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BIOFILMS: REMOVING MARINE BIOFOULING FROM HULLS, AND CLEANING WOUNDS (AND GROWING SKIN BACK OVER THEM) IN HUMANS, USING JUST AIR SOUND AND WATER

ABSTRACT

The growth of marine biofouling on ship hulls, and chronic infections in wounds, have a common foundation in the initial growth of bacterial biofilms over the surface. Biofilms are communities of bacteria that form living 'aggregates' that are far more resistant to removal (by chemicals, antibiotics, or mechanical scrubbing) than single (planktonic) bacteria. As it matures, the biofilm forms the foundation in which other species can grow, leading to marine biofouling on ship hulls, and chronic infections in human and animal wounds. The existence of chronic wounds in humans shows that current treatments are not wholly effective (the estimated cost of healthcare services in the UK for chronic wounds alone was £5.6 bil-

lion in 2017/18). The toxicity of antifoul for hulls, and the effort required to mechanically remove marine biofoulant, has led to the development of through-hull ultrasonic deterrents to reduce biofouling growth, but variable performance has stopped widespread adoption.

This report introduces new technology that has combined air, sound, and saltwater, to reduce the growth of marine biofouling on hull materials, and removed biofilm from wounded skin, and even promoted skin regrowth over the wounds.

1. INTRODUCTION

1(A) INTRODUCTION TO THE PROBLEM

The historical view of bacteria as individual cells, often free-floating or loosely attached to a substrate, has been replaced in recent years by the recognition of the importance of biofilms. In a biofilm, colonies exist within a matrix, into which other species of bacteria and fungi can co-exist, and this leads to greater resistance to anti-microbial agents. Repeated ineffective uses of anti-microbials against biofilms can promote further growth of anti-microbial resistance, as the wider environment (e.g. natural waterways, waste management infrastructure etc.) contains a vast reservoir of microbial species in which resistance can develop if diluted, sub-therapeutic doses of the agent pass from the water infrastructure into the wider environment.

This paper presents two inventions that use mechanical forces, as opposed to chemical or anti-microbial drivers, to remove biofilms. In each case, the mechanical force is developed when an appropriate acoustic field encounters a bubble, and as such that combination does not extend into the wider environment, and hence does not present opportunities to promote anti-microbial resistance.

The first device is an invention to clean marine biofouling from ship hulls. Whereas a previous design[1] used non-inertial cavitation (thereby avoiding damage to the rubber anechoic on which the fouling grew) to avoid the establishment of mature biofilms on the hull into which macroscopic biofouling can grow, this paper

uses the device to clean established macroscopic biofouling from steel hull material using non-inertial cavitation. The cleaning footprint of the device is around 10 cm by 10 cm, in a design that is easily scalable to larger footprints through the tessellation of units.

In the second invention, the substrate is delicate (human wound tissue) and requires a smaller footprint to accommodate the wound topography, and inertial cavitation is avoided. The removal of the biofilm occurs because of small-scale (of order of a few bubble radii) liquid currents and shear that can be generated on the substrate by microscopic air bubbles.[2] The bubbles generate shear and liquid currents when they are excited by an appropriate acoustic wave in the liquid: the acoustic waves stimulate ripples on the wall of the bubble,[3],[4] and these ripples in turn stimulate liquid currents and shear close to the bubble. Furthermore, the sound field generates acoustic radiation forces that drive the bubbles (and their cleaning action) into crevices that are difficult to clean using traditional brushes and wipes.[5]-[6]-[7]-[8] Suitable technology can pass both bubbles and sound down the liquid stream onto the substrate to be cleaned.[9]

1(B) THE SURFACES TO BE CLEANED

The two inventions are aimed at two types of surface substrates: ship hulls (which are substantially flat over the 10 cm scale, but will contain varying topography on the microscopic scale) and wounds (which are have undulating and varying topography on both the macroscopic and microscopic scales). The total area to be cleaned is greater with hulls, and the stand-off distance for wounds must be greater, and so the architecture of the two devices differ, even though both exploit the same acoustical phenomenon.

