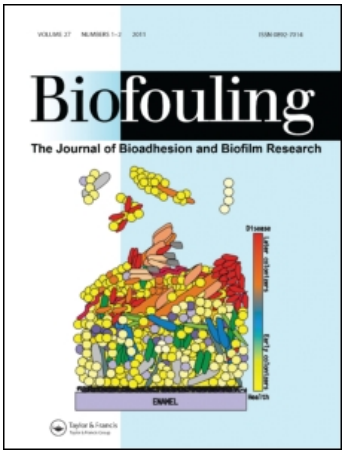

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Effect of ultrasound on cyprids and juvenile barnacles

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Settlement inhibition of barnacle (*Amphibalanus amphitrite*) cypris larvae resulting from exposure to ultrasound was measured at three frequencies (23, 63, and 102 kHz), applied at three acoustic pressure levels (9, 15, and 22 kPa) for exposure times of 30, 150, and 300 s. The lowest settlement was observed for 23 kHz, which also induced the highest cyprid mortality. Cyprid settlement following exposure to 23 kHz at 22 kPa for 30 s was reduced by a factor of two. Observing surface exploration by the cyprids revealed an altered behaviour following exposure to ultrasound: step length was increased, while step duration, walking pace, and the fraction of cyprids exploring the surface were significantly reduced with respect to control cyprids. The basal area of juvenile barnacles, metamorphosed from ultrasound-treated cyprids was initially smaller than unexposed individuals, but normalised over two weeks' growth. Thus, ultrasound exposure effectively reduced cyprid settlement, yet metamorphosed barnacles grew normally.

Keywords: ultrasound; barnacle cyprid; exploration behaviour; *Amphibalanus amphitrite*; marine fouling prevention; juvenile barnacle growth

Introduction

Marine fouling significantly increases global carbon emissions by increasing fuel demand and generating additional maintenance costs, which can run into billions of dollars annually for marine industries (Yebra et al. 2004; Schultz et al. 2010). Barnacles are among the most problematic macrofoulers, due to their size and gregarious colonisation of solid surfaces (Crisp and Meadows 1962). This incurs significant hydrodynamic drag and can potentially damage the protective coatings on steel hulls (Christie and Dalley 1987; Schultz 2007).

The lifecycle of the striped barnacle, *Amphibalanus amphitrite* (= *Balanus amphitrite* (Darwin 1854)), includes six planktonic naupliar stages, a non-feeding cypris larval stage, and a sessile adult stage. Cyprids explore solid surfaces and select a settling location, where the adult barnacle grows. Therefore, barnacle fouling prevention research often focuses on dissuading or inhibiting cyprid settlement (Chambers et al. 2006; Aldred and Clare 2008), which is considered to be the key to preventing surface colonisation by this organism.

Cyprids are capable of swimming, which brings them into contact with solid substrata. They perform a tactile exploration of solid surfaces by forming temporary anchoring points with their antennules. This adhesion is mediated by secreted footprint protein (Phang et al. 2009). Cyprids integrate their surface sensing with environmental cues in determining the final settlement choice (Aldred and Clare 2008). Several studies have measured cyprid exploration behaviour, correlating it with their settlement preferences. Berntsson et al. (2000) showed that cyprids spend more time exploring smooth surfaces and tend to swim when exposed to micro-textured surfaces. The Etho Vision 3.0 software (Marechal et al. 2004) was developed to quantify total exploration time and the total distance covered by cyprids during their substratum exploration. Chaw and Birch (2009) developed a complementary fine scale microscopic observation of cyprid exploration, thus implementing a behavioural assay that quantifies the step length and step duration. This study revealed that cyprids explore hydrophilic surfaces using longer steps of shorter duration and correlated this behaviour with a higher settling affinity on hydrophilic *vs* hydrophobic surfaces.

Several approaches have been explored for preventing barnacle cyprid settlement. While biocides are highly effective (Rudolf et al. 1997; Billinghurst et al. 1998; Kem et al. 2003), they are generally damaging to the environment and are consequently subject to regulations limiting their widespread implementation.

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Perez et al. (2008) introduced a promising strategy, which avoids the use of biocides by using a localised, pulsed electrical field to efficiently inhibit cyprid settlement. Mechanical forces generated by acoustic waves have been shown to be effective for inhibiting marine fouling. Branscomb and Rittschof (1984) reported that low frequency (30 Hz) sound waves reduce barnacle settlement, while Donskoy and Ludyanskiy (1995) describe exposure to low acoustic frequencies (<200 Hz) preventing the settlement of juvenile and adult mussels. However, these frequencies fall within the audible spectrum of humans and may thus generate noise pollution, limiting their application. Ultrasound frequencies >20 kHz, which are beyond the human audible range, may offer a more widely accepted solution.

