Buzz Off! An Evaluation of Ultrasonic Acoustic Vibration for the Disruption of Marine Microorganisms on Sensor Housing Materials

Abbreviated Headline: Ultrasonic Bio-fouling for Sensors

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Significance and Impact of the Study

In this study, ultrasonic acoustic vibration is presented as a chemical-free, ecologically friendly alternative to conventional methods for the perturbation of microbial attachment to submerged surfaces. The results indicate the potential of an ultrasonic anti-bio-fouling method for the disruption of microbial bio-films on marine sensor housings, which is typically a principle limiting factor in their long-term operation in the oceans. With increasing deployment of scientific apparatus in aquatic environments, including further off-shore and for longer duration, the identification and evaluation of novel anti-fouling strategies that don't employ hazardous chemicals are widely sought.

Abstract

Bio-fouling is a process of ecological succession which begins with the attachment and colonisation of microorganisms to a submerged surface. For marine sensors and their housings, bio-fouling can be one of the principle limitations to long-term deployment and reliability. Conventional anti-bio-fouling strategies using biocides can be hazardous to the environment, and therefore alternative chemical-free methods are preferred. In this study, custom made testing assemblies were used to evaluate ultrasonic vibration as an anti-bio-fouling process for marine sensor housing materials over a 28-day time course. Microbial bio-fouling was measured based on (1) surface coverage, using fluorescence microscopy, and (2) bacterial 16S rDNA gene copies, using Quantitative PCR. Ultrasonic vibrations (20 KHz, 200 ms pulses at 2 s intervals; total power 16.08 W) significantly reduced the surface coverage on two plastics, PMMA and PVC for up to 28 days. Bacterial gene copy number was similarly reduced, but the results were only statistically significant for PVC, which displayed the greatest overall resistance to bio-fouling, regardless of whether ultrasonic vibration was applied. Copper sheet, which has intrinsic biocidal properties was resistant to bio-fouling during the early stages of the experiment, but inhibited measurements made by PCR and generated inconsistent results later on.

Keywords

Bio-fouling

Anti-fouling

Ultrasonic

Sensor

Propidium Monoazide

Introduction

Environmental monitoring networks increasingly rely upon the long-term deployment of sensor equipment in the ocean (Hart and Martinez, 2006). However, the accuracy and reliability of these systems are rapidly impaired by bio-fouling. This is a process of ecological succession, which begins with the formation of a conditioning film containing dissolved organic compounds (proteins and polysaccharides) leading to the establishment of a bacterial biofilm. This paves the way for the recruitment and settlement of single-celled eukaryotes and, later, marine invertebrates (Callow and Callow, 2002). The gradual build-up of bio-material on sensors and their housings can obstruct water movement, modify the microenvironment around the sensor head, obscure optical windows and electrodes, and increase the rate of corrosion (Videla and Characklis, 1992). At the end of the deployment lifetime the apparatus must be removed to dry dock and cleaned, often requiring hazardous chemicals, and leading to increased maintenance costs and "down-time". Sensitive equipment such as sensor electrode arrays require specialist cleaning or must be replaced after each deployment.

Current methods to reduce bio-fouling on sensors include, but are not limited to, the use of biocidal materials and coatings, the controlled release or generation of biocidal chemicals, and physical removal using wipers, scrapers or water jets (Manov et al., 2004; Whelan and Regan, 2006; Delauney et al., 2010). Each method has unique advantages and limitations; for example biocides contaminate the environment being monitored (Terlizzi et al., 2001), coatings can erode or become damaged and wipers require complicated moving parts, which must remain water-tight. Acoustic vibration, either within the audible (20 Hz – ≤ 20 KHz) or ultrasonic (≥ 20 KHz) frequency range, has been demonstrated as a promising, ecologically friendly alternative to conventional methods for the removal of organic and in-organic material attached to submerged surfaces (Gittens et al., 2013; Legg et al., 2015). For