(I) SHIP HULLS

Biofilm formation occurs within hours of an object being placed in seawater, and as the biofilm matures, it provides the foundation and nutrients for the attachment and growth of

macroscopic marine fauna and flora. The use of copper and lead on marine vessels, centuries ago, to deter such fouling was an early, if unrecognised at the time, method of combatting biofilms.[10]-[11]-[12]

Marine biofouling reduces the efficiency of shipping by up to 50% due to increased mass and drag, with associated fuel penalties and implications for the carbon footprint of the vessel.[13]-[14]-[15] Additionally, the increased turbulence and attenuation reduces the performance of any sensors fitted to the hull (in particular sonars, but also optical sensors used for measurements of water properties and chemical sensors that rely on sample collection).[16] Turbulent flow over a vessels hull will also increase the radiated broadband noise, an effect of critical importance to warships[16] (Figure 1).

(II) CHRONIC WOUNDS

Chronic wounds differ from hull surfaces in a number of ways. Whereas the hull is substantially hard and flat (on the cm scale), wounds are generally more soft (giving less acoustic reflection), have a complicated topographical profile with multiple pockets and crevices, the patient might move during treatment, and the substrate of value is living and needs to be stimulated to optimise its self-healing properties. A device with a smaller footprint accommodates this change in substrate profile and requires a greater stand-off distance (necessitating a longer water stream).

The very existence and prevalence of chronic, non-healing wounds indicates that state-of-the-art treatments are insufficient. In the UK alone, non-healing wounds, particularly of the lower limb, affect more than 2 million patients per annum and cost the NHS an estimated £5 billion each year.[17]

Chronic wounds fail to progress through the normal stages of healing in a timely manner. The largest remediable cause of chronicity is infection in the form of a multispecies biofilm that is present in most chronic wounds. In addition, the biofilm phenotype of bacterial infection is implicated in many types of chronic, difficult to treat infections including cystic fibrosis, implanted device infections and periodontitis.[18] The biofilm

Figure 1. Biofoulant on the hull of the USS Pittsburgh (SSN 720) in Bremeton, Washington, as she awaits inactivation in dry dock at Puget Sound Naval Shipyard and Intermediate Maintenance Facility. Photograph by *Wendy Hallmark*. *This US Navy photograph is considered public domain and has been cleared for release via the Department of Defense.*

phenotype, apart from impacting wound healing, protects the microorganisms against host defences, increases adhesion and provides a relative immunity to antibiotics. Removing the biofilm without damaging the underlying tissue should improve healing.

2. METHODS

2(A) HULL PLATE CLEANING METHOD

(I) DESIGN OF THE MARINE DEVICE

Figure 2(a) shows a schematic of the ship hull cleaner. The device[1] works by non-inertial cavitation to avoid damaging anechoic linings. The study was designed to examine how non-inertial cavitation hinders the establishment of the biofilm on the hull into which macroscopic marine biofouling takes hold following the initial development of a bacterial biofilm on the hull. The device inhibits the maturation of the biofilm, so reducing the subsequent establishment of macroscopic marine biofouling. It was tested on aluminium, steel and rubber substrates.[1]

Commercial systems are available that attempt to use ultrasound to prevent antifouling, although their performance is mixed, and these methods radiate strong ultrasound into the