Although laboratory studies have shown that frequencies in the order of tens of kHz efficiently kill barnacle larvae (Mori et al. 1969; Suzuki and Konno 1970), these do not report their influence on barnacle settlement. Seth et al. (2010) used ultrasound cavitation to pulverise barnacle nauplii. The ultrasound energy was estimated by calorimetric absorption, without allowing for potential differences in energy adsorbed by cyprids *vs* their aqueous environment. Kitamura et al. (1995) probed the effect of ultrasound on naupliar mortality and cyprid settlement, exploring three frequencies and using specific acoustic pressure levels. Total irradiation (kPa.s) was used to represent the acoustic energy, but the study did not specify the acoustic pressure experienced by the cyprids. Fischer et al. (1981) found that devices emitting frequencies in the 20–100 kHz range maintained a surface free of colonisation by macroscopic marine organisms. More recently, Shipsonic (Netherlands) and Ultrasonic Antifouling (UK) have commercialised ultrasound-based products, which are marketed for marine fouling prevention on berthed pleasure craft. While these are presumed effective, no information is available on the frequency and acoustic pressure needed to generate marine fouling prevention.

Although ultrasound is a promising marine fouling prevention tool, the scarcity of information regarding the required power and exposure time range limits its effective implementation. The application of ultrasound in marine fouling prevention will benefit from a better understanding of its impact on cyprid mortality and the inhibition of barnacle settlement. Therefore, a comprehensive and systematic study of the influence of frequency, acoustic pressure and exposure time on cyprid settlement, in combination with a cyprid exploration behavioural assay, is needed to provide essential information for the application of ultrasound in marine fouling prevention. The present study examines the efficiency of three frequencies (23, 63, and 102 kHz), and the influence of exposure time and acoustic pressure on barnacle cyprid settlement. Moreover, it quantifies the effect of ultrasound exposure on cyprid mortality and cyprid exploration behaviour. In addition, it explores the growth of juvenile barnacles, which were metamorphosed from ultrasound-treated cyprids.

Materials and methods

Cyprid culture

Barnacle larvae were reared at the marine laboratory of the Tropical Marine Science Institute (TMSI), National University of Singapore. Adult *A. amphitrite*, collected from Kranji mangroves, Singapore, were kept in running seawater at 25–30°C in an open circulating marine aquarium and fed daily with freshly hatched *Artemia*. Larvae spawned from the adults were reared on an algal mixture of 1:1 v/v of *Tetraselmis suecica* and *Chaetoceros muelleri* at 25°C, at a density of approximate 5×10^5 cells ml^{-1} (Rittschof et al. 2003). The seawater and algae were replaced every 2 days to ensure an adequate food supply. Barnacle larvae developed into the cyprid stage within 5–7 days. The cyprids were stored at 4°C and used for experiments after 3 days. Cyprids were acclimated to room temperature for 30 min before initiation of the experiment.

Ultrasonic irradiation system

A diagram of the experimental setup is shown in Figure 1. A sinusoidal wave from a function generator (Agilent 33210A, USA) was fed into a power amplifier (HSA4051, Japan). This signal was used to drive an 80 mm ceramic piezoelectric transducer (Fuji Ceramics, Japan) at selected resonant frequencies of 23, 63, and 102 kHz. The transducer rested on the bottom of a

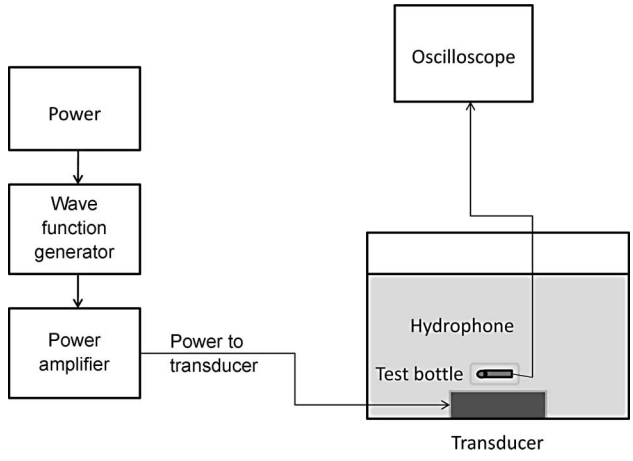

Figure 1. Schematic diagram of the ultrasound system.

transparent acrylic water tank with its emission axis pointing vertically upwards.

An open polyethylene snap-cap vial (Ref. 18 09 0906, Alpha analytical, Singapore) was placed with its axis horizontal at a height of 20 mm above the transducer. The acoustic pressure inside the vial was measured using a hydrophone (GRAS 10-CT, G.R.A.S Sound & Vibration, Denmark) and adjusted to the desired value. The vial was then removed and rinsed with filtered seawater (FSW, passed through 5 and 1 μm double open filter cartridges: Cole-Palmer, 255482-43 and 255481-43, respectively). Sixty to seventy cyprids in 10 ml FSW were then placed in the vial, which was capped and re-positioned at the same location above the transducer.

Cyprid settlement and mortality assay

Changes in cyprid settlement due to the ultrasound were studied under different acoustic pressures and exposure times using three ultrasound frequencies, viz. 23, 63, and 102 kHz. Acoustic pressures of 9, 15, and 22 kPa were used for an exposure time of 30 s to probe the effect of ultrasound pressure on settlement. To assess the effect of exposure time, cyprids were exposed to an acoustic pressure of 20 kPa for 30, 150, and 300 s. An acoustic pressure of 20 kPa was selected for these assays on the basis that this pressure level would be high enough to elicit a response from the cyprids, but not so high as to be the dominant factor. After treatment with ultrasound, the exposed and control (untreated) cyprids were incubated in their respective capped vials for 24 h at 28°C, on a 15:9-h light/dark cycle. All settled cyprids were counted, including those that were permanently attached, but not metamorphosed, and fully metamorphosed barnacles.