clarity, the term “ultrasonic” is used herein to describe the acoustic vibration used in this study, 20 kHz, which lies on the border between the audible and ultrasonic frequencies. The cavitation induced by ultrasonic pulses propagating through water can inhibit bio-fouling on solid sub-strata, including the removal or destruction of bacterial biofilms (Qian et al., 1997; Mermillod-Blondin et al., 2001), and reduction in the settlement rate of barnacle larvae (Branscomb and Rittschof, 1984; Kitamura et al., 1995; Guo et al., 2011a; Guo et al., 2011b). The latter is most pronounced at a frequency of approximately 20 KHz (Kitamura et al., 1995; Guo et al., 2011b). Not surprisingly, there are numerous patents relating to the use of acoustics for the removal of bio-fouling, and ultrasonic cleaning systems are already underdevelopment (Mazue et al., 2011) or commercially available for “in the water” maintenance of ship / boat hulls (e.g. SonicShield by CMS Marine Ltd, SHIPSONIC by Globus Benelux, Sonihull by NRG Marine Ltd, UltraSystem by Ultrasonic Antifouling Ltd and ClearHull by Ultrasonic Works Ltd). Recently, a multi-national European collaboration, the “Cleanship” project, undertook field trials of an ultrasound-based method for the detection and prevention of bio-fouling on submerged steel plates (De Carellan et al., 2014), and there have been reports of successful long-term sea trails of acoustic anti-biofouling systems fitted to commercial and military vessels, although these references are not readily available.

Despite the clear potential, and increasing popularity of ultrasonic anti-fouling methods, there is a paucity of data relating to the potential application for the protection of marine sensing. In this study, ultrasonic acoustic vibration is evaluated as an anti-bio-fouling method on three types of material used for the fabrication of marine sensor housings; Poly(methyl methacrylate) (PMMA), Polyvinyl chloride (PVC) and Copper (Cu). The tests were carried out on small pieces of each material, the size of a microscope slide, which were fixed to stainless steel plates via nylon spacers. The plates were submerged at 1.5 m depth, in

baffled housings, in a busy industrial dock for 28 days during the summer of 2015. One plate was fitted with a commercially available ultrasonic transducer, the ClearHull 110 system, which produced ultrasonic acoustic vibrations (20 KHz 200 ms pulses at 2 s intervals; total power 16.08 W) over the duration of the experiment; another was deployed without ultrasonic acoustic vibration as a control. The extent of micro-fouling on each material was quantified after 1, 3, 14, 21 and 28 days during the deployment using two principle methods; (1) surface coverage using DAPI staining with fluorescence microscopy, and (2) bacterial 16S rDNA gene copy number using Quantitative Polymerase Chain Reaction (qPCR). The demonstration of ultrasonic anti-fouling is timely considering the increasing use of sensor and measurement apparatus in the ocean, and the increasing preference for environmentally friendly, biocide-free anti-fouling methods that don't contaminate the environment. To the best of our knowledge this is the first quantitative, time course evaluation of ultrasonic anti-fouling for the protection of marine sensor housings.

Results and Discussion

The effects of ultrasonic vibration on the bio-fouling of marine sensor housing materials were evaluated using custom made testing assemblies, as shown in Figure 1. The extent of bio-fouling was measured over a 28 day deployment using two parameters; surface coverage and bacterial 16S rDNA gene copy number. From the determination of the surface coverage using the DAPI staining and fluorescence microscopy, shown in Figure 2, the application of ultrasonic vibrations to the materials led to a significant (t-test, $P = <0.05$) reduction in DAPI staining, particularly during the later stages of the experiment (from day 14). These effects were also apparent on the steel plates to which the materials were mounted, and the assembly fitted with ultrasonic anti-fouling remained mostly clear throughout the deployment, whilst the control became colonised extensively by barnacles (not shown). Cu had the lowest levels of DAPI staining during the early stages of the deployment (day 1 and 3), in line with its intrinsic anti-fouling properties, but had high levels of DAPI staining from day 14. This coincided with the formation of a blue / green patina over the Cu surface which prevented proper examination, and therefore the results for Cu from day 14 onwards were inconclusive. PVC displayed the greatest resistance to bio-fouling, and had significantly lower surface coverage than PMMA, both on the control and with ultrasonic disruption.