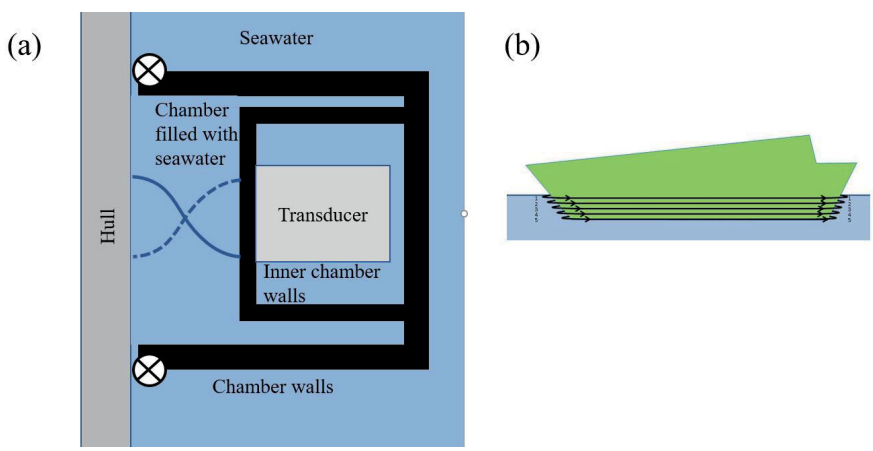

Figure 2. (a) Schematic of the hull cleaning device. A half-wavelength (λ/2) spacing is indicated as a simple illustration of the creation of an acoustic pressure antinode on the hull by making use of the acoustically rigid walls of the chamber, but in fact it is often convenient to tune the device to generate alternative modal patterns on the hull. (b) The method for covering a ship hull. The typical footprint of a single cell is around 0.1 m by 0.1 m although they can be made larger with multiple transducers. Cells like this can also tesselate to cover a larger overall footprint.

surrounding sea. Acoustic pressure as high as 214 dB re: 1 μPa have been measured close to the source, causing inertial cavitation, at 23 kHz)[19]. The leakage of sound away from devices is also an increasing concern with conventional hull treatment technologies, as lower amplitudes radiated to distance might still adversely affect behaviour (foraging, social interactions, breeding etc).[20],[21] The device design presented here reduces the likelihood for adverse effects on marine life (fish, [22] cetaceans[23] and benthic species[24]). It operates at 70 kHz (higher ultrasonic frequencies in general being less likely to produce adverse subjective response in fauna). Moreover, the design of the device uses features to contain the region of intense ultrasound to within the device, minimising the escape of acoustical radiation into the surrounding seawater. For example, the chamber walls reduce the leakage of sound into the environment. The device provides a water-filled cavity to the surface and within it creates a modal sound field that ensures an acoustic pressure antinode is present over the surface to be cleaned (the 'target').

(II) TEST DESIGN

A previous study[1] examined the ability of the device to prevent the formation of the biofilm substrate on which macroscopic biofoulant could grow, and so reduced the establishment and growth of macroscopic biofouling. In this current study, mature biofouling was allowed to grow, and was then removed using the hull-cleaning invention. This was compared to the cleaning achieved by a mechanical rotating brush system.

Samples of steel, aluminium, and rubber plates (50 samples of each material) of surface area 10 cm by 10 cm were submerged in a seawater dock for periods of 36, 41 and 50 days respectively. During these periods, mature biofouling was established on the surfaces of the plates.

The samples were then removed from the seawater and the thickness of the biofouling was measured in 10 regions on each plate, with an average thickness calculated.

The plates were then treated to remove the mature biofouling for a treatment period of 1 minute using the apparatus of Figu-

re 2(a). A single chamber was used for the reported testing, powered by a single transducer. The transducer was driven in continuous wave mode at 70 kHz, with a power amplifier supplying 100 W to the single transducer. The target surface area that was covered by the cleaning chamber at any given time was 36 cm^2, whilst the area of the sample plates was 100 cm^2 (the plates measured 10 cm by 10 cm). That means that, to cover the entire plate, the device was continuously moved over the plate containing established mature biofoulant placed for 1 minute, during which time any section might be covered for 25% of the time.

The rms acoustic pressure amplitude that could be generated over the treated surface was measured prior to testing, using a plate of the material through which a hydrophone just protruded into the water, such that its active element was aligned with the surface to be cleaned. The rms acoustic pressure over steel was 16.8 kPa, over aluminium was 17.3 kPa, but over rubber was much less, at 11.2 kPa.

