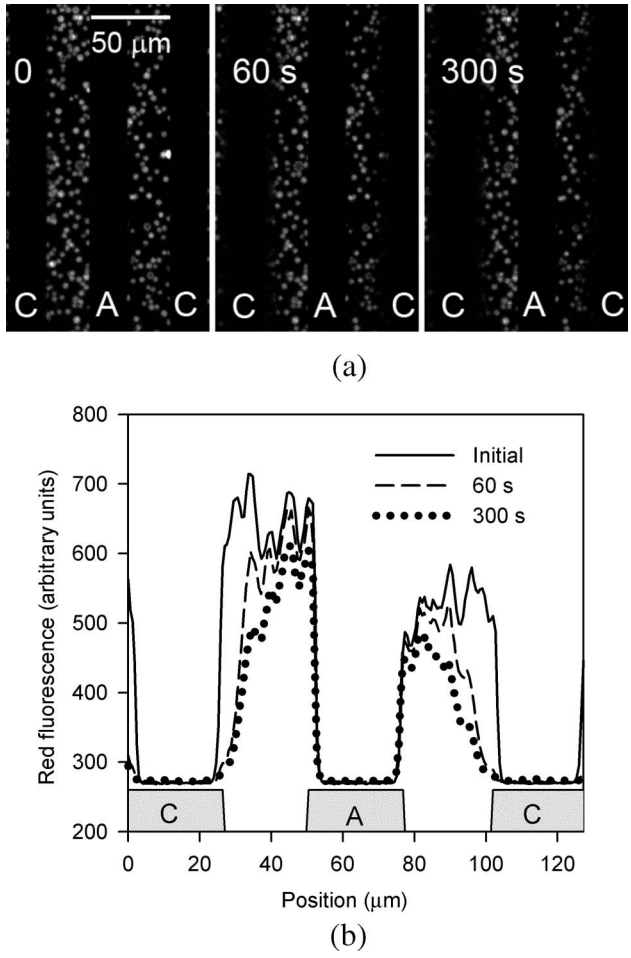

Figure 3. (a) Time series microscopy images showing cathodic oxidation of BODIPY$^{581/591}$ labeled beads upon electric activation. The images correspond to fluorescence emission of the reduced form of the dye before electric treatment (left), after 60 s (center), and after 300 s of signal application. The small spheres correspond to the beads located in between the electrode strips. Anodic and cathodic strips are intercalated and indicated by the letters A and C, respectively. (b) Average fluorescent signal corresponding to the microscopy images, showing that the fluorescence signal fades near the cathodic strips as electric delivery proceeded, whereas the fluorescence signal from beads near the anode maintained their fluorescence.

Quantification of the degree of oxidation by ROS was performed using the R/G ratio (see Materials and methods section). After an incubation period of 3 days, the R/G ratio in unwired IDEs was 1.10 $\pm$ 0.05, while in IDEs exposed to pulsed electric signals, the R/G ratio was 0.65 $\pm$ 0.08, which is a statistically significant difference ($p < 0.001$), confirming the oxidation of the BODIPY$^{581/591}$ dye in wired IDEs exposed to pulsed electric signals.

A confirmation that OH• was produced in the pulsed IDEs was obtained using RNO. This aniline compound is an efficient hydroxyl radical scavenger

(Comninellis 1994), whose oxidation can be easily tracked by the disappearance of its natural absorbance at 440 nm and, therefore, RNO degradation serves as an indirect measurement of OH• production. For these experiments, the seawater drop placed on the IDEs contained 20 μM of RNO. The totality of the RNO was consumed after electric pulsing for 3 h, whereas no evidence of RNO bleaching was detected in IDEs not subject to electrical stimulation, even after incubation for 3 days.

In addition, chlorine formation was not detected in any of the experiments, and the tests with bromide-free artificial seawater showed no evidence of ozone generation in the IDEs. The detection limits for these compounds were 10 $\mu g\, l^{-1}$ as Cl_2 for free chlorine and 10 $\mu g\ 1^{-1}$ for ozone, respectively (American Public Health Association et al. 1995).

Barnacle settlement experiments

With a demonstration that ROS are produced upon the application of pulsed electric signals to the IDE surfaces, an evaluation of the effect of ROS on barnacle settlement was undertaken. If ROS are responsible for AF behavior, then it should be possible to inhibit the AF effect of electric stimulation by adding ROS scavengers directly in barnacle settling experiments.