The number of bacterial 16S rDNA gene copies on each material was quantified as an additional measure of bio-fouling during the later stages of the deployment, from day 14. This analysis included the biological material that accumulated outward from the surface of each sample, and which could not be measured in 2-dimensions as surface coverage. In these experiments the bio-fouling on each material was removed into sterile dock water with a sterile swab and divided into two representative samples. One of these was left untreated, whilst another was exposed to PMA, a photo-reactive compound, which diffuses across broken cell membranes and intercalates with the genome. Subsequent photo-activation of

PMA-DNA complexes leads to irreversible covalent modification of DNA, preventing its amplification by PCR. Thus, using membrane integrity as a proxy for cell viability, only viable cells are included in subsequent amplification of genomic DNA by PCR. The number of 16S rDNA gene copies detected on PMMA and PVC, with or without ultrasonic anti-fouling is shown in Figure 3, along with the estimated bacterial viability calculated by comparing the gene copy number with and without PMA pre-treatment. The results for Cu are not shown; the presence of labile copper in the samples inhibited PCR amplification, requiring additional DNA purification leading to loss of sample and making quantification unreliable.

On both plastics, the ultrasonic vibration led to a reduction in the number of bacterial gene copies. This indicates a reduction in bio-fouling in support of the results from DAPI staining, shown in Figure 2. However, the difference was only statistically significant (t-test, $P = > 0.05$) for PVC. PMMA presented with more DAPI staining than PVC throughout the experiment (with or without anti-fouling), but conversely the material recovered from the surface contained significantly fewer bacterial gene copies. Bacterial colonisation typically occurs during the primary stages of bio-fouling followed by the settlement of other microorganisms and small invertebrates, which may influence the bacterial component of the biofilm, for example through competition for nutrients and predation. These processes may contribute to the discrepancy between total bio-fouling estimated using DAPI staining, and bacterial bio-fouling estimated using qPCR.

Two principle mechanisms of action for ultrasonic disruption to biofilms include (1) detachment / dislodging of biological material from the surface and (2) damage / death of living cells from sheer stress induced by cavitation. PMA is a state of the art method for differentiating between total and viable bacterial populations on the basis of cell wall / plasma membrane integrity. On PMMA, ultrasonic anti-fouling had no significant effect on

bacterial viability over the experimental time course. In contrast, on the PVC there was an increase in the proportion of viable bacteria over time; specifically at day 28 the bacteria remaining on the surfaces had a greater than 90 % viability. This implies the formation of a sparse, but healthy community of organisms, with mechanism (1) predominating.

This preliminary investigation indicates that ultrasonic anti-fouling can significantly inhibit bio-fouling on PMMA or PVC marine sensor housings for up to 28 days, which may enhance the deployment lifetime and accuracy of the sensors contained within them. PVC showed the greatest resistance to biofouling indicating that it may be preferred for sensor housing fabrication. Although the results for Cu were inconclusive, it is likely that, due to the intrinsic biocidal properties of this material, it would have displayed the greatest overall resistance to bio-fouling, regardless of ultrasonic vibration. However, it is also relatively expensive and cannot be readily moulded into complicated designs, unlike the thermoplastics PMMA and PVC. Furthermore, there is an increasing preference for environmentally friendly, biocide-free anti-fouling methods that don't contaminate the environment or interfere with measurements; as in, for example, the inhibition of the PCR analysis in this particular study from Cu-exposed samples.

The results presented in this study were generated using ultrasonic acoustic vibration with a frequency of 20 kHz, which has also been demonstrated to be effective at perturbing the settlement of barnacle larvae (Kitamura et al., 1995; Guo et al., 2011b), with short 200 ms pulses every 2 s. Comprehensive optimisation of these ultrasonic acoustic parameters, made according to the material type and architecture, may achieve greater reductions in colonisation rate. Crucially, the use of short, intermittent pulses may alleviate interference to sensor measurements caused by structural vibrations, where these measurements are made in between pulses. Another, important consideration is the power consumption of ultrasonic anti-fouling over a long deployment, which is a potential limitation of this method compared

to, for example, the use of biocidal coatings. The Clear Hull 110 system configuration used for this study operated with a total power of 16.08 W from a 12 V transformer, connected to the mains supply (240 V). This generated a consumption of 134 mAh (based on 200 ms pulses every 2 s), which could be sustained for a period of 30 days using a battery pack containing 24 D-cell batteries (assuming 3.6 V / 17 Ah cells working with 4 batteries in series, with 6 series in parallel), or equivalent. This is within reasonable specifications, however significant reductions in power consumption could be achieved by further optimisation of the electronics and the ultrasonic acoustic parameters. The findings of this investigation show considerable promise for the development and commercialisation of ultrasonic anti-fouling systems for sensor housings and support structures, in addition to the systems already available for the protection of marine vessels.