These values confirm that the cleaning process here occurred by non-inertial cavitation (since inertial cavitation would have required an rms acoustic pressure in excess of 100 kPa). The rubber significantly reduced the rms pressure that could be generated across it, as expected because it is more absorbent than the metals (both in terms of not reflecting the sound back into the water to the same extent that the metals do, and absorbing a portion of the sound that enters it by converting it to heat). Nevertheless, good cleaning was still possible because the rigid walls of the chamber assisted in the formation of 11.2 kPa rms acoustic pressure on the surface of the rubber.

By design, the device is capable of cleaning samples while submerged. For this study, however, a risk assessment required that samples be removed from the sea water to be cleaned as the amplifier and high-voltage power supply did not have a sufficiently high water-proofing rating.

The thickness of the remaining biofouling after the treatment was measured in 10 places on each plate, and an average thickness calculated. The difference between the initial and final thickness measurements was calculated and expressed as a percentage value for the biofoulant that was removed from the plate surfaces.

A subset of plates were subjected to a conventional cleaning treatment using an electrically powered rotary brush for a period of 1 minute rather than acoustic energy in accordance with the present invention. The % thickness reduction was again calculated, and the results shown in Table 1.

(III) MEASUREMENT METHODS FOR THE MARINE SAMPLES

The thickness of mature marine biofouling before and after treatment was measured at multiple sites on each tested plate using an Episcopic Differential Interference Contrast (EDIC) microscope. This microscope works by having the light source and DIC prisims above the sample, meaning it is possible to measure growth on solid surfaces. The depth of field on an EDIC microscope is very narrow and as a result it is possible to focus on the top of the biofouling, the sample or anywhere in between. What this allows is for a technique whereby focussing the microscope on the base of the sample and setting an origin, it is then possible to move the sample down until the top of the sample is in focus. The difference between the 2 points, which is measured by the microscope stage, is the thickness of the foulant at this point. As the biofouling spread is heterogenous over the surface of the sample it was necessary to measure the thickness at several points. Doing this for 10 points on every plate was sufficient to quantify statistically significant results.

2(B) WOUND TREATMENT METHOD

Both devices described in this paper transmit sound and microbubbles through water to the target. However, whereas the water for the marine biofoulant invention takes the form of a cushion, giving a few millimetres stand-off of the solid cleaner from the target, the second device increases the stand-off to several centimetres by using a water stream. This LAS (Liquid Acoustic Stream) device uses a gentle stream of saline,[25] flowing to the surface at around 2 litres/minute, with a stand-off distance of around 1 cm (Figure 3). This is so that the device can efficiently treat the varying topography of a wound bed, whilst

avoiding contact with the wound. By doing this, as opposed to immersing the wound in saline, the device can treat the wound *in situ*.

Until regulatory approvals are obtained to use the device on patients, the studies in this paper are performed on a recognised model for human skin used for *in vitro* wound studies. In this study, two types of wound model were used, pig trotters and human full thickness EpiDerm tissues, to demonstrate cleaning. Pig trotter wounds infected with *Pseudomonas aeruginosa* pMF230 were cleaned with LAS and compared to untreated control samples.

