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Original Article

Acoustic disturbance in blue mussels: sound-induced valve closure varies with pulse train speed but does not affect phytoplankton clearance rate

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Anthropogenic sound has increasingly become part of the marine soundscape and may negatively affect animals across all taxa. Invertebrates, including bivalves, received limited attention even though they make up a significant part of the marine biomass and are very important for higher trophic levels. Behavioural studies are critical to evaluate individual and potentially population-level impacts of noise and can be used to compare the effects of different sounds. In the current study, we examined the effect of impulsive sounds with different pulse rates on the valve gape behaviour and phytoplankton clearance rate of blue mussels (*Mytilus* spp.). We monitored the mussels' valve gape using an electromagnetic valve gape monitor and their clearance rate using spectrophotometry of phytoplankton densities in the water. We found that the mussels' valve gape was positively correlated with their clearance rate, but the sound exposure did not significantly affect the clearance rate or reduce the valve gape of the mussels. They did close their valves upon the onset of a pulse train, but the majority of the individuals recovered to pre-exposure valve gape levels during the exposure. Individuals that were exposed to faster pulse trains returned to their baseline valve gape faster. Our results show that different sound exposures can affect animals differently, which should be taken into account for noise pollution impact assessments and mitigation measures.

Keywords: bivalve, experimental exposure, habituation, invertebrate, noise pollution, sound.

Introduction

The marine soundscape is changing due to increasing amounts of human activity at sea (Andrew *et al.*, 2002; Duarte *et al.*, 2021). Such activities include shipping, pile driving, and seismic surveys, which all generate high-intensity sound that is audible to marine life (Slabbekoorn *et al.*, 2010; Popper and Hawkins, 2019). Anthropogenic noise has the potential to mask biologically relevant sounds, cause stress, and disrupt critical activities (Codarin *et al.*, 2009; Filiciotto *et al.*, 2016). Sound impact studies mostly focussed on marine mammals and fish (e.g. Ellison *et al.*, 2012; Harris *et al.*, 2018; Erbe *et al.*, 2019; Slabbekoorn *et al.*, 2019; de Jong *et al.*, 2020), while invertebrates received only limited attention (Morley *et al.*, 2014; Solan *et al.*, 2016). Nevertheless, invertebrates make up a significant part of the overall marine biomass and are important for all trophic levels (Hatton *et al.*, 2021). Mussels may be an interesting study species for sound impact studies because of their ecological importance in terms of habitat construction, water quality impact, critical part of the food chain, and general abundance. It is also relatively easy to keep them in the lab and monitor their behaviour (Borthagaray and Carranza, 2007).

Bivalves have been shown to respond to natural sounds and to be disturbed by artificial and anthropogenic sound. For example, planktonic larvae of the eastern oyster (*Crassostrea virginica*) showed increased settlement during playbacks of oyster reef recordings when compared to other ambient playbacks (Lillis *et al.*, 2013). Swash-riding clams (*Donax variabilis*) respond to the sound of waves by jumping out of the sand to ride on waves to migrate along the shoreline with rising and falling tides (Ellers, 1995a; Ellers, 1995b). Anthropogenic noise has been shown to affect bivalve physiology through increases in several biochemical stress parameters, such as single-strand breaks in DNA, reduced oxygen consumption, and adjustments in metabolism and hemolymph parameters (Peng *et al.*, 2016; Vazzana *et al.*, 2016; Day *et al.*, 2017; Wale *et al.*, 2019). Even increased mortality after seismic survey passes has been observed (Day *et al.*, 2017). Behaviourally, bivalves have been shown to respond to noisy human activities by increased and deeper digging behaviour and a reduction in closing movements, "coughs", and locomotory behaviour (Mosher, 1972; Peng *et al.*, 2016; Day *et al.*, 2017).

Various studies examined noise impact on mussels' valve gape and filtration rate. Valve gape and filtration rate have been found to be positively correlated, likely due to physical constraints of the valve gape on the pumping capacity (Jørgensen *et al.*, 1988; Jørgensen, 1996; Riisgård *et al.*, 2003; Maire *et al.*, 2007). However, a dissociation of valve gape and pumping capacity has also been found (Kramer *et al.*, 1989; Maire *et al.*, 2007). Insight into the effects of sound on valve gape opening and filtration rate in bivalves is highly relevant to estimate energy budgets and, consequently, fitness consequences (Riisgård and Randløv, 1981; Riisgård and Larsen, 1995; Redmond *et al.*, 2017). Sound has been found to cause immediate valve closure (Roberts *et al.*, 2015; Charifi *et al.*, 2017; Hubert *et al.*, 2022), as well as an increased mean valve gape (Wale *et al.*, 2019), and both a higher clearance rate and

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a lower filtration rate (Spiga *et al.*, 2016; Wale *et al.*, 2019). These results seem to be contradicting but may be explained by different experimental set-ups and different sound stimuli in different studies. Sound impact studies that measure valve gape and feeding rate simultaneously will provide more insight into the correlation between the two measures during sound exposures and into the effects of sound on mussels' energy budget.

Anthropogenic sound vary in several acoustic characteristics, which may all influence the impact on animals, including bivalves. The pulse rate interval (PRI) is a well-known example of relevant acoustic variation that may affect the impact of noise pollution on animals. Pile driving and seismic surveys generate impulsive sound with a consistent PRI. For pile driving, this varies between 1 and 4 s (Matuschek and Betke, 2009; Hall, 2013), and for seismic surveys, between 5 and 15 s (McCauley *et al.*, 2000; Slabbekoorn *et al.*, 2019). Such impulsive sounds have been shown to affect marine mammals (Ellison *et al.*, 2012; Harris *et al.*, 2018), fish (Hubert *et al.*, 2020; van der Knaap *et al.*, 2021), and invertebrates (Solan *et al.*, 2016; Day *et al.*, 2017). The pulse rate interval can be set to fit the goals of a project (for example, the seismic survey resolution) and results in different sound exposure levels, which are relevant for regulations. However, behavioural impact is not necessarily negatively correlated with the sound energy level. There is still limited insight into the effects of the variation in pulse rates. Humans and rats (*Rattus norvegicus*) showed a faster decrease in startle-like responses to artificial pulse trains with lower PRIs (humans: 20 versus 100 s; rats: 2 versus 16 s) (Davis, 1970; Gatchel, 1975). Similar studies have been done with European seabass (*Dicentrarchus labrax*) in the context of pile driving: some indication for an effect of pulse rate was found in an indoor basin set-up (Neo *et al.*, 2015), but these results were not congruent to those from an outdoor floating pen set-up (Hubert *et al.*, 2020). Insight into the effects of pulse rates will aid environmental impact assessment and potentially yield mitigation measures for sound impact problems.