Materials and Methods

Testing Equipment. The effect of ultrasonic acoustic vibration on the adhesion of microorganisms to three sensor housing materials, Poly(methyl methacrylate) (PMMA), Polyvinyl chloride (PVC) and Copper (Cu), was evaluated using submersible assemblies, shown in Figure 1. Each assembly was fabricated from a 3 mm thick stainless steel plate (Marine Grade 316), onto which were mounted an array of removable "Packets" supporting a sample of each material, cut to the equivalent size of a conventional glass microscope slide (25 mm by 75 mm). The packets were constructed from rigid, glass-filled Nylon blocks (100 mm by 95 mm) secured to the steel plate at each corner using 20 mm A4 grade M4 hex head machine screws (Din 933) with A4 grade M4 Nylock nuts. The materials were secured within a recessed portion of the Nylon surface by two lateral stainless steel bars, screwed into the Nylon using 12 mm A4 stainless steel M4 machine screws. Each slide was cleaned with detergent and 70 % (v/v) ethanol solution to remove any residual grease and debris prior to deployment. The entire assembly was housed within an opaque polypropylene box featuring baffled openings to allow water movement and prevent large marine life from entering, and secured to the back plate using a 12 mm A4 stainless steel M4 machine screw at each corner. Two testing assemblies were deployed simultaneously, either with or without the application of ultrasonic vibration provided by a Clear Hull 110 ultrasonic anti-fouling system (Ultrasonic Works Ltd, UK). The Clear Hull 110 features an aluminium transducer attached to a control unit by a water-proof cable, and powered from a 12 V transformer connected to a 240 V (mains) electrical supply. The transducer was fixed to the stainless steel back plate through a central 11 mm hole. It was set to emit 200 ms pulses at 2 s intervals, tuned to a frequency of 20 kHz with a total power of 16.08 W. A sacrificial Zn anode was attached to the back plate containing the aluminium transducer to prevent corrosion. The equipment was hung at a depth of 1.5 meters from a pontoon within the Empress Dock, Southampton (UK)

between the 8th of July and the 5th of August 2015. During this time the Empress Dock water had a temperature of between 18.6 and 19.1 ^{o}C, and a mean salinity of 31 ± PSU.

Sample Recovery. Three "Packets" were recovered from each assembly after 1, 3, 14, 21 or 28 days post deployment. At each time point, the assemblies were removed from the water and the back plates were released from their housings. The packets were unbolted and immersed in 0.2 μm filtered dock water for later analysis. The equipment was re-assembled and submerged within five minutes to minimise disruption to the remaining samples.

Fluorescence Microscopy. Each sample of material was washed three times by immersion in Phosphate Buffered Saline (PBS) (pH 7.4). Then, the materials were immersed in 5 mL of PBS containing 100 μL of 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, UK) in darkness for 5 minutes. These were removed to air, and kept in darkness, at room temperature for 20 minutes prior to observation using an EVOS™ FL cell imaging microscope (Thermo Fisher Scientific) with a DAPI filter set (344-357 nm Excitation; 447-460 nm Emission). Images were captured using a 10x objective from ten locations on each slide, which were selected at random by deriving coordinates using a random number generator. Images were collected from each material sample, at each time point over the course of the deployment. Each image was analysed using the thresholding function of ImageJ Image Analysis Software (Schneider et al., 2012) to estimate the percentage coverage. Equivalent thresholding limits were applied to each image in order to make a fair comparison.