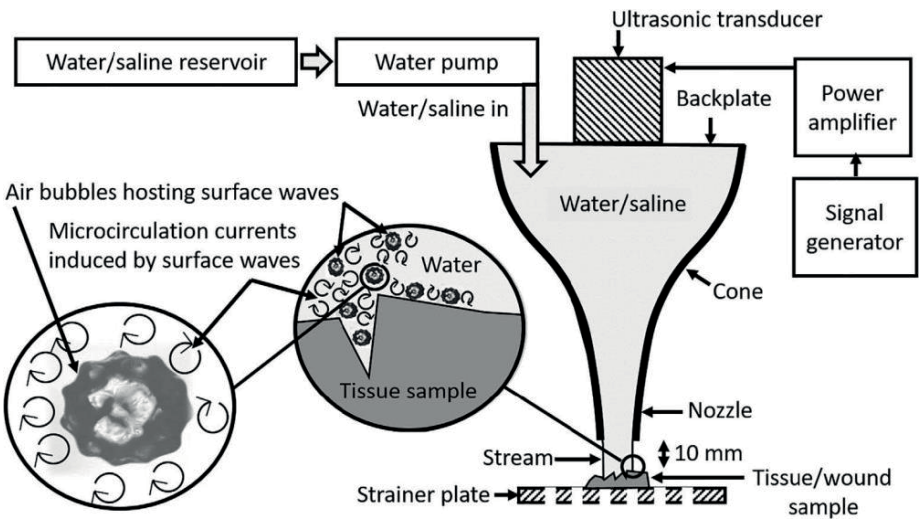

Figure 3. Schematic of liquid acoustic wound stream (LAS) system. Schematic diagram of the experimental set up for the LAS system cleaning a tissue or wound sample. The two inserts demonstrate the ultrasonically induced activity of the air bubbles that is associated with the cleaning effects of the LAS. The diagram is adapted from Malakoutikhah et al. 2020,[8] Chong et al. 2021[32] and Secker et al. 2022.[25]

It is well-recognised in clinical practice that if a wound is cleaned by removal of a biofilm, healing becomes more likely. However, one question of key importance is whether additional healing mechanisms can be stimulated during treatment, effectively enhancing the skin's own healing mechanisms that have stalled during the inflammatory stage within a chronic wound. To do this, the cleaning element must be removed from the LAS test,

since the aim was to observe whether there is any healing over and above that aided by the cleaning. To demonstrate healing, pig trotters are unsuitable, as they are dead and will no longer heal. Hence for the healing tests, pre-wounded reconstituted human epithelial tissues (EpiDerm Full Thickness, MatTek Corp., Ashland, Massachusetts) were wounded and kept in sterile conditions for 7 days. One set were untreated as a control, another set of alternative controls were treated with 0.9% saline only flowing through LAS (without the acoustics), and one set were subjected to LAS treatment. The cost of the EpiDerm Full Thickness samples meant that only triplicate repeats were affordable in this experiment.

After treatment, the EpiDerm Full Thickness samples were histologically processed and examined microscopically following Haematoxylin and Eosin (H&E) staining.

The main set-up for the LAS device, as used in the *in vitro* tests of this paper, is detailed in Figure 3, with full details of the experiment given in an earlier paper.[25] Confirmation that this device does not produce inertial cavitation on the target, was obtained through observation that it produced no sonoluminescence, no foil pitting, and no release of free iodine from KI solution.

Such stream technology had been used on a range of substrates prior to testing on this model of human skin. Previously, its 132 kHz ultrasound efficacy to remove contaminants from hard inert surfaces was shown for a range of applications, including cleaning baby equipment,[9] railway components[26]-[27]-[28], surgical instruments[29],[30] and tools,[26] bone prior to transplant[30] and pipework/packaging associated with food and beverages.[9,5,26,][31] Food itself has been cleaned without damage (including salad[32] and hay[33]), as have other soft targets including hands.[26]

Both hard (e.g. particulate[26-27-28]) and softer contaminants have been removed. Softer ones include glues,[26] greases[27] and lubricants[8], amyloid prion in brain tissue,[29,30] and biofilms (including those associated with dental[30,][34], marine[35] and gastronomic[32,33] surfaces). The effective tackling of biofilms using only sound, air and water meant that, unlike the use of conventional antimicrobial treatments (antibiotics, antivirals, antifungals etc.), the use of such technology should not so readily pro-

mote the rise of AntiMicrobial Resistance (AMR), which is projected to be killing more people than cancer by 2050, and have cost the world economy more than the current size of the global economy.[31,][36],[37]

3. RESULTS

3(A) HULL PLATE CLEANING RESULTS

Table 1 shows that, for each of the three materials tested (steel, aluminium and rubber), the treatment time of only 1 minute achieved a high percentage removal of the mature macroscopic biofouling.