Marine animals are likely to be exposed to anthropogenic sound repeatedly and for extended periods of time. Sound propagates very well underwater and is therefore hard to avoid. This is especially the case for animals with a sessile or sedentary lifestyle, like most bivalves. Initially, animals may respond behaviourally to sound, but during repeated exposure, animals may resume pre-exposure behaviour (Neo *et al.*, 2018). Such an attenuation in responsiveness can be explained by habituation, sensory adaptation, or motor fatigue (Rankin *et al.*, 2009). The mechanism in place can provide insight into the consequences of noise pollution. Habituation indicates that an animal can still detect a stimulus and respond to it but ceases to do so, while sensory adaptation and motor fatigue indicate that an animal is less able to detect and respond to the stimulus (Domjan, 2010). Nevertheless, behavioural habituation does not imply the absence of any negative effect, and it is critical to understand the long-term effects of disturbances on animals (Bejder *et al.*, 2009). Several animals have been shown to habituate to sound (Rankin *et al.*, 2009; Neo *et al.*, 2015). Bivalves have also been shown to reduce responsiveness over repeated tactile, visual, and acoustic stimuli (Wilkens, 1986; Dehaudt *et al.*, 2019; Hubert *et al.*, 2022). Our previous sound impact study on mussels aimed to gain insight into the mechanism underlying a decay in responsiveness to sound. The results indicated that motor fatigue is an unlikely explanation for the decay in responsiveness in mussels, but we could not conclusively attribute it to habituation or sensory adaptation (Hubert *et al.*, 2022). Fading response patterns show that it is important to not only study initial responses to sound but also recovery, potential compensation behaviour, and long-term effects in general.

Blue mussels are an ecologically important species because they are filter-feeders (essential to nutrient recycling), provide habitat as reef-builders, and are an important prey species (Kautsky, 1981; Jørgensen, 1990; Borthagaray and Carranza, 2007). Additionally, they are commercially important (Eurostat, 2019) and abundant in both intertidal and subtidal habitats (Gosling, 1992). Mussels are semi-sessile, with limited possibilities to move away from acoustic disturbance. This lifestyle makes them a suitable species for lab studies because their behaviour may be less affected by confinement than other species. Mussels have been shown to detect and respond to sound (Roberts *et al.*, 2015; Hubert *et al.*, 2022) and are expected to detect the particle motion aspect of sound, as they lack gas-filled cavities. The perceptual mechanism of sound detection is not fully understood, but the abdominal sense organ, short cilia on the mantle and tentacles, and statocysts have been suggested to play a sensory role (Haszprunar, 1983; Zhadan, 2005; Roberts *et al.*, 2015; Charifi *et al.*, 2017). Mussels have been shown to respond to substrate-borne tonnes from 5 to 410 Hz (no tonnes outside this range tested, Roberts *et al.*, 2015) and responded well to water-borne tonnes of 150 and 300 Hz in an earlier study (Hubert *et al.*, 2022). Mussels have been used for ecotoxicology studies extensively, providing ample opportunity for comparisons between the impacts of sound exposures and other stressors.

In the current study, we examined the effect of impulsive sound pulse trains with different pulse intervals on the valve gape behaviour and clearance rate of blue mussels. We logged the mussels' valve gape using a valve gape monitor and their clearance rate using spectrophotometry of phytoplankton densities in the water. The mussels were exposed to impulsive sound with different pulse intervals. We aimed to answer the following questions: (1) Are the valve gape and clearance rate of blue mussels correlated? (2) Do artificial impulsive sound exposures affect the valve gape and clearance rate? (3) Does the pulse rate of the sound affect the recovery of the mussels' valve gape to pre-exposure levels?

Material and methods

Subjects

In this study, we used 354 wild-caught blue mussels (*Mytilus* spp.) of 3.5–6.0 cm in length. They were collected from the poles of the Scheveningen Pier (52.117°, 4.280°; lat, long) in the inter-tidal area of the North Sea coast in Scheveningen, the Netherlands. The study population probably consisted of pure *M. edulis*, hybrids with *M. galloprovincialis*, and occasional hybrids with *M. trossulus* (Coolen *et al.*, 2020). The hybrids are not easily discriminated from pure *M. edulis* mussels, and molecular techniques are needed to do this.

The mussels were kept at the Leiden University sea water facility in stock tanks (200 × 40 × 50 cm; L × W × H) with continuously filtered artificial sea water (Instant Ocean, Aquarium Systems) of 18–20°C at a 13:11 h light–dark schedule. They were fed with phytoplankton (*Nannochloropsis* spp., Colombo) 2–3 times per week. We did not feed them in the