Propidium Monoazide (PMA) Pre-treatment and DNA Extraction. PMA pre-treatment was carried out according to published methods (Magiopoulos et al., 2016). Each sample of material was immersed in 10 mL of a sterile PBS (pH 7.4) and the surface bio-fouling was removed using a sterile Buccal swab (IsoHelix, UK). The PBS and swab head

were transferred to a sterile, tube, vortexed, and divided into two samples. One sample was mixed with 2.5 μL of a 50 μM solution of PMA, and the other was mixed with 2.5 μL of sterile water. The samples were left in darkness for 5 minutes and then, on ice, irradiated with white light from two 650 W lamps (FLASH 2000 L, DTS, Italy) placed approximately 20 cm from the samples for 3 min. Each sample was washed three times in sterile PBS prior to DNA extraction using the PowerWater DNA isolation kit (MoBio Inc., USA) according to the manufacturer's recommended protocol.

Quantitative Polymerase Chain Reaction (qPCR). The DNA recovered from each sample of material, with or without PMA pre-treatment, was used to quantify the number of 16S rRNA gene copies using qPCR. PCR was carried out using universal bacterial primers, 341F and 785R and 5 PRIME HotMaster Taq DNA Polymerase (5 PRIME GmbH, Germany) according to the manufacturer's recommendations. Each reaction was spiked with EvaGreen DNA binding dye (Biotium, USA) and 5 μL of template DNA; the total volume was 20 μL. The thermal cycling parameters were 94 ^{o}C for 2 minutes, followed by 40 cycles of 94 ^{o}C for 20 seconds, 46 ^{o}C for 10 seconds and 65 ^{o}C for 50 seconds with a final elongation step of 65 ^{o}C for 10 minutes. The reactions were carried out in triplicate, and measured in real-time using an Mx3005P real-time thermalcycler (Agilent Technologies, USA). A post-amplification, high-resolution melting curve was plotted to ensure product specificity. The efficiency of the PCR amplification was determined according to the method of Pfaffl (Pfaffl, 2001), and was found to be 97 %. Quantification of 16S rRNA gene fragments was achieved by comparing the threshold cycle (Ct) values obtained from the samples with those from a dilution series of custom made 16S rRNA gene DNA standards with concentrations ranging from 2,000,000 to 200 copies per reaction, as described by Magiopoulos *et al* (Magiopoulos et al., 2016).

Statistical Analysis. The results of the DAPI-fluorescence analysis, and qPCR analysis were tested, where appropriate, for statistical significance using the Paired T-test with a 95 % confidence. Statistical tests were carried out using Minitab (Minitab Ltd, UK; version 17).

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Conflicts of Interest

None declared.

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Figure Legends.

Figure 1. Photographs of the ultrasonic anti-fouling testing apparatus. The apparatus was carefully designed to enable the synchronous evaluation of bio-fouling on three materials, using a single ultrasonic transducer, and wherein a sample of each material could be recovered at regular intervals, and in a short period of time to minimise disruption to the experiment. “Packets” were prepared to contain small samples of marine sensor housing materials, Cu (top), PMMA (middle) and PVC (bottom), fixed to glass-filled Nylon blocks by stainless steel lateral support plates (Panel A). The packets were bolted to a stainless steel back-plate featuring a central hole (Panel B), used to affix a ClearHull 110 ultrasonic transducer (Panel C). This was connected to a control module on the pontoon, via a waterproof cable, and powered by a 12 V transformer connected to a mains (240 V) power supply (not shown). The assemblies were enclosed within a plastic housing featuring baffled openings to restrict the entry of large debris / marine life, whilst allowing water circulation over the surfaces (shown in Panel D with or without the steel plate attached). Two identical assemblies were deployed, with or without the ClearHull 110 ultrasonic transducer.

Figure 2. Percentage cover by DAPI-stained biological matter on the surfaces of sensor housing materials. The materials were deployed for up to 28 days, with or without ultrasonic anti-fouling. The results represent the mean from at least 3 replicate samples, recovered at each time point. The error bars show the standard deviation of the mean. Statistically significant differences (t-test, $P > 0.05$) between control and ultrasound are denoted with an asterisk.

Figure 3. Top: bacterial 16S rDNA gene copy number measured on PMMA and PVC, after 14, 21 or 28 days with or without ultrasonic anti-fouling. The results show the mean from at least 3 material samples, and the error bars show the standard deviation. Statistically significant differences (t-test, $P > 0.05$) between control and ultrasound are denoted with an

asterisk. Bottom: the table shows the estimated percentage of viable bacteria on each material sample based on a comparison between PMA pre-treated and untreated samples.