Glutathione is a nucleophilic thiol ubiquitously present in living cells at concentrations between 0.1 and 5 mM (Kosower and Kosower 1976). Among the many reactions in which glutathione is involved in biological systems, its ability to act as a one-electron reductant makes it a ROS scavenger, donating protons to most radicals centered in carbon, nitrogen and oxygen atoms. Reactions with superoxide, H_2O_2, and hydroxyl radical are well documented (Kosower and Kosower 1976; Rosen 1999). The oxidized form (glutathione disulfide) is composed of two molecules linked by a covalent disulfide bond, and is usually found in healthy cells at concentrations ranging between 0.006 and 0.2 mM (Kosower and Kosower 1976). Higher concentrations of the oxidized form are linked to oxidative stress and cell damage (Kosower and Kosower 1976). In barnacle settlement tests in which aliquots of a glutathione solution were added every 12 h (Figure 4), barnacle fixation significantly increased with the addition of 0.2 and 1.0 mM glutathione compared to settlement occurring in control experiments not receiving any glutathione ($p = 0.008$, Kruskal–Wallis test), providing evidence that ROS are involved in the AF properties of pulsed electric signals. Parallel experiments with similar glutathione doses, but no electric stimulation, showed no effect of glutathione concentration on settlement. In the wired IDE receiving the highest dose of glutathione tested (5.0 mM), significant inhibition of biofouling was not observed. Although it is not clear why this occurred, a potential explanation for this observation is that glutathione disulfide might have accumulated to concentrations that became toxic to the larvae, thus affecting the overall settlement activity. Nevertheless, larval mortality was not evidenced in these experiments.

Experiments using catalase as an ROS scavenger specifically targeting H_2O_2 (it catalyzes the dismutation of H_2O_2 to water and oxygen (Rosen 1999)) revealed that addition of catalase also inhibited the biofouling properties of the electric stimulation (Figure 5). Periodic additions of 0.5 and 1.0 units of catalase resulted in significantly higher barnacle settlement ($p < 0.001$, ANOVA) as compared with control experiments not receiving any catalase. As observed with the glutathione experiments, the largest catalase dose tested (10 units per dose) resulted in low barnacle settlement. However, in this case, larval mortality was visually evident at the moment of counting unsettled organisms. This observation was interpreted as a

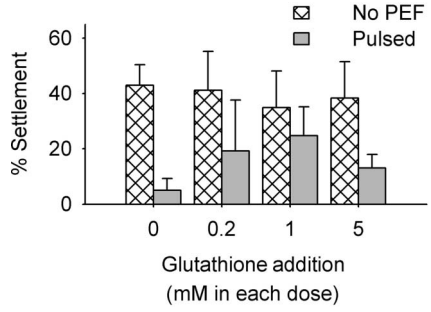

Figure 4. Settlement of barnacle larvae on IDEs as a function of electric stimulation and ROS scavenging by glutathione addition. Error bars show 95% confidence intervals.

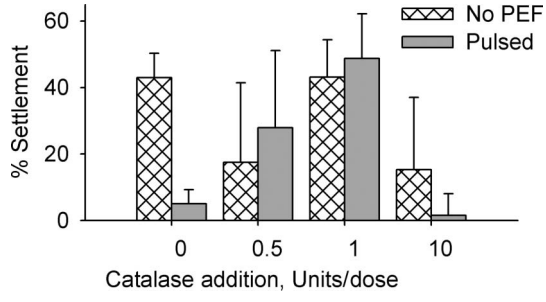

Figure 5. Settlement of barnacle larvae on IDEs as a function of electric stimulation and ROS scavenging by catalase addition. Error bars indicate mean 95% confidence intervals.

toxicity event not representative of ROS scavenging. Thus, as catalase reduces H_2O_2 to water, these results support the hypothesis that electrochemically generated H_2O_2 played a major role on preventing barnacle settlement.

Searching for further experimental evidence capable of discerning whether H_2O_2 or hydroxyl radicals (and the products of hydroxyl radical chemistry) had more influence on preventing barnacle settlements, settlement experiments with the addition of H_2O_2, but no electric stimulation, were performed. This experiment provided an environment in which H_2O_2 was abundant, but OH• formation was minimized. Figure 6 shows that barnacle settlement was not affected in experiments with initial H_2O_2 concentrations as high as 0.3 mM, but higher doses resulted in significant inhibition of barnacle settlement. Moreover, visual inspection of larvae at the conclusion of the experiments showed that the decrease in barnacle settlement was associated with larval mortality. When there was no electric stimulation, the concentrations of H_2O_2 needed to inhibit barnacle settlement were an order of magnitude higher than the equilibrium concentrations observed in IDEs subjected to electric stimulation (Figure 2), and no larvae mortality was observed in any of the control tests not receiving the addition of external H_2O_2. Thus, it appears that at the low concentrations of H_2O_2 accumulating in wired IDE experiments, this specific ROS is not responsible for the AF properties of the electric signals.