Cyprid mortality resulting from ultrasound exposure was quantified. In this assay, 50–70 cyprids were transferred to FSW-filled polyethylene snap-cap vials and exposed to three frequencies, viz. 22, 63 and 102 kHz for exposure times of 30, 150 and 300 s, at a fixed acoustic pressure of 20 kPa. After 24 h, the vials were emptied into a counting tray and dead cyprids were scored as described in Kem et al. (2003). The mortality of the control cyprids (not exposed to ultrasound) was recorded for comparison. Both settlement and mortality assays were conducted in triplicate.

Cyprid exploration behaviour assay

Cyprid exploration behaviour tests were performed on ultrasound-treated cyprids and control cyprids. As no significant cyprid settlement differences were observed following exposure to 63 and 102 kHz ultrasound (see Results below), the cyprid exploration behaviour assays were conducted with 23 and 102 kHz, which represent the lowest and highest frequencies, respectively. An acoustic pressure of 20 kPa was applied to separate batches of cyprids for times of 30 and 300 s' duration. These were then transferred to a FSW-filled polycarbonate multi-well plate (Nalge Nunc International, USA) and the behaviour of 15–20 individual cyprids for each condition was recorded using the imaging method described in Chaw and Birch (2009). As above, the behaviour of control cyprids was measured in the same way. After a 2 min acclimation period, the observation and recording time was set to 5 min (Marechal et al. 2004). Image analysis was used to determine the step length, defined as the distance between two sequential temporary anchoring points, and the step duration, defined as the time elapsed from the detaching of a trailing antennule to its reattachment, forming a new temporary anchoring point. Step length and step duration data were pooled for all cyprids within each condition (Chaw and Birch 2009). The cyprid walking pace was defined as the number of steps taken by cyprids in 5 min.

The cyprid exploration rate was calculated as a ratio of the exploring cyprids to the total number of cyprids. A microscope (IX51, Olympus, Japan) was used at low magnification ($\times 40$) to generate a field of view covering one well of the plate. After adding 50–60 cyprids to each well and allowing 2 min for acclimation, the number of exploring cyprids was counted over a period of 5 min. Cyprids exploring the surface were scored only once, even if they resumed surface exploration after swimming.

Barnacle growth assay

To examine whether cyprid exposure to ultrasound compromises the growth of metamorphosed barnacles, these were cultured in the laboratory and their growth compared with that of barnacles developed from control cyprids (not exposed to ultrasound). Cyprids treated with frequencies of 23, 63, and 102 kHz for 300 s at an acoustic pressure of 20 kPa, respectively, were transferred to Petri dishes (Alpha analytical, Singapore), where they were allowed to settle. Metamorphosed juvenile barnacles were maintained in an aquarium with running seawater, with supplemental feeding using the same algal mixture as for the culture of nauplii for 3 h per day. The underside of 20–25 juvenile barnacles within each Petri dish was photographed every 2 days over a 2-week period (Skinner et al. 2007) using a digital camera (Olympus E330, Japan) attached to a microscope (Olympus SZX 10, Japan). The images were processed with ImageJ software (Version 1.43), which used edge contrast to define the perimeter and thereby calculate the total basal area.

Statistical analysis

All statistical comparisons were performed using GraphPad Prism 5 (GraphPad Software Inc.). Two main factors were considered in cyprid settlement, mortality, and exploration behavioural assays, viz. ultrasound frequency and acoustic pressure, or exposure time and frequency. Data were analysed with a two-way analysis of variance (ANOVA) to evaluate the significant influence of these parameters. This was followed by a one-way ANOVA together with a Tukey *post* test to determine differences between treated and control cyprids. Barnacle growth rate data were analysed with a one-way ANOVA, followed by a Tukey *post* test. All data are reported as mean $\pm$ standard error (SE). For all comparisons, p-values ≤ 0.05 were considered as statistically significant.

Results

Cyprid settlement assay

Ultrasound exposure significantly reduced cyprid settlement compared to untreated control cyprids (one way ANOVA, $p < 0.05$). Tukey pair-wise comparisons revealed that all ultrasound treatments differed significantly from the untreated control ($p < 0.05$), as shown in Figure 2. Significant differences were also found among ultrasound frequencies (two-way ANOVA, $p < 0.05$; Figure 2). The number of settled cyprids was lowest for cyprids exposed to 23 kHz (Tukey test, $p < 0.05$), with no significant difference between 63 and 102 kHz at equal exposure times (Tukey test, $p > 0.05$).

Settlement differed significantly with acoustic pressure levels (two-way ANOVA, $p = 0.0008$), with higher acoustic pressure leading to lower settlement (Tukey test, $p < 0.05$, Figure 2A). At 23 kHz, an acoustic pressure of 22 kPa applied for 30 s reduced settlement by a factor of two (Figure 2A). Different settlement was similarly observed with exposure times (two-way ANOVA, $p = 0.001$). Settlement decreased with ultrasound exposure and was lower than for control cyprids (Tukey test, $p < 0.05$, Figure 2B).