Furthermore, Table 1 shows that for the steel and rubber plates the use of acoustic energy provided improved cleaning as compared to brushing. For aluminium, the biofouling thickness reductions were similar for ultrasonic and brush cleaning, except that the aluminium surface was significantly damaged, demonstrating scratches caused by the bristles of the brush (Figure 4). The brush was also mechanically damaged and required replacement. The rubber plate was also damaged by the bristles of the brush (Figure 5(a)), and the brush similarly damaged by the rubber (Figure 5(b)).

Table 1. Comparison of the effectiveness of removing macroscopic marine biofouling, from three hull materials. The results for the ultrasonic invention are compared with those from an electrically powered mechanically rotating brush.

	Percentage thickness reduction of mature biofoulant:	
	…as a result of ultrasonic treatment (+/- 1 standard deviation)	**…as a result of using a mechanically rotating brush device (+/- 1 standard deviation)**
Steel	91 (+/- 4)	40 (+/- 12)
Aluminium	94 (+/- 3)	97 (+/- 1)
Rubber	80 (+/- 1)	64 (+/- 1)

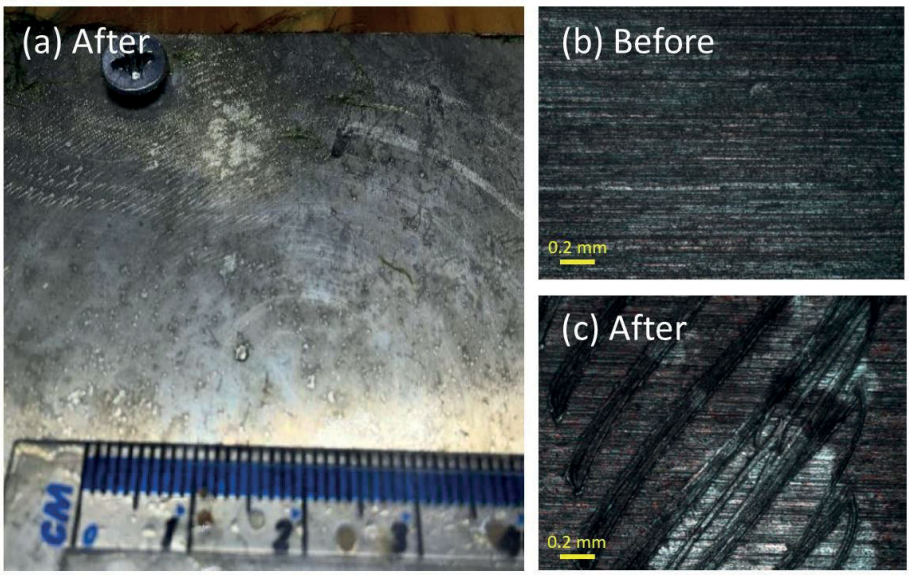

Figure 4. Photographs of surface damage to the aluminium caused by rotary brushing. Panels (a) and (c) show the condition of the aluminium surface after cleaning, at low and high magnification respectively. For comparison, panel (b) shows the aluminium surface before cleaning, at high magnification.

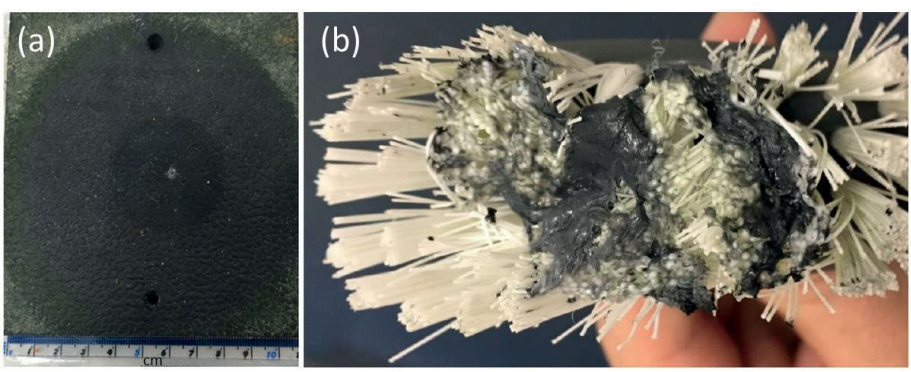

Figure 5. Photographs of (a) surface damage to the rubber caused by rotary brushing, and (b) the damage done to the brush by the cleaning undertaken in (a).