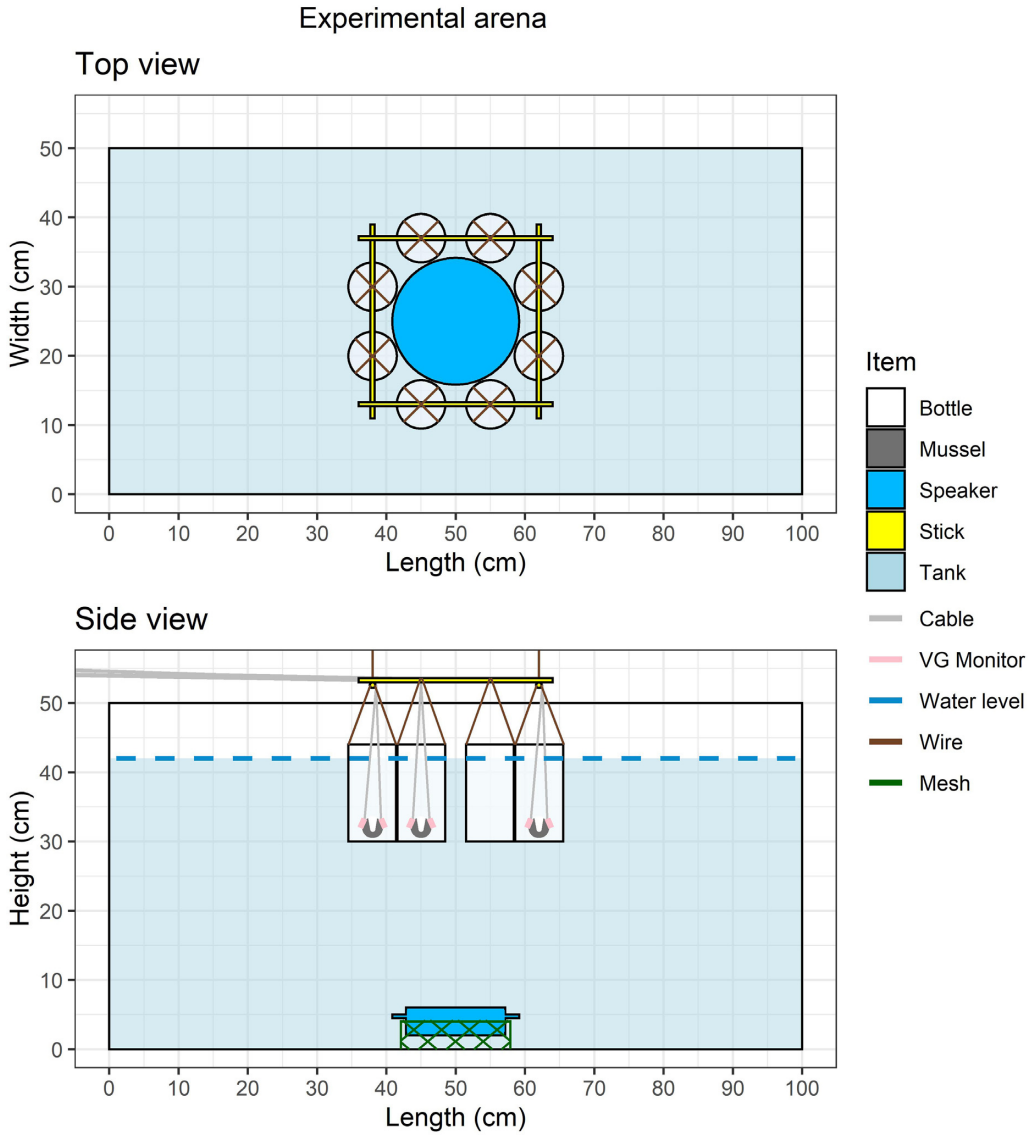

Figure 1. Schematic views of the experimental arena (100 × 50 × 50 cm; L × W × H) from above (top view) and the front (side view). During the experiments, six mussels were placed in plastic bottles hanging in the rectangular tank, and two empty bottles served as controls for changes in phytoplankton density without mussel impact. The eight bottles were hung at equal distances from the speaker (see Top view). The mussels were exposed to sound using a speaker on a mesh frame at the bottom of the tank.

stock tank on the day that they were used in the experiments to promote feeding activity during the experiment when phytoplankton was present. The animals were collected on 2 and 5 November 2020 and the experiments were conducted from 24 November until 23 December 2020. The mussels were released back into the wild after the experiment.

Experimental set-up

The trials were performed in a glass tank (100 × 50 × 50 cm; L × W × H) with sea water (salinity ~33 g/kg) and an underwater speaker on a mesh frame at the centre of the bottom. We hung eight plastic 0.5 l bottles in the tank at equal distances around the speaker (Figure 1). We cut the top ~4 cm of each bottle and used wires to hang them below a square frame of wooden sticks, which was hanging under a truss above the experimental tank. This hanging structure was used to prevent the transmission of mussel movement from one bottle to another. The bottles allowed us to test six mussels (gape + clearance) and two empty bottles (control clearance) simultaneously while excluding chemical communication or physical contact between the individuals (see Supplementary Figure S3 for empirical evidence for the independence). For this reason, we also cleaned the bottles after each trial by rinsing them in a sea water aquarium without animals and filled the bottles with filtered water from the same filter system that was also connected to the stock tanks. The positions of the two empty bottles were counterbalanced. The water temperature was identical to the stock tanks, and the experiment was conducted during the hours with artificial daylight with light sources that were identical to the stock tank.

Valve gape measurements

We used a valve gape monitor to log the valve gape behaviour of the mussels (c.f. Ballesta-Artero *et al.*, 2017; Hubert *et al.*, 2022). The valve gape monitor consisted of multiple pairs of electromagnetic coils coated in epoxy. The active coil of each

pair generated an electromagnetic field, which resulted in a current in the responsive coil. The strength of the measured electromagnetic field is determined by the distance between the coils and thus reflects the valve gape. The valve gape monitor yielded on average 46.7 (range: 46.6–46.9) datapoints per minute for each individual. The raw data from the measured electromagnetic field strengths were converted to absolute distances between the sensors using the calibration of the monitor. By subtracting the minimum distance of each mussel from all measured distances of the same mussel, we determined the valve gape (at the location of the sensors). We attached the coils of one pair on opposite valves of an individual mussel using a combination of hot glue and cyanoacrylate glue. Immediately after attaching the coils, the individual mussels were hung in the centre of one of the bottles at 12–13 cm below the water surface. After we placed the last mussel in a bottle, we added 6 ml of phytoplankton (on average ~13.54 e^7 cells/l, *SD*: ~2.79 e^7) in each bottle and started the playback, which started with 25 min of silence before the first sound exposure (in the non-control conditions).

Phytoplankton measurements

We took three 2 ml water samples at 0, 60, and 95 min after the start of each trial (Figure 3), from each of the eight bottles (nine samples per bottle), to determine the absorption, a measure for the phytoplankton (cell-chlorophyll) density in the water. We first resuspended a sample to increase the homogeneity of the phytoplankton in the bottle and then took the three samples. The absorption of each individual water sample was measured in triplicate using a spectrophotometer (GENESYS 30, Thermo Scientific) at a wavelength of 430 nm. A spectrophotometer shines light through a sample solution and measures the light intensity that is absorbed by the sample. To link the measured absorption to the cell density, we made a calibration of absorption and cell density. To do this, we made solutions of 300 ml of sea water with 0, 1, 2, 3, 4, 5, and 6 ml phytoplankton in triplicate, from which we took a single 2 ml sample per solution (21 samples). We measured each 2 ml sample in triplicate using the spectrophotometer and took three samples from each 2 ml sample to count the number of phytoplankton cells using a microscope (Axioplan 2 imaging microscope, Zeiss) and hemocytometer. This was done twice during the experimental period, yielding 42 unique 2 ml samples (measured in triplicate) and 126 unique microscope samples.

Sound exposure

During the experiments, we exposed the mussels to sequences of pure tonnes of 200 and 350 Hz with silence intervals. We chose these frequencies because mussels have been shown to respond to sound from 5 to 410 Hz (Roberts *et al.*, 2015), responded well to pure tonnes of 150 and 300 Hz in an earlier study (Hubert *et al.*, 2021), and because there is no overlap in the harmonic structure of the two stimuli. A sound treatment consisted of a sequence of 5 s pure tonnes of one of the two frequencies (200 or 350 Hz), followed by a single 5 s exposure of the other frequency. The silent interval between the 5 s tonnes was consistent within a trial and was either 5, 10, 20, 40, 80, or 160 s. For each interval, we used a treatment that started with a 200 Hz pulse train and a treatment that started with a 350 Hz pulse train. Each treatment started with 25 min of silence to allow the mussels to acclimate to ambient sound

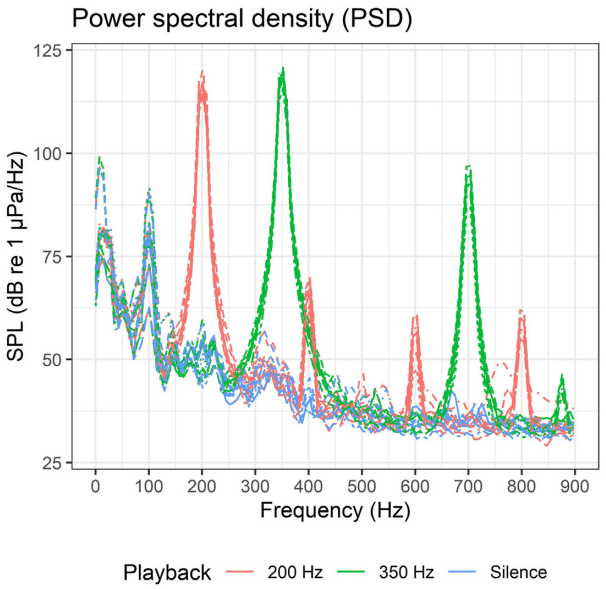

Figure 2. Power spectral density (window length: 6144, window type: Hann) of recordings of the two pure tonnes (red and green) and the ambient conditions of the silence playback (blue) at all mussel locations (different lines).

conditions before the start of the experimental exposure of the pulse trains, which lasted for 65–66 min (depending on the pulse rate), and was followed by 4–5 min of silence playback until the end of the trial (95 min). We also used two control treatments in which the pulse trains were replaced by silence and only the last pure tonne remained. This resulted in 14 different sound treatments with six different pulse rates and a control (Figure 3). We counterbalanced the order of the treatments. The sound treatments were created with Audacity (The Audacity Team, version 2.3.3) and played back with an underwater speaker (UW30, Lubell labs) from a solid-state recorder (DR-07, TASCAM), through an amplifier (M033N, Kemo).