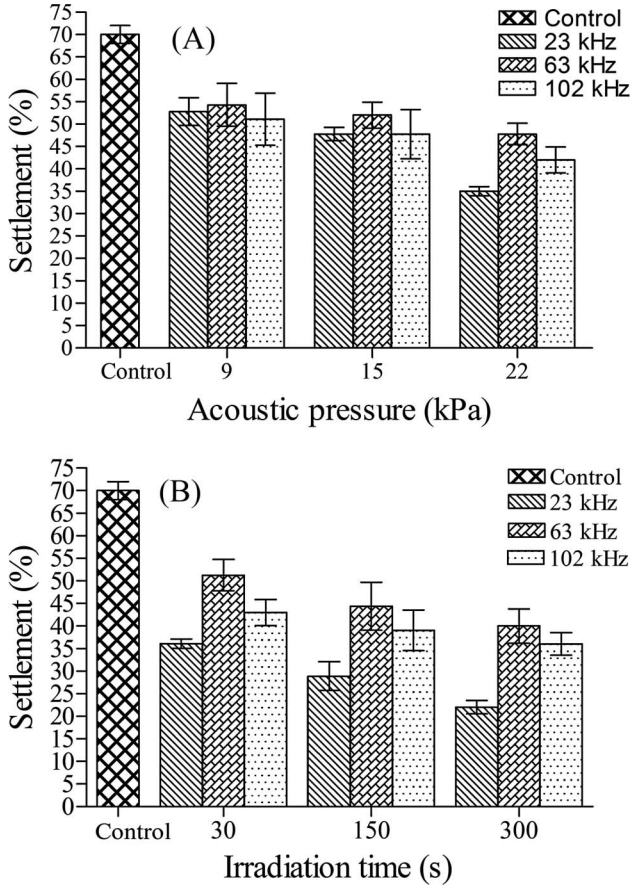

Figure 2. The effect of ultrasound exposure on cyprid settlement. (A) Tested acoustic pressures of 9, 15, and 22 kPa for an exposure time of 30 s. (B) Exposure time of 30, 150, and 300 s at a pressure of 20 kPa.

Cyprid mortality

Mortality assays showed that exposure to ultrasound increased cyprid mortality (one-way ANOVA, $p < 0.05$), with both exposure time and frequency having a significant effect (two-way ANOVA, $p < 0.05$, Figure 3). For 63 and 102 kHz, a significant increase in mortality was not recorded for 30 and 150 s exposures (Tukey test, $p > 0.05$). Increased mortality was observed for 300 s exposure (Tukey test, $p < 0.05$) and no differences were observed in mortality induced by 63 and 102 kHz (Tukey tests, $p > 0.05$). The highest mortality rates were induced by 23 kHz (Tukey test, $p < 0.05$) and its effect increased with exposure time

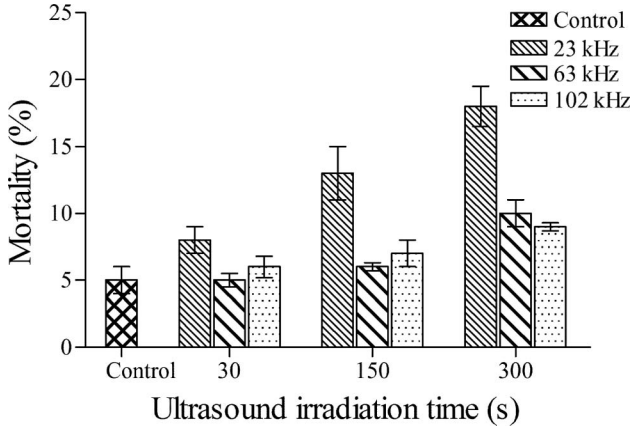

Figure 3. The effect of ultrasound exposure on cyprid mortality at an acoustic pressure of 20 kPa.

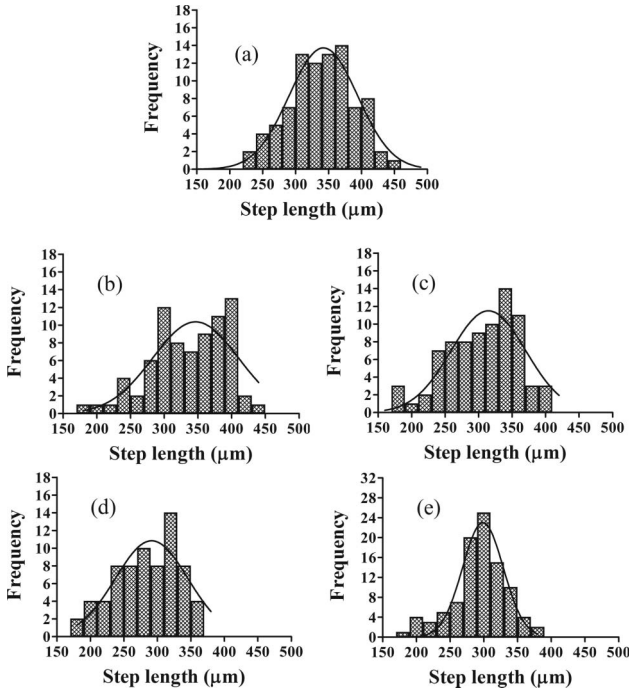

Figure 4. Histograms of step length data for: (a) untreated control cyprids; (b) and (c): cyprids exposed to 23 kHz for 30 and 300 s, respectively; (d) and (e): cyprids exposed to 102 kHz for 30 and 300 s, respectively. Ultrasound was applied at an acoustic pressure of 20 kPa.