An additional scavenging experiment was performed with superoxide dismutase, an enzyme that catalyzes the degradation of superoxide to H_2O_2 and oxygen (Rosen 1999). In this experiment, when two units of enzyme were periodically added to IDEs receiving electric signals, barnacle settlement was 11% ± 4%, compared to 5% ± 4%, (mean ± 95% confidence interval) in experiments not subjected to electric stimulation. This difference was not statistically significant, and thus, it is concluded that the addition of superoxide dismutase did not inhibit the AF properties, suggesting that this specific ROS was not a key component of the AF process.

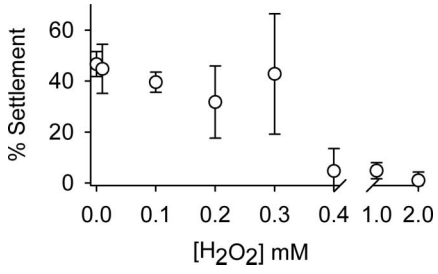

Figure 6. Influence of externally added H_2O_2 on barnacle larvae settlement without electrical stimulation. Titanium plates were used as substratum. For each case, $n = 4$ except for 0 mM ($n = 12$) and 1 mM ($n = 8$). Bars show 95% confidence intervals.

Discussion

The abiotic experiments demonstrate that ROS are formed at the IDE surface during pulse electric stimulation. Direct evidence of H_2O_2 accumulation was obtained, and hydroxyl radical formation was indirectly suggested by the microscopic visualization of BODIPY$^{581/591}$ oxidation and confirmed with the RNO scavenging test. Furthermore, the microscopic observations of BODIPY$^{581/591}$ oxidation provided evidence that oxidation of the dye took place near the surface of the cathode, indicating that hydroxyl radical formation was a cathodic process.

Based on these observations, a mechanism for ROS formation and activity on the IDE surface is proposed (Figure 7). At the cathode, two main reactions are proposed to take place, namely, the production of hydroxyl radicals from reduction of H_2O_2, and the formation of H_2O_2 by reduction of molecular oxygen. Evidence for the former reaction was obtained by microscopic observations, as described above (Figure 3). Evidence for the second reaction was obtained in experiments described elsewhere (Pérez-Roa 2008). Briefly, in a two cell electrochemical chamber in which

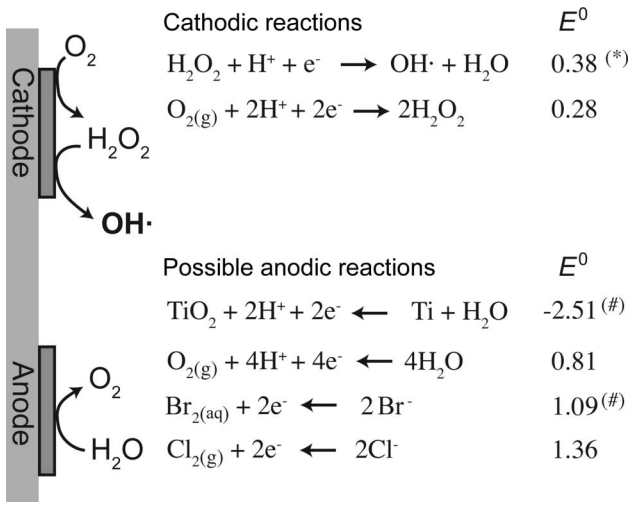

Figure 7. Proposed reactions occurring in the cathode and anode of IDEs subjected to pulsed electric signals. The reactions involved, H_2O_2 and hydroxyl radical generation, are schematically shown in the diagram. Potentials are expressed at standard conditions and pH = 7. Unmarked potentials were obtained from Oldham and Myland (1994), while those marked with (*) were from Rosen et al. (1999) and with (#) from Latimer (1952).

titanium electrodes were placed in the anodic and cathodic chambers (same material as the electrodes in the IDEs), and similar electric pulses as those used in this research were applied, H_2O_2 accumulation was only observed in the cathodic chamber. Moreover, when oxygen was removed from the cathodic chamber, H_2O_2 accumulation was not observed.

It is likely that the high reactivity of OH• would result in other radical-involving reactions, which could help facilitate the propagation of the oxidative effect beyond the surface of the cathode (Figure 3b). In a seawater environment, hydroxyl radicals might have generated either peroxyl radicals (ROO•, with R an organic chain) upon reaction with dissolved organic matter or bromide radicals upon reaction with bromide ions. Further reactions of these radicals may have also occurred. Certainly, it is known that BODIPY$^{581/591}$ oxidation can also be catalyzed by peroxyl radicals (Drummen et al. 2004). Reactions involving bromide oxidation by radical attack are likely to happen, but a further quantification of bromine-containing byproducts was not performed in the context of this work.