After the trials, we recorded both pure tonnes and the ambient conditions of silence playback with a calibrated hydrophone (96-min, HTI) and digital recorder (DR-100MKII, TASCAM). We made recordings in all eight bottles at the location of the mussel and generated power spectral density plots using a custom-made R-package (Figure 2). The rms SPL (sound pressure level, geometric mean of all locations in the 100–600 Hz bandwidth) was 141.8 dB re 1 μPa of the 200 Hz stimulus playback, 143.2 dB re 1 μPa of the 350 Hz stimulus, and 102.8 dB re 1 μPa for the ambient conditions during silence playback.

The sound propagation in the experimental tank can be expected to differ substantially from the propagation of the same stimuli at sea. The proximity of the tank walls and water surface affects the ratio between sound pressure and particle motion and the directionality of particle motion (Rogers *et al.*, 2016; Campbell *et al.*, 2019). We aimed to minimize the impact of these artificial sound field conditions on the experiment by placing the mussels as far as possible from the water surface and tank walls, but the sound is still expected to be substantially different from the same playback in natural water bodies. This does not pose a problem for the current study, as we aimed to gain insight into the effects of pulse rate and the relation between valve gape and clearance, and not

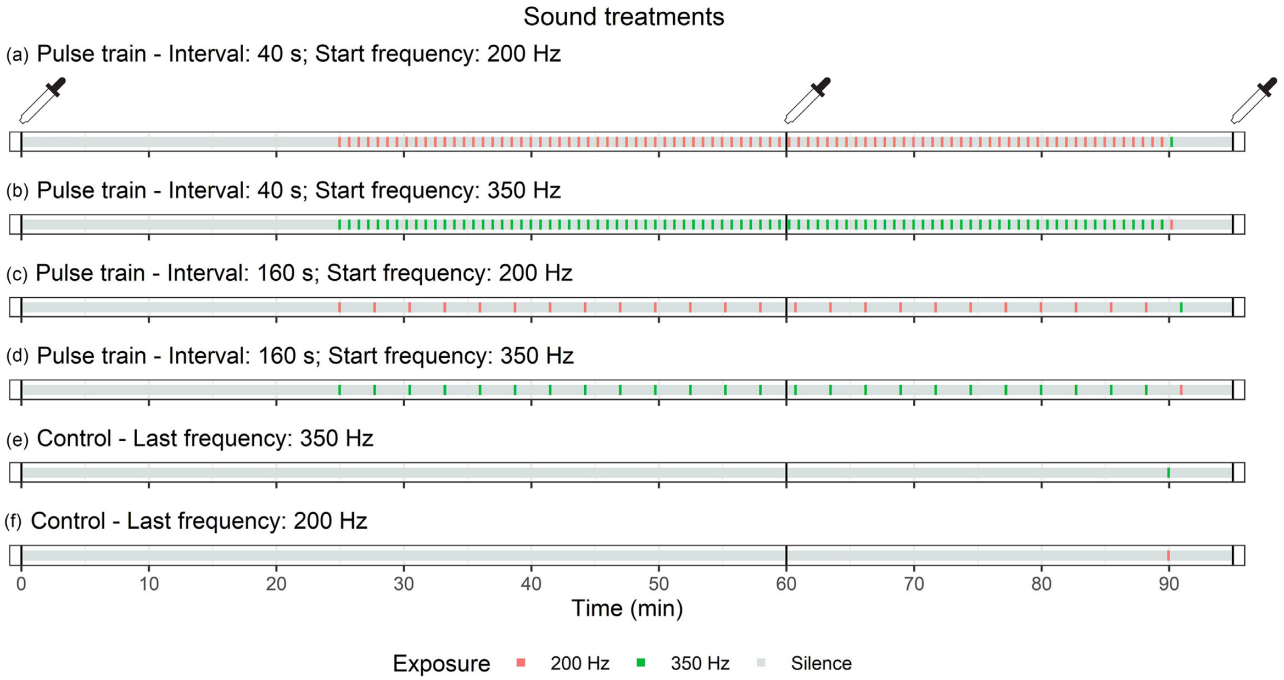

Figure 3. Schematic overview of the temporal and spectral variation in the experimental playback stimuli. We exposed the mussels to one of the 12 pulse train playback tracks (represented by a–d) or one of the two control tracks (e–f). The pulse trains started after 25 min of silence and consisted of 5 s pulses with a consistent interval of 5, 10, 20, 40, 80, or 160 s. The pulses were 200 or 350 Hz pure tonnes (with some energy at higher harmonic tonnes, see Figure 2). All pulses within a pulse train were of the same frequency, except for the last pulse (at 90–91 min, depending on the interval). We made duplicate tracks for all intervals, one that started with 200 Hz tonnes and one that started with 350 Hz tonnes (a–b and c–d). The control playbacks only contained the last pulse (at 90 min). The other pulses were replaced by silence (e–f). The black vertical lines at 0, 60, and 95 min represent the moment at which we took the water samples for determining the phytoplankton density in the bottles. The length of the sound pulses is not to scale in this figure.

to determine absolute response levels to a particular realistic anthropogenic sound.

Data processing and statistics

We used the paired absorption (spectrophotometer) data and cell counts to validate the absorption measures as a proxy for the phytoplankton density. We examined the relationship between cell counts and absorption measures using a generalized linear model (GLM) with the absorption as response variable and cell counts as dependent variable. For all models, we checked normality of the residuals and we calculated the standardized coefficients as a measure of effect size. We reported the standardized coefficient, 95% confidence interval, and *p*-value.

We analysed the valve gape and absorption (spectrophotometer) data from 59 trials. Individuals from which the valve gape monitor got loose during the trial were excluded from the dataset, resulting in 347 individuals that were included. Additionally, we collected absorption data from 119 replicates of bottles without a mussel during the same 59 trials. To examine the relation between the valve gape and clearance rate, we constructed another GLM with the decrease in absorption as the response variable and the mussel shell size and mean valve gape as dependent variables. We also reported the R^2_{adj} to gain insight into the proportion of the variance in the decrease in absorption that is explained by the model.

