From DNA to Ecological Performance: Effects of Anthropogenic Noise on a Reef-Building Mussel

Matthew A. Wale[1,3*], Robert A. Briers[1], Mark G. J. Hartl[2], David Bryson[1], Karen Diele[1, 3]

[1] School of Applied Sciences, Edinburgh Napier University, Edinburgh, UK

[2] Centre for Marine Biodiversity & Biotechnology, Heriot-Watt University, Edinburgh, UK

[3] St Abbs Marine Station, St Abbs, UK

Corresponding Author

*Matthew Wale – M.Wale@napier.ac.uk

ABSTRACT: Responses of marine invertebrates to anthropogenic noise are insufficiently known, impeding our understanding of ecosystemic impacts of noise and the development of mitigation strategies. We show that the blue mussel, *Mytilus edulis*, is negatively affected by ship-noise playbacks across different levels of biological organization. We take a novel mechanistic multi-method approach testing and employing established ecotoxicological techniques (i.e. Comet Assay and oxidative stress tests) in combination with behavioral and physiological biomarkers. We evidence, for the first time in marine species, noise-induced changes in DNA integrity (six-fold higher DNA single strand-breaks in haemocytes and gill epithelial cells) and oxidative stress (68% increased TBARS in gill cells). We further identify physiological and behavioral changes (12%

reduced oxygen consumption, 60% increase in valve gape, 84% reduced filtration rate) in noise-exposed mussels. By employing established ecotoxicological techniques we highlight impacts not only on the organismal level, but also on ecological performance. When investigating species that produce little visually obvious responses to anthropogenic noise, the above mentioned endpoints are key to revealing sublethal effects of noise and thus enable a better understanding of how this emerging, but often overlooked stressor, affects animals without complex behaviors. Our integrated approach to noise research can be used as a model for other invertebrate species and faunal groups, and inform the development of effective methods for assessing and monitoring noise impacts. Given the observed negative effects, noise should be considered a potential confounding factor in studies involving other stressors.

KEYWORDS: *Mytilus edulis*; DNA damage; Oxidative stress; Algal clearance; Ecological performance; Marine

1. INTRODUCTION: The globally increasing levels of anthropogenic noise in our oceans caused by shipping, oil and gas exploration, and the installation of renewable energy devices, are of growing environmental and societal concern (Williams *et al*., 2015). Lower frequency noise (20 to 200 Hz), for example, propagates and persists over large distances and time scales, and shipping alone has led to a 10 to 100-fold rise in the oceans noise floor (Tyack, 2008). The full extent to which noise affects biota is not yet fully understood, particularly for marine invertebrates, one of the least studied groups in this context. Their ability to "hear", by perceiving the particle motion component of sound, has long been ignored. Given that invertebrates constitute approximately 60% of eukaryotic marine species (Ausubel *et al*., 2010), play pivotal roles in marine ecosystems

(Glynn and Enochs, 2011; Queirós *et al*., 2013) and are growing in commercial importance (Eddy *et al*., 2017; Fisheries F A O, 2016), there is an urgent need for more in depth studies, as highlighted in reports by the Convention of Biological Diversity (CBD, 2012) and OSPAR (Gotz *et al*., 2009) on the impacts of noise on invertebrates in the marine environment.

Here, we determine in controlled laboratory experiments how underwater noise affects the commercially (Marine Scotland Science and Mss, 2016) and ecologically (Borthagaray and Carranza, 2007) important blue mussel *Mytilus edulis*. A model species for ecotoxicological studies, *M. edulis* is a biogenic reef builder (Borthagaray and Carranza, 2007; Widdows and Brinsley, 2002), creating habitat for other organisms. Through filter-feeding these animals remove particulates from the water column improving overall water quality (Officer *et al*., 1982), and transporting essential nutrients to the benthos (Widdows and Brinsley, 2002). As a benthic marine invertebrate, *M. edulis* is able to perceive noise through contact with both substrate and surrounding water. Although this noise detection is not "hearing" as we humans perceive it, it still affords these animals a way of detecting changing noise levels in the environment. *M. edulis* (Roberts *et al*., 2015) and also its close relative *M. galloprovincialis* (Vazzana *et al*., 2016) are known to be sensitive to anthropogenic noise, however, how noise affects much of their biology is unknown. We take a novel approach testing and employing established ecotoxicological techniques (i.e. Comet Assay and oxidative stress tests) in combination with behavioral and physiological biomarkers to detect sublethal stress effects of noise exposure. This mechanistic, multi-method approach (Kight and Swaddle, 2011) enables the identification of subtle, visually hidden biochemical (structural DNA