3(B) WOUND TREATMENT RESULTS

Figure 6 shows the results of cleaning biofilm of *Pseudomonas aeruginosa pMF230* from wounded pig trotters, after it had been cultured on the wound bed for 5 hours. Compared with the unwashed control wound beds, washing with saline alone had

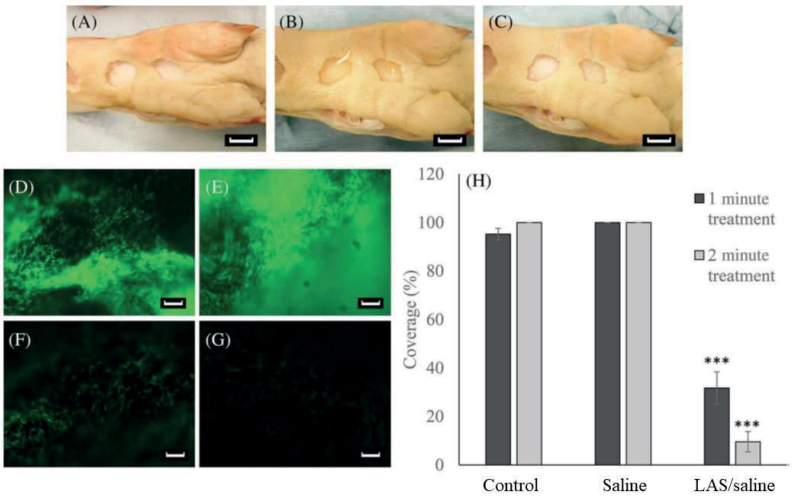

Figure 6. Pig trotter wound model. Example images of (A) _2 cm diameter wounds produced within frozen/thawed pig trotters before inoculation, (B) post inoculation of Pseudomonas aeruginosa pMF230 incubated at 37 Celsius for 5 hours, and (C) post 2 min liquid acoustic wound stream (LAS) treatment. Scale bars represent 2 cm. Representative episcopic differential interference contrast/EpiFluorescence (EDIC/EF) micrographs of green fluorescent protein (GFP)-tagged P. aeruginosa biofilms in (D) the control (untreated wounds), (E) after a 1 minute saline wash at a flow rate of 2 L/min, (F) after a 1 minute LAS treatment at a flow rate of 2 L/min, and (G) after a 2 minutes LAS treatment at a flow rate of 2 L/min. Scale bars represent 10 μm. Image analysis (H) of the EDIC/EF micrographs demonstrating the residual percentage coverage of GFP tagged P. aeruginosa pMF230 within the pig trotter wounds after 5 hour incubation at 37 Celsius: control (untreated wounds), after a 1 or 2 minutes saline wash at a flow rate of 2 L/min (saline) and after a 1 or 2 minutes LAS treatment at a flow rate of 2 L/min (LAS/saline). Error bars represent the SEM (n = 3), One-Way analysis of variance/Tukey post-hoc test demonstrated ***P ≤ .001 when compared with the untreated controls. Reproduced from Secker et al. 2022.[25]

no significant effect on the residual coverage of GFP tagged bacteria in the model. Washing with LAS for 1 minute reduced the coverage by 73% and washing for 2 minutes resulted in a 90% reduction.