To examine the phytoplankton consumption by the mussels, we analysed the absorption over time and the effect of the presence of a mussel on this. We constructed a full model GLM with the absorption measurements as the response variable and the sampling time and mussel presence (*Y*/*N*) and the interaction between them, as covariates. We evaluated the inclusion of both covariates and the interaction based on model selection using AICc, and finally ran the model with the lowest AICc. Next, we examined the effect of the sound treatments on phytoplankton consumption. For this, we only used the absorption data from the bottles that contained a mussel. We analysed the absorption data in a GLM with the mussel shell size, the sound treatment, sampling time, and the interaction of time with treatment as covariates. We again selected the best model based on AICc and ran it.

To examine the valve gape response of the mussels to the sound exposure, we compared the valve gape 20 s before and 20 s after the onset of the sound. We analysed the response to the first pulse to examine the response of naïve mussels; the second last pulse, to examine the response of persistently exposed mussels; and the last pulse of a different frequency, to examine whether mussels responded again to a novel stimulus. For this, we used three GLMs with the valve gape as the response variable and the period (before/after) as the dependent variable. We only included mussels that opened for at least 0.5 mm during the entire trials and the ones that were exposed to pulse trains with an interval of 20 s or higher for these analyses.

To gain insight into the effect of the pulse rate, we determined the time it took the mussels to return to (or exceed) their pre-exposure valve gape levels: the recovery time. For this, we used the fraction open before (4 min) as the pre-exposure level and used a moving average of 4 min for the data after the onset of the sound to determine when the pre-exposure level was reached again. We plotted an overview of the

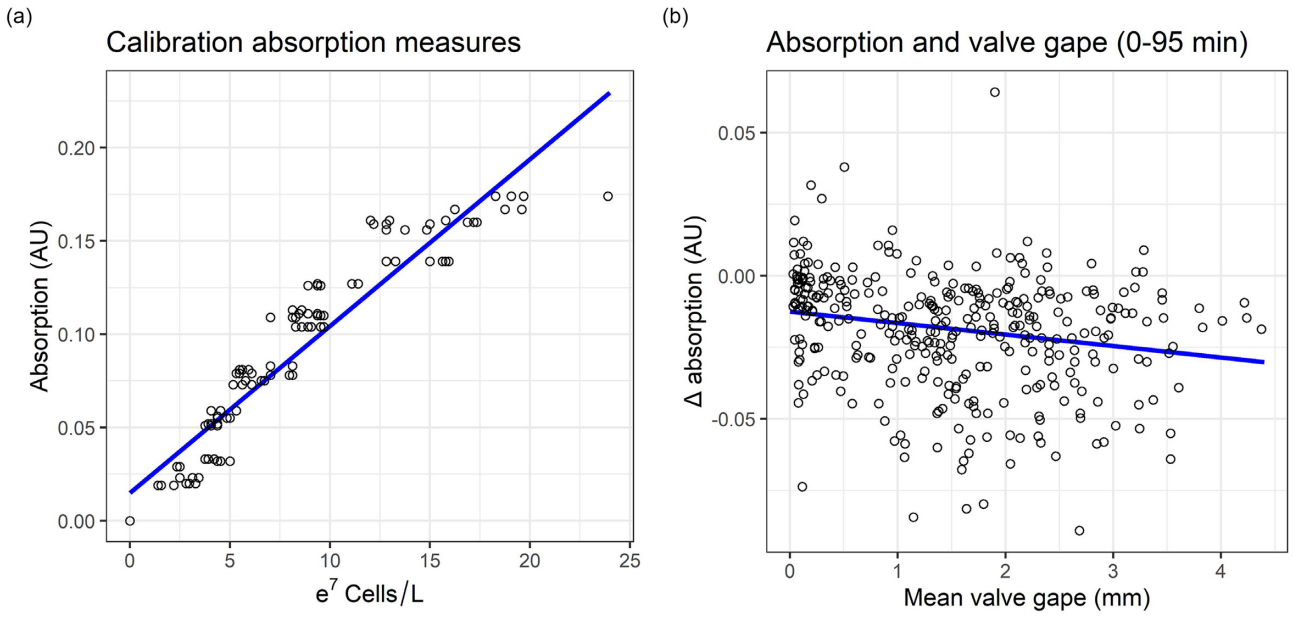

Figure 4. (a) Correlation between absorption measures and microscope cell counts of the same water samples ($n = 126$ paired counts). (b) Significant correlation between change in absorption and the mean valve gape during the entire trial ($n = 347$ individuals). A trend line is drawn for mussels of 4 cm, the median of the subjects. See Supplementary Figure S2 for plots on various mussel size classes. The open circles indicate the data points, and the blue lines indicate significant correlations.

number of mussels that did not close upon the onset of the sound, that partially closed and returned to pre-exposure levels during the trial, and that partially closed and did not return to pre-exposure levels. Based on this overview, we decided to examine the effect of pulse rate on the recovery time of the mussels that did recover (further justification in Results). We used a GLM with the recovery time as the response variable and the interval as a covariate. We only used mussels that opened for at least 0.5 mm during the entire trial and that were at least 10% open right before the first sound pulse. All data processing and analyses were done using R (R Core Team, 2016) and the packages MuMIn (Barton, 2016), effect size (Ben-shachar *et al.*, 2020), and rsq (Zhang, 2020).

Results

Visual observation of the mussels' valve gape over the course of the entire trial (Supplementary Figure S1) reveals that mussels recovered from handling and acclimated to the experimental tank in the first 25 min. The control mussels remained at this level for a while, and the mussels that were exposed to sound responded to the sound by partially closing their valves. Mussels in both treatment conditions responded to the water sampling at ~60 min, but the control mussels seem to have responded stronger. The control mussels also clearly responded to a single sound pulse at 90 min, while the exposure mussels only seem to respond mildly to a novel sound pulse played back between 90 and 91 min.

The absorption measured using the spectrophotometer was positively correlated with the number of cells in the same water samples counted using a microscope (cell count slope: 0.95; 95% *CI*: 0.89, 1.01; p-value < 0.001; Figure 4a), and we thereby validated absorption as a measure for the phytoplankton density in the water. The reduction in absorption from the start to the end of the trial was negatively correlated with the shell size of the mussels (size slope: −0.24; 95% *CI*: −0.34, −0.14; p-value < 0.001). The change in absorption was also negatively correlated with their mean valve gape (valve gape slope: −0.20; 95% *CI*: −0.30, −0.10; p-value < 0.001; Figure 4b), which indicates that mussels with a larger valve gape removed more phytoplankton from the water. However, only a part of the change in absorption was explained by the model (R^2_{Adj}: 0.10), meaning that the valve gape and mussel size were not the only factors determining the clearance rate of the mussels.