Table 1. Step length for control cyprids and cyprids exposed to an ultrasound pressure of 20 kPa.

Ultrasound treatments	Average step length ± SE(μm)
Control	341 ± 5
23 KHz for 30 s	300 ± 6
23 KHz for 300 s	305 ± 6
102 KHz for 30 s	288 ± 8
102 KHz for 300 s	291 ± 4

(Tukey test, $p < 0.05$). When applied for 300 s at an acoustic pressure of 20 kPa, 23 kHz led to a three-fold increase in cyprid mortality (Figure 3).

Cyprid exploration behaviour assay

For cyprids exposed to ultrasound, both step length and step duration data yielded a distinguishable response. Figure 4 shows histograms of control and ultrasound-treated cyprids and Table 1 reports mean ± SE values extracted from these data. Step duration, exploration rate, and walking pace are summarised in the histograms shown in Figure 5.

Cyprid exploration behaviour was significantly affected by ultrasound frequency (two-way ANOVA, $p < 0.01$). From Figure 4, step lengths <230 μm were

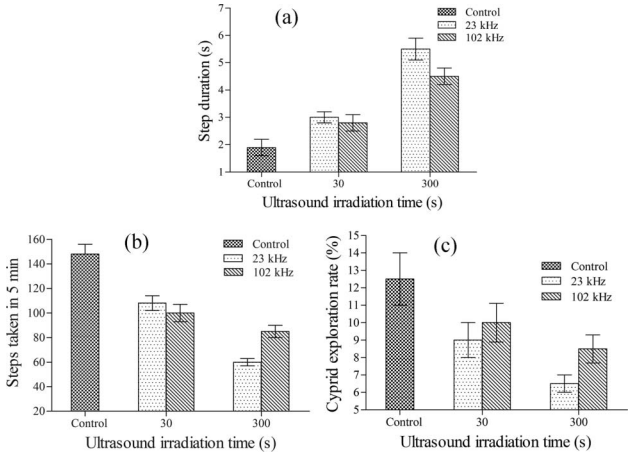

Figure 5. Histograms of cyprid behaviour data for: (a) Step duration; (b) Walking pace (c) Exploration rate. Ultrasound was applied at an acoustic pressure of 20 kPa.

not observed on control cyprids, whilst step lengths <170 μm were obtained for ultrasound treated cyprids. From Table 1, the mean step length was significantly reduced for cyprids exposed to ultrasound (one-way ANOVA, $p < 0.05$). However, there was no significant difference in the step lengths of cyprids subjected to 23 or 102 kHz ultrasound (Tukey test, $p > 0.05$).

Step duration increased with ultrasound exposure time (two-way ANOVA, $p < 0.0001$; Figure 5a), with insignificant differences between frequencies with exposure times of 30 s (Tukey, $p > 0.05$). For 300 s' exposure, 23 kHz increased step duration significantly more than 102 KHz (Tukey test, $p < 0.05$). Ultrasound exposure also diminished the cyprid walking pace (two-way ANOVA, $p < 0.0001$; Figure 5b). This reduction followed a similar trend to the step duration changes: the influence of 23 and 102 kHz was indistinguishable for 30 s exposure and 23 kHz was more effective than 102 kHz, when ultrasound was applied for 300 s (Tukey test, $p < 0.05$, Figure 5b). The cyprids' exploration rate decreased with ultrasound exposure time (two-way ANOVA, $p = 0.0074$; Figure 5c) and followed the same trend: 23 and 102 kHz generated an indistinguishable effect at 30 s exposure time, while 23 kHz generated a more substantial reduction than 102 kHz at 300 s exposure time (Tukey test, $p > 0.05$).

Barnacle growth assay

Barnacles newly metamorphosed from ultrasound-treated cyprids initially showed smaller basal areas than those metamorphosed from control, untreated cyprids (one-way ANOVA, $p < 0.05$, Figure 6). There were no differences in size between the newly

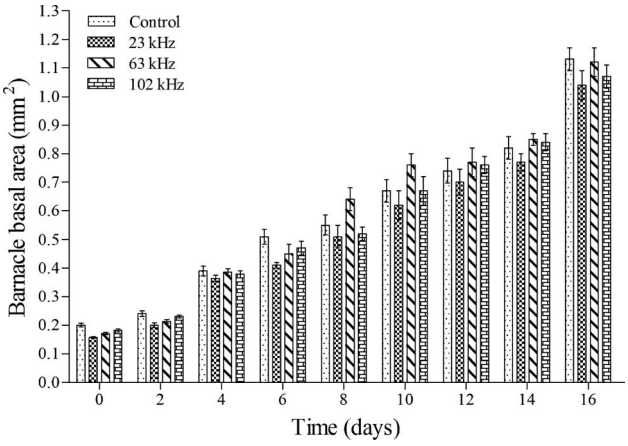

Figure 6. Growth of juvenile barnacles, metamorphosed from untreated control cyprids and cyprids exposed to ultrasound frequencies of 23, 63, and 102 kHz, for 300 s with an acoustic pressure of 20 kPa.

metamorphosed adults obtained from cyprids exposed to 23, 63, and 102 kHz ultrasound frequencies, all applied at 20 kPa for 300 s (Tukey test, $p > 0.05$). For all barnacles, the basal area increased with time. After culture for 10 days, the differences in basal area between ultrasound-treated and control barnacles diminished and were no longer significant (Tukey test, $p > 0.05$).