Figure 7 shows the results of examination of the H&E-stained sections of uninfected wound models, which allowed measurement of the length of the tongue of reepithelialisation. The EpiDerm FT wound model is known to heal with the addition of hu-

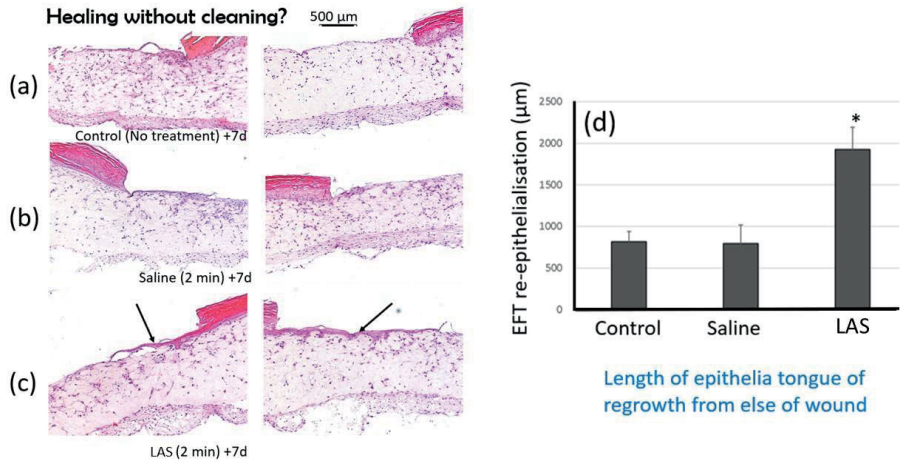

Figure 7. Epiderm full thickness (EFT) wound models that have been wounded (note there is only partial coverage of each sample by the upper (outer) layer), and presented here as Haematoxylin and Eosin (H&E) stained sections from the EFT wound models. The sections are 4 µm thick. The wounds were kept clean after wounding and imaged here after 7 days. The upper row (a) shows two control samples having no treatment. The middle row (b) shows two samples that were treated for 2 minutes after wounding by a saline stream run at 2 L/minute through the device, but without the sound activated. The lower row (c) is treated exactly the same as for row (b), but this time with the sound activated. The black arrows in the micrographs in row (c) highlight the re-epithelialisation tongue observed in these sections. Panel (d) shows data from image analysis measurements (E) of the extent of reepithelialisation 7 days post treatment are shown. Error bars represent the SEM (n = 3), One-Way analysis of variance/Tukey post hoc test demonstrated *P ≤ .05 when compared with the non-treated controls. Reproduced from Secker et al. 2022.25

man growth serum, and measurement of the length of the tongue is a method of quantifying healing within this model. There was no significant difference in tongue length between the control (no wash) and saline washed samples but the difference between the LAS treated models and the controls was significant (P ≤ .05). No acoustically-derived damage to the EpiDerm FT was seen in microscopic examination of the histological sections.

4. CONCLUSIONS

This paper introduced two inventions. The first is a marine hull cleaner to remove macroscopic biofouling. Having previously[1] tested the ability of the device to prevent the build-up of mature marine biofouling, the current study tested its ability to remove mature biofouling.

The data in Table 1 demonstrates that the method and apparatus of the present invention is highly effective at removal of mature biofouling. The removal by the ultrasonic device was significantly greater than removal observed by the rotating brush for the same treatment time, except for aluminium, where statically there was no difference in their cleaning performance.

After use, the brush was so badly damaged that it could no longer be used. Furthermore, the samples that were brushed showed scratches and damage to all three materials, particularly the rubber. No damage could be detected, either through visual or microscopic examination, of the samples that were cleaned using the ultrasonic device, and no damage was sustained to the ultrasonic device during these tests.

The second device was a wound cleaner, with a smaller contact footprint and a stand-off distance of 1 cm, to cope with the varying topography of a wound and the safety requirements of the patient and usability for the healthcare worker. It operates using only non-inertial cavitation to avoid damage to the wound bed. In addition to cleaning wounds, there was preliminary evidence that the device could stimulate wound healing over and above the beneficial treatment caused by cleaning.

ACKNOWLEDGMENTS

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