Over time, the absorption (measure for phytoplankton density) of the water in the bottles in the experimental set-up decreased. The decrease in phytoplankton was significantly faster when a mussel was present (with mussel: −0.49; 95% *CI*: −0.63, −0.34; p-value < 0.001; Figure 5a) and the rate in which the phytoplankton decreased was higher during the first period of 0–60 min than in the period thereafter of 60–95 min (60–95 min: 0.16; 95% *CI*: 0.04, 0.29; p-value = 0.011; Figure 5a). The decrease in phytoplankton was not significantly affected by the sound treatment (sound exposure: 0.03; 95% *CI*: −0.23, 0.29; p-value = 0.815; Figure 5b). The shell size of the mussels was significantly correlated with the decrease in phytoplankton (size slope: −0.22; 95% *CI*: −0.30, −0.14; p-value < 0.001). Their valve gape was also significantly correlated with their shell size (size slope: 0.11; 95% *CI*: 0.04, 0.18; p-value = 0.003), was larger in the exposure condition (sound exposure: 0.37; 95% *CI*: 0.15, 0.58; p-value < 0.001; Figure 5c), and larger during the 60–95 min period than the 0–60 min period (60–95 min: 0.27; 95% *CI*: 0.12, 0.41; p-value < 0.001; Figure 5c).

The mussels partially closed their valves after the onset of the sound (after: −0.53; 95% *CI*: −0.74, −0.32; p-value < 0.001; Figure 6a). They did not close anymore after the second last sound exposure (after: −0.07; 95% *CI*: −0.29, 0.15; p-value = 0.541; Figure 6b). The last sound exposure had a deviating frequency. A non-significant trend indicated that the mussels tended to respond again by partially

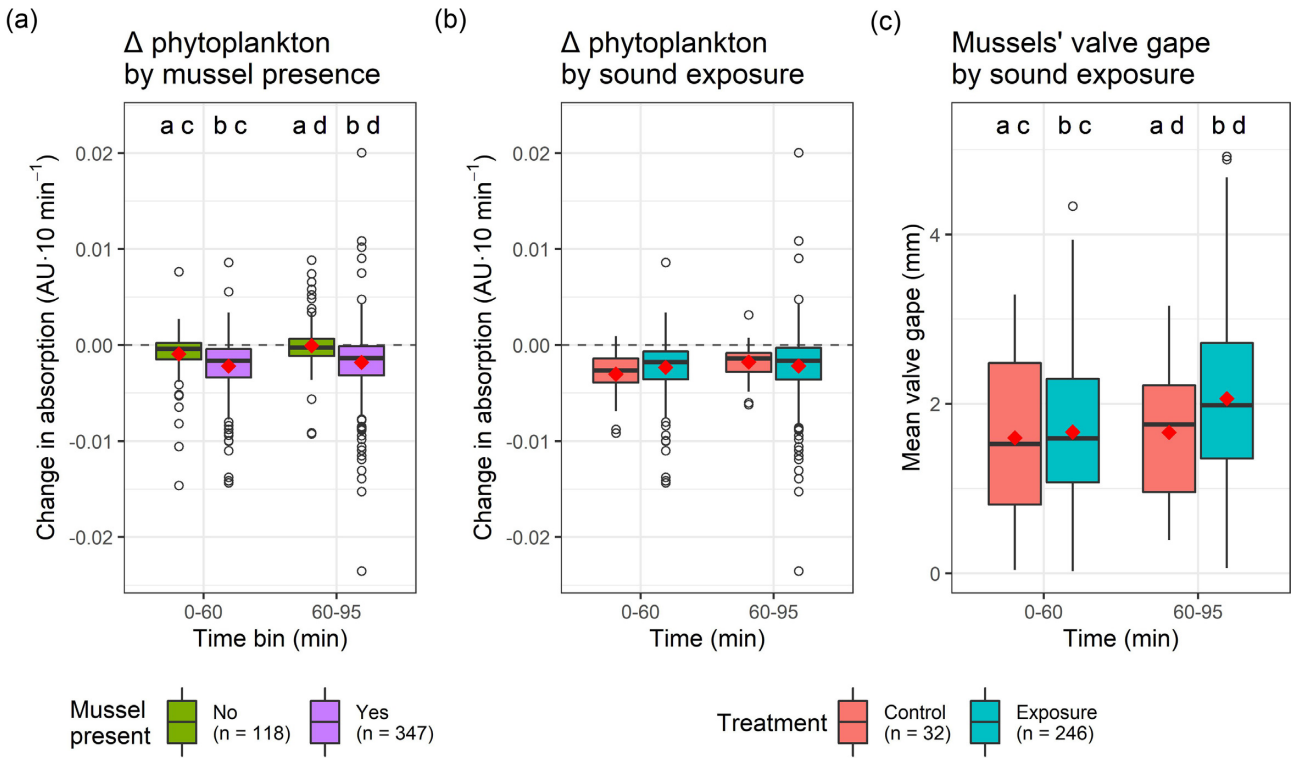

Figure 5. (a) The rate of decline in absorption (measure for the phytoplankton density in the water) was significantly larger in the first experimental period (0–60 min) and when a mussel was present in the bottle. (b) The sound treatment did not affect the decrease in absorption in the bottles with a mussel. (c) The mean valve gapes of the mussels were significantly higher in the exposure condition, and their valve gapes were higher in the second experimental period, irrespective of treatment (60–95 min). The box-whisker plots indicate the median, first, and third quartiles, min and max excluding outliers, and outliers. The red diamonds indicate the means. Different letters above boxplots indicate a significant difference.

closing their valves (after: −0.20; 95% *CI*: −0.42, 0.04; *p*-value = 0.077; Figure 6c).

The majority of the mussels that were exposed to a pulse train responded by (partially) closing their valves, and also returned to pre-exposure levels during the trial (Figure 7a). For those individuals, there was a positive correlation between recovery time and the time interval between pulses ($\log_e$ pulse interval slope: 0.19; 95% *CI*: 0.02, 0.37; *p*-value = 0.032; Figure 7b). We did not include the effect of pulse rate on the number of individuals that did not close and did not recover in the analysis (Figure 7a, like a zero-inflated model). The lack of closure after the onset of the first pulse is likely not affected by pulse rate. The number of individuals that did not recover (per interval) is rather limited and, based on visual inspection of the data (Figure 7a), we concluded that if any pattern will emerge from the individuals that did not recover, it would be in line with the pattern we found for the individuals that did recover (Figure 7b).

Discussion

The mussels fed on phytoplankton during the trials, and the reduction in phytoplankton in the water was correlated to their valve gape and shell size. The impulsive sound did not negatively affect the phytoplankton clearance rate or valve gape during the entire trial. Nevertheless, at a shorter term, the mussels responded to the sound by partially closing their valves. They did not respond anymore to the second-last sound pulse, but a non-significant trend indicated that they responded again to the last deviating sound pulse, which suggests that they habituated to the preceding tonne series of identical frequency. After their initial response, mussels that were exposed to slower pulse rates recovered (returned to baseline valve gape levels) more slowly than mussels that were exposed to fast pulse rates.