Discussion

Cyprids exposed to ultrasound exhibited lower settlement rates and higher mortality. Settlement inhibition was enhanced with increasing acoustic pressure and longer exposure times, which suggests a progressive degradation in the condition of the cyprids, although no obvious visible damage was observed. Of the ultrasound frequencies used, 23 kHz was most effective, with 63 and 102 kHz generating a similar, lower response. Mortality did not increase significantly when cyprids were exposed to 63 or 102 kHz for up to 150 s and it only increased moderately for cyprids exposed to these frequencies over 300 s. Interestingly, settlement rates for these two frequencies were reduced significantly for the different exposure times but did not induce higher mortality. The most effective configuration was obtained with application of 23 kHz at 20 kPa for 300 s, which reduced cyprid settlement by a factor of two and induced a three-fold increase in their mortality. Kitamura et al. (1995) reported a similar increased efficacy of lower ultrasound frequencies, in the region of 20 kHz, and that the impact of ultrasound increased with total irradiation. Their study also confirms the lethal effect of ultrasound exposure, with an enhanced impact at 19.5 kHz. These results are consistent with the data reported in this study and other similar work by Mori et al. (1969) and Suzuki and Konno (1970).

Ultrasound treatment presents several benefits over chemical-based biocidal strategies. Biocide-based antifouling coatings function through the gradual release of molecules from the surface of the coating, a portion of which may accumulate in, and adversely affect, the marine environment (Chambers et al. 2006). In contrast, ultrasound exposure does not engender a cumulative effect, as molecules are not released into the marine environment. Ultrasound as a method of fouling prevention confers additional advantages. It can be applied at will in a highly controlled manner, as opposed to biocides that are released continuously based on the chemistry of the coating (Chambers et al. 2006). For example, ultrasound could be applied in a regimented pulsed fashion, as has been described for electric fields (Perez et al. 2008), or could be turned on while in port and turned off once a ship has reached cruising speed, thus potentially saving power and other resources. Moreover, ultrasound may be conveniently applied to surfaces with low liquid shear forces (eg low-flow areas of the hull and the sea chest), where fouling-release non-stick coatings have limited effectiveness. Undoubtedly, a thorough assessment of the effect of ultrasound on the marine environment would be prudent before its widespread implementation. With an optimised engineering design, its implementation will ideally be confined to surface treatment, while limiting the propagation of sound waves into the marine environment.

The decreased settlement and increased mortality observed following exposure to ultrasound is likely a result of physical injury to the cyprid. Ultrasound pressure fluctuations are efficiently transmitted through liquids and can dissipate their energy in biological tissues, which can be altered and damaged by this process (Brondum et al. 1998). High intensity ultrasound, which develops significant levels of cavitation, can be used to disintegrate barnacle larvae (Seth et al. 2010). Cavitation induced by ultrasound has been cited as responsible for compromising the viability of several organisms, including bacteria and algae growing on solid substrata (Scherba et al. 1991; Lagsir et al. 2000; Liang et al. 2009). Cavitation is followed by implosion, which generates high liquid shear forces. Microstreaming, induced by gas bubbles generated during cavitation, is also capable of injuring cells (Suslick 1988). These liquid shear forces can damage organisms. Given that cavitation threshold generally increases with ultrasound frequency (Hao et al. 2004; Ma et al. 2005; Kratochvíl and Mornstein 2006), this may explain the enhanced efficiency of 23 kHz for compromising cyprid viability. Further exploration of the ultrasound-induced physical damage is expected to shed light on the

causes underlying the reduction in cyprid settlement and the increased mortality.

Interestingly, exposing cyprids to ultrasound also alters their subsequent surface exploration behaviour. Given the similar response generated by 63 and 102 kHz, only 23 and 102 kHz frequencies were used in these experiments. While the step length was changed significantly following ultrasound exposure, significant differences in step length were not observed either between 23 and 102 kHz or when the exposure time was increased from 30 to 300 s (Table 1). In contrast, both frequency and exposure time generated significant changes in step duration, walking pace, and exploration rate. Following ultrasound exposure, step duration increased and both walking pace and exploration decreased. All three parameters followed similar trends: the influence of ultrasound increased with exposure time, with 23 and 102 kHz applied for 30 s generating the same change, while 23 kHz was more effective than 102 kHz, when applied over a period of 300 s (Figure 5). This increasing change in the exploration behaviour with ultrasound exposure may be indicative of a physiological change in the condition of the cyprids. As for the settlement and mortality assays, 23 kHz generated the largest impact.