Several reactions can be speculated to occur at the anode after the naturally occurring TiO_2 formation on the anodic surface upon contact with water. These reactions include the oxidation of water with formation of oxygen and the oxidation of chloride or bromide ions (Figure 7). The experiments conducted in this study did not provide direct evidence favoring any of these reactions, although in earlier research with similar IDEs, a color change at the surface of the electrodes was observed, suggesting oxidation of the electrode surface (Pérez-Roa et al. 2008), and in the current study, chlorine formation was not detected in any of the experiments. According to the standard potential for each of these reactions (Latimer 1952; Oldham and Myland 1994), and considering that TiO_2 formation on the anodic surface is limited by the surface area, oxidation of water would be likely the easiest reaction to take place, and therefore, it is proposed that this is a plausible reaction on the anode of the IDEs (Figure 7). Furthermore, diffusion of oxygen from the anode to the cathode could be also an important process for the continuous production of H_2O_2 at the cathode. These reactions previously have been observed in a two-cell electrolytic cell designed for drinking water disinfection studies, where oxygen was produced at the anode and H_2O_2 resulted from oxygen reduction at the cathode (Drogui et al. 2001). Still, the oxidation of bromide ions is likely to happen in seawater and it cannot be fully discarded, although bromine species were not measured in these experiments.

The effective inhibition of barnacle settlement in wired IDEs at H_2O_2 concentrations that do not affect barnacle settling when electric stimulation is not used is an indication that H_2O_2 is not directly involved in barnacle settlement inhibition. Furthermore, direct evidence of significant hydroxyl radical formation in the IDEs was obtained and, therefore, the experimental evidence suggests that either hydroxyl radicals, or the products of hydroxyl radical reactions, have a more direct effect on preventing barnacle settlement than H_2O_2. The results presented in this study also supported the conclusion that hydroxyl radical formation is a cathodic process resulting from the electrochemical production of H_2O_2, also at the cathode. These observations provide a mechanistic framework for the potential use of electric signals as a biofouling control strategy that produces short-lived chemicals that act directly at the surfaces where they are produced and do not have lasting negative effects on the environment, which is one of the main disadvantages of current AF coating technologies (Yebra et al. 2004). Interestingly, the mechanistic explanation of barnacle biofouling presented here is similar to a proposed mechanism of electrochemical bacterial disinfection, in which hydroxyl radical generation contributed to the inactivation of *Escherichia coli*, whereas the presence of H_2O_2 and superoxide had negligible bactericidal effects (Jeong et al. 2006). Previously, Liu et al. (1997) had shown the involvement of cathodic H_2O_2 production in the electrochemical inactivation of *Staphylococcus epidermidis* and *Staphylococcus aureus* cultured in agar plates using catalase as scavenger, although they did not investigate the involvement of other ROS, and therefore, a direct comparison cannot be made.

The use of electric signals for biofouling control has not only been aimed at invertebrates, but also at bacteria (Nakasono et al. 1993; Matsunaga and Lim 2000; van der Borden et al. 2004; Pérez-Roa et al. 2006; Hong et al. 2008), including the application of the IDE microelectrodes similar to those used in this study for the prevention of biofouling by *Pseudomonas aeruginosa* biofilms (Pérez-Roa et al. 2006). van der Borden et al. (2004) presented evidence of increased current-induced detachment of *S. epidermidis* biofilms, with up to 80% of detachment after the currents induced a voltage across the electrodes of 2 V. Furthermore, Hong et al. (2008) applied currents resulting on a voltage across electrodes of 2.4 V, investigating the effect of these signals on the adhesion and viability of attached *P. aeruginosa*. These results indicate that anodic currents enhanced bacterial mortality upon adhesion to the surface, while cathodic currents induced bacterial detachment. In a related study, Chang et al. (2007) demonstrated the involvement of ROS in the bactericidal action of an Al_2O_3 catalyst when *E. coli* cells were in contact with the catalytic surface.

Taking the observations with invertebrate biofouling control described here, together with the existing studies implicating ROS on controlling bacterial fouling, it is possible to suggest that ROS generation at an electrically treated surface could be effective at simultaneously preventing bacterial and invertebrate biofouling, which seems desirable for any marine AF strategy. The challenge remains in the scale-up of electrochemical biofouling control technologies, which have been mainly limited by the lack of suitable and durable materials for long-term biofouling control applications (Wake et al. 2006). Hydrodynamic flow considerations would also play an important role on larger coatings, as the surface concentration of radicals might be limited by convective flow patterns. Nevertheless, the recognition that hydroxyl radicals are the main ROS involved in biofouling prevention should inspire additional developments of these technologies.

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