Valve gape correlated to clearance rate

The decrease in phytoplankton density was significantly correlated with the mussels' valve gape. Larger mussels and mussels with a larger valve gape removed more phytoplankton from the water, and this is in line with previous research (Riisgård and Seerup, 2003; Sylvester *et al.*, 2005; Maire *et al.*, 2007) and can be explained by an increased pumping capacity (c.f. Jørgensen *et al.*, 1988). Not all variation in the clearance rate could be explained by the valve gape. This is both in line with previous studies that found a correlation between valve gape and filtration or pumping rate (Jørgensen *et al.*, 1988; Riisgård *et al.*, 2003; Maire *et al.*, 2007). Exhalant siphon area has been shown to be a better proxy for pumping rate than valve gape (Newell *et al.*, 2001; Maire *et al.*, 2007). This confirms the possibility for dissociation between valve gape and pumping capacity (Kramer *et al.*, 1989; Newell *et al.*, 2001; Riisgård *et al.*, 2003) and may explain part of the variation in the decrease in phytoplankton that could not be explained by valve gape in the current study. Newell *et al.* (2001) suggested that valve gape is a general indicator of active feeding and respiration while exhalant siphon area is a useful index of pumping rate (Jørgensen *et al.*, 1988; Newell *et al.*, 2001; Maire *et al.*, 2007).

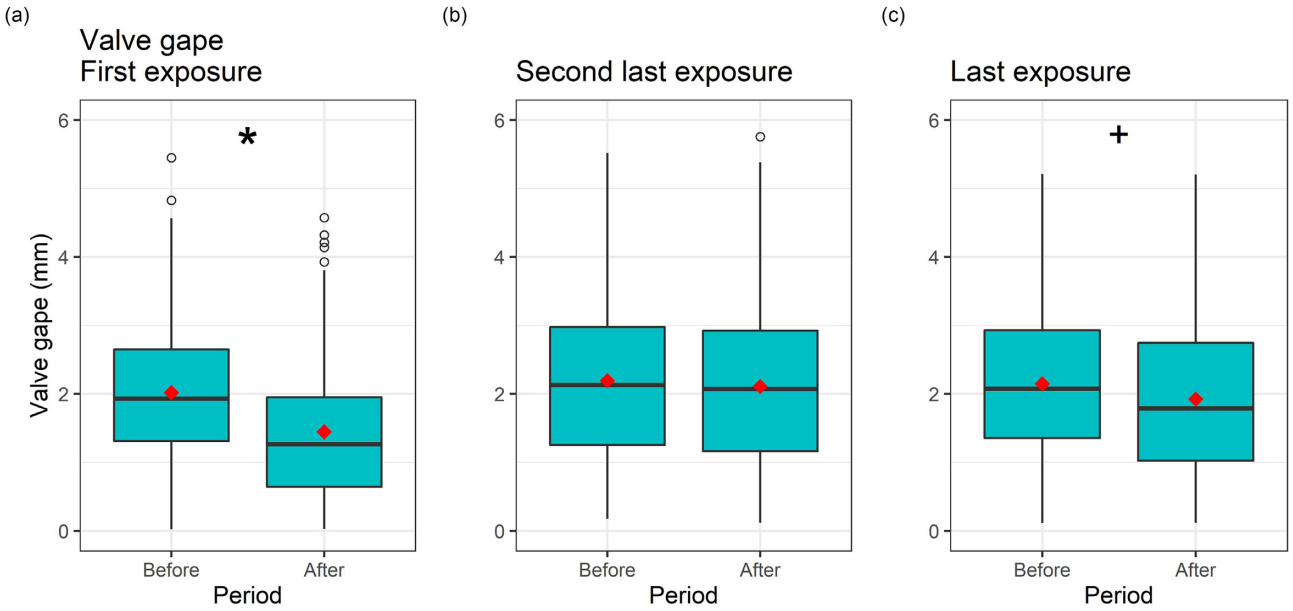

Figure 6. The valve gape of the mussels 20 s before and after the onset of the (a) first, (b) second last, and (c) last (deviating) sound pulse. The mussels significantly closed their valves after the onset of the first exposure. This was not the case at the second last exposure, and there was a non-significant trend at the last exposure. Only mussels that were exposed to pulse trains with a pulse interval of 20 s and higher, and mussels that opened >0.5 mm during the trial were included in this analysis ($n = 161$). A significant difference is indicated by an asterisk, and a non-significant trend is indicated by a plus.

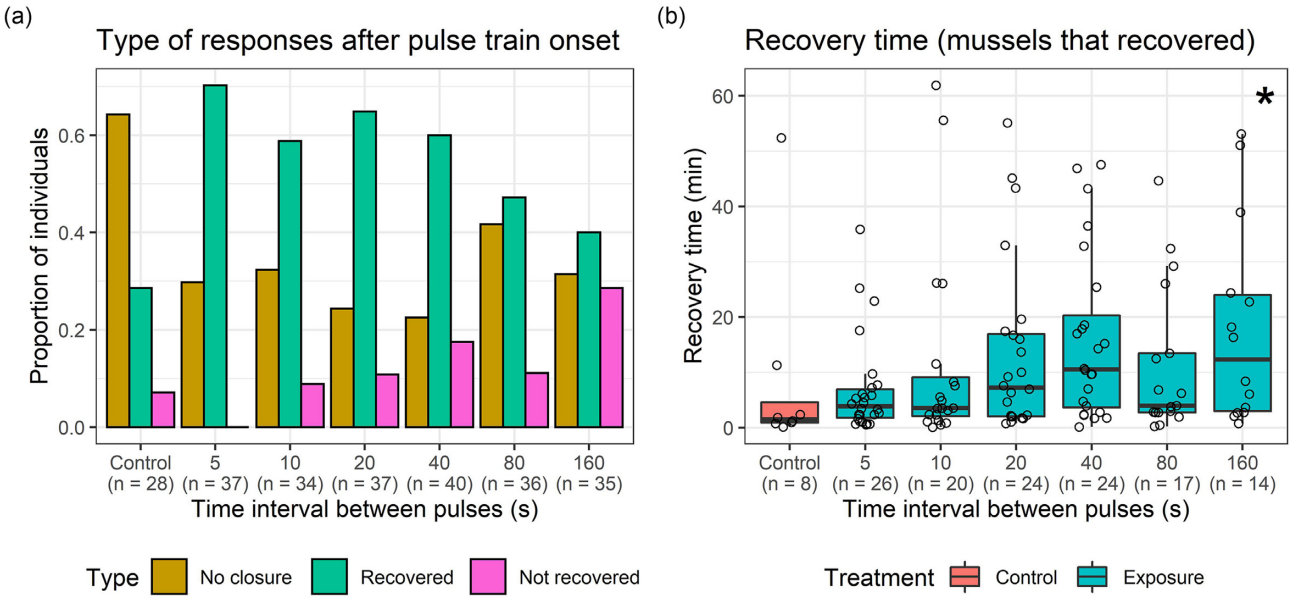

Figure 7. (a) The proportion of mussels that did not (partially) close their valves after the onset of the pulse trains, and from the ones that did (partially) close their valves, the proportion of individuals that returned and did not return to pre-exposure levels in the 70 min after the onset. Separate counts for the control and all pulse rates are depicted. Only mussels that opened >0.5 mm during the entire trial and that were at least 10% open before the first pulse were included in this plot ($n = 247$). (b) The recovery time of the mussels that did recover during the trial (up to 70 min after the pulse train onset) was significantly longer for individuals that were exposed to treatments with slower pulse rates ($n = 125$), as indicated by the asterisk in the upper-right corner.