A previous study by Chaw and Birch (2009) observed that when cyprids explore a surface that is less favoured for settlement, they decrease their step length and increase their step duration, which may be considered as performing a more detailed and prolonged inspection of the surface. Cyprids exposed to ultrasound, which compromises their viability, also decreased their step length and increased their step duration when exploring the same surface. However, the cause and mechanism leading to this change in behaviour may be different. Specifically, a change in surface properties cannot directly be compared with ultrasound-induced damage to the cyprids' condition. With the possibility that the exploration behaviour on a less favoured surface was caused by weakened temporary adhesion points, the likelihood of ultrasound exposure weakening cyprid adhesion to the surface can be surmised. However, no adhesion data have been gathered from cyprid behaviour assays to validate this hypothesis. With respect to settlement, the reduced exploration rate and walking pace exhibited by cyprids exposed to ultrasound generate fewer temporary anchoring points in a given time. This behaviour may lead to a lower surface density of footprint protein, which acts as a settlement-inducing cue (Aldred et al. 2008; Aldred and Clare 2008).

Metamorphosis of cyprids exposed to ultrasound yields barnacles with a smaller basal area than those from control cyprids. Exposure to ultrasound has been shown capable of accelerating or inhibiting the growth of organisms (Matsuura et al. 1994; Ahn et al. 2003; Pitt and Ross 2003). As the juvenile barnacles grew over 2 weeks, with regular feeding, the size of barnacles of the two populations converged (Figure 6). Thus, despite a clear reduction in cyprid viability following ultrasound exposure, barnacles metamorphosed from these larvae were able to compensate and grow normally.

Ultrasound could therefore be applicable for barnacle fouling prevention by using an efficient transducer, which can generate an acoustic pressure of about 20 kPa at a frequency just above the audible threshold of 20 kHz. As illustrated in the present study, even short exposure times were efficient in reducing cyprid settlement, by half at 30 s and by one-third after exposure for 5 min.

Conclusions

Exposure to ultrasound significantly affected barnacle cyprid settlement, viability, and exploration behaviour. Both the highest efficacy against settlement and the highest mortality were shown for 23 kHz. When exposed to ultrasound at 23 kHz for 300 s at a pressure of 20 kPa settlement was reduced by a factor of two and mortality was increased by a factor of three. These changes are probably a consequence of the organisms sustaining physical damage, presumably induced by cavitation and the liquid shear forces it generates. Juvenile barnacles, metamorphosed from cyprids exposed to ultrasound, grew normally. Further studies are needed to elucidate any damage induced by ultrasound to the cyprid's carapace and its antennules, including their sensory organs and attachment discs. These may shed light on the mechanisms responsible for reduced cyprid viability and settlement inhibition. The use of ultrasound shows significant promise as a fouling prevention technology, particularly as a replacement for biocidal coatings on surfaces with low shear flow. Incorporating a proper understanding of the mechanisms of action into engineering design should allow optimisation of the ultrasound application, while limiting any unwanted environmental effects. This could result in a practical and environmentally-safe antifouling prevention strategy.

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References

Ahn CY, Park MH, Young SH, Kim HS, Jang KY, Oh HM. 2003. Growth inhibition of Cyanobacteria by ultrasonic radiation: laboratory and enclosure studies. Environ Sci Technol 37:3031–3037.

Aldred N, Clare AS. 2008. The adhesive strategies of cyprids and development of barnacle-resistant marine coatings. Biofouling 24:351–363.

Aldred N, Phang IY, Conlan SL, Clare AS, Vancso GJ. 2008. The effects of a serine protease, Alcalase, on the adhesives of barnacle cyprids (*Balanus amphitrite*). Biofouling 24:97–10.

Billinghurst Z, Clare AS, Fileman T, Mcevoy J, Radman J. 1998. Inhibition of barnacle settlement by the environmental oestrogen 4-nonylphenol and the natural oestrogen. Mar Pollut Bull 36:833–839.

Berntsson KM, Jonsson PR, Lejhall M, Gatenholm P. 2000. Analysis of behavioral rejection of micro-textured surfaces an implication for recruitment by the barnacle *Balanus improvises*. J Exp Mar Biol Ecol 251:59–83.

Branscomb ES, Rittschof D. 1984. An investigation of low frequency sound waves as a means of inhibiting barnacle settlement. J Exp Mar Biol Ecol 79:149–154.

Brondum J, Egebo M, Agerskov C, Busk H. 1998. Online pork carcass grading with the auto-form ultrasound system. J Anim Sci 76:1859–1868.

Chambers LD, Stokes KR, Walsh FC, Wood RJK. 2006. Modern approaches to marine antifouling coatings. Surf Coat Technol 201:3642–3652.

Chaw KC, Birch WR. 2009. Quantifying the exploratory behaviour of *Amphibalanus amphitrite* cyprids. Biofouling 25:611–619.

Christie AO, Dalley R. 1987. Adhesion in barnacles. Barnacle Biol 5:419–433.

Crisp DJ, Meadows PS. 1962. The chemical basis of gregariousness in cirripedes. Proc R Soc Lond B 156: 500–520.