We measured the phytoplankton density in sea water using a spectrophotometer and validated and calibrated this approach using cell counts with a microscope. The phytoplankton density went down over time, and the decrease was faster when a mussel was present. The decrease in phytoplankton density in our controls (without mussel) can probably be explained by phytoplankton (potentially partly dead) sinking to the bottom, which happened predominantly in the first 60 min. On the other hand, light in the experimental room could have allowed the phytoplankton (*Nannochloropsis* spp.) to grow and reproduce. The applied methodology seems to be working well and provides a time-efficient tool to assess clearance rate-related variation in phytoplankton density, similar to a coulter counter (e.g. Riisgård *et al.*, 2003).

No effect of sound on phytoplankton clearance

The sound treatments did not affect the phytoplankton clearance rate of the mussels (the decrease in phytoplankton density in the water), but the mussels that were exposed to sound had a larger valve gape throughout the trial. Based on the short-term partial valve closure due to the sound exposure, we did expect a (potentially short term) decrease in clearance rate due to the sound treatments. However, the decrease in valve gape was typically relatively short compared to the duration of the entire trial. Other studies have reported variable results, with higher and lower feeding rates of mussels during sound exposure conditions (Spiga *et al.*, 2016; Wale *et al.*, 2019). High temporal resolution of monitoring of phytoplankton density or the exhalant siphon area may provide additional insight into initial effects and potential compensation and may also help to explain the variable pattern of results.

The larger valve gape of mussels that were exposed to sound may be explained by a slightly larger valve gape before the start of the sound and a weaker response to collecting the second water sample and to the last (deviating) sound that was also played back in the control condition (visual observation from Supplementary Figure S1). We did not statistically test whether mussels in the control condition responded stronger to the water sampling because of the variation in the precise timing and duration of the sampling and the lack of trials without sampling. However, in future experiments, it would be relevant to examine whether mussels respond less to visual, water movement, or tactile cues during sound exposures. Sound may affect such responses through cross-modal interference, shifts in risk-taking behaviour, or compensatory behaviour (Halfwerk and Slabbekoorn, 2015; Hubert *et al.*, 2021). Experiments on cross-modal interference will increase insight into the effects of sound on anti-predator behaviour of prey and are needed to understand overall effects of sound on animals (Morris-Drake *et al.*, 2016).

Attenuation in valve gape response

The majority of the mussels that initially closed their valves upon sound exposure, returned to pre-exposure levels during the 65–66 min pulse trains. Attenuation in responsiveness to repeated exposures can potentially be explained by habituation, sensory adaptation, or motor fatigue. Habituation can be discriminated from sensory adaptation and motor fatigue by being specific to a particular stimulus (Bejder *et al.*, 2009; Rankin *et al.*, 2009). Our previous study with a similar design showed that motor fatigue was an unlikely explanation for the attenuation in responsiveness in mussels, but we could not conclusively attribute it to habituation or sensory adaptation (Hubert *et al.*, 2022). To determine whether the attenuation in responsiveness could be attributed to habituation, we exposed the mussels to a single novel sound stimulus of a different frequency at the end of the pulse train (a stimulus specificity test). The mussels did not significantly close their valves after the second-last exposure, but a non-significant trend indicated some closure after the novel exposure. Because of the non-significant trend, we are again cautious to attribute the attenuation in responsiveness to either habituation or sensory adaptation. It would still be highly relevant to further examine this because habituation indicates that a subject still senses the stimulus and is still able to respond, but nevertheless ceases to do so (Domjan, 2010). While sensory adaptation indicates a decrease in the responsiveness of the sensory system, which also affects the reception of other—potentially relevant—stimuli in the same sensory modality (Rankin *et al.*, 2009; Domjan, 2010). Both explanations have different ecological implications and therefore warrant further examination (Bejder *et al.*, 2006; Bejder *et al.*, 2009).

Low pulse rates have higher impact

We found a positive correlation between the time interval between pulses of the sound pulse trains and recovery time of the mussels' valve gape; this means that mussels that were exposed to lower pulse rates took longer to return to pre-exposure valve gape levels. This is in line with previous studies in rats (*Rattus norvegicus*) and humans that also showed a faster decrease in startle-like responses to pulse trains with faster pulse rates (Davis, 1970; Gatchel, 1975). The reported effects of different pulse rates on fish were less straightforward and consistent (Neo *et al.*, 2015; Shafiei Sabet *et al.*, 2015; Hubert *et al.*, 2020). However, the tested range in pulse rates was relatively small in the fish studies when compared to the rat, human, and current mussel studies. This might be the reason that the latter three studies found more clear effects of pulse rate variation than the experiments with fish. More studies with a larger test range are needed in fish to know whether the taxonomic congruence in response patterns, from rats and humans to mussels, also encompasses fish.

Interestingly, our results of relatively low pulse rates having relatively long impacts mean that mussels that were exposed to a lower cumulative sound exposure level (SEL) were affected more than individuals that were exposed to treatments with a higher SEL. This is in line with previous studies on European seabass, showing more impact from intermittent sound with relatively low SELs than continuous sound with relatively high SELs (Neo *et al.*, 2014; Neo *et al.*, 2016). This implies that SEL is not a suitable predictor for behavioural impact, as temporal patterns of sound exposure matter and should therefore be taken into account for impact assessments across taxa.

Conclusions

We used spectrophotometry to determine the phytoplankton clearance rate of the mussels and found a correlation between their valve gape and the reduction in phytoplankton. We did not find an effect of the sound pulse trains on the mussels' clearance rate or a negative effect on their valve gape, even though they initially responded to the acoustic exposure by closing their valves. Most mussels returned to pre-exposure valve gape levels during the remainder of the exposure, and individuals that were exposed to faster pulse rates recovered faster. This indicates that sound exposures with variable temporal patterns can also vary in impact, which should be taken into account in impact assessments and mitigation measures. Future studies should examine whether actual anthropogenic sound also affects mussels *in situ*, whether mussels habituate to sound, and whether acoustic disturbance or habituation makes them more vulnerable to predation or parasitism. Fitness effects on individual mussels from anthropogenic noise may be relevant for population development, also in the context of pressure from other stressors, their role as an ecosystem engineer, and in aquaculture.

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Supplementary data

Supplementary material is available at the ICESJMS online.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Data availability statement

All data and scripts used for the analyses reported in this article are available from the Zenodo Repository, doi:10.5281/zenodo.7154170.

Author contributions

All authors contributed to the conceptualization and methodology. JH conducted the formal analysis, made the visualizations, and wrote the draft. RM performed the experiment. HS supervised the project. All authors contributed to revising the manuscript and approved the final draft.

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