Donskoy DM, Ludyanskiy ML. 1995. Low frequency sound as a control measure for zebra mussel fouling. In: Proceedings of the 5th International Zebra Mussel and Other Aquatic Nuisance Organisms Conference, Toronto, Canada, February 1995. Available *via* the National Invasive Species Information Center, Beltsville (MD). p. 103–112.

Fischer EC, Castelli VJ, Rodgers SD, Bleile HR. 1981. Marine biodeterioration: an interdiciplinary study. Annapolis (MD): Naval Institute Press. p. 261.

Hao HW, Wu MS, Chen YF, Tang JW, Wu QY. 2004. Cavitation mechanism in cyanobacterial growth inhibition by ultrasonic exposure. Colloids Surface B 33: 151–156.

Kem WR, Soti F, Rittschof D. 2003. Inhibition of barnacle larval settlement and crustacean toxicity of some hoplonemertine pyridyl alkaloids. Biomol Eng 20: 355–361.

Kitamura H, Takahashi K. 1995. Inhibitory effect of ultrasound waves on the larval settlement of the barnacle, *Balanus amphitrite* in the laboratory. Mar Fouling 12:9–13.

Kratochvíl B, Mornstein V. 2006. Monitoring the effects of cavitation ultrasound on *Artemia salina* larvae. Scripta Med 79:3–8.

Lagsir NO, Gros AM, Boistier E, Blum LJ, Bonneau M. 2000. The development of an ultrasonic apparatus for the noninvasive and repeatable removal of fouling in food processing equipment. Lett Appl Microbiol 30:47–52.

Liang H, Nan J, He WJ, Li GB. 2009. Algae removal by ultrasonic exposure-coagulation. Desalination 23:191–197.

Ma BZ, Chen YF, Hao HW, Wu MS, Wang B, Lv HG, Zhang GM. 2005. Influence of ultrasonic field on microcystins produced by bloom-forming algae. Colloids Surf 41:197–201.

Marechal JP, Clare H, Sebire M, Clare AS. 2004. Settlement behaviour of marine invertebrate larvae measured by EthoVision 3.0. Biofouling 20:211–217.

Matsuura K, Hirotsune M, Nunokawa Y, Sotoh M, Honda K. 1994. Acceleration of cell growth and ester formation by ultrasonic wave irradiation. J Ferment Bioeng 77:36–40.

Mori ET, Yamaguchi Y, Nishikawa A. 1969. The antifouling effect of ultrasonic waves on hulls. Japan: Mitsubishi Heavy Industries. Technical Review 6:1–9.

Perez RE, Anderson MA, Rittschof D, Orihuela B, Wendt D, Kowalke GL, Noguera DR. 2008. Inhibition of barnacle (*Amphibalanus amphitrite*) cyprid settlement by means if localized, pulsed electric fields. Biofouling 24:177–184.

Phang IY, Chaw KC, Choo SSK, Kang RKC, Lee SSC, Birch WR, Teo SMT, Vancso GJ. 2009. Marine biofouling field tests, settlement assay and footprint micromorphology of cyprid larvae of *Balanus amphitrite* on model surfaces. Biofouling 25:139–147.

Pitt WG, Ross SA. 2003. Ultrasound increases the rate of bacterial cell growth. Biotechnol Prog 19:1038–1044.

Rittschof D, Lai CH, Kok LM, Teo SLM. 2003. Pharmaceuticals as antifoulants: concept and principles. Biofouling 19:207–212.

Rudolf SS, Paul KS, Zhou BS. 1997. A settlement inhibition assay with cyprid larvae of the barnacle *Balanus amphitrite*. Bull Environ Contam Toxicol 35:1867–1874.

Scherba G, Weigel RM, O'Brien Jr WD. 1991. Quantitative assessment of the germicidal efficacy of ultrasonic energy. Appl Environ Microbiol 57:2079–2084.

Schultz MP. 2007. Effects of coating roughness and biofouling on ship resistance and powering. Biofouling 23:331–341.

Schultz MP, Bendick JA, Holm ER, Hertel WM. 2010. Economic impact of biofouling on a naval surface ship. Biofouling 27:87–98.

Seth NS, Chakravarty P, Khandeparker L, Anil AC, Pandit AB. 2010. Quantification of the energy required for the destruction of *Balanus amphitrite* larva by ultrasonic treatment. J Mar Biol Assoc UK 90:1475–1482.

Skinner LF, Siviero FN, Coutinho R. 2007. Comparative growth of the intertidal barnacle *Tetralita stalactifera* (Thoracica: Tetraclitidae) in sites influenced by upwelling and tropical conditions at the Cabo Frio region, Brazil. J Tropical Biol 55:71–78.

Suslick KS. 1988. Ultrasound: its chemical, physical, and biological effects. New York (NY): VCH Publishers Inc. p. 287–303.

Suzuki H, Konno K. 1970. Basic studies on the antifouling by ultrasonic waves for ship's bottom fouling organisms. J Tokyo Univ Fish 56:31–48.

Yebra DM, Kiil S, Dam-Johansen K. 2004. Antifouling technology – past, present and future steps towards efficient and environmentally friendly antifouling coatings. Prog Org Coat 50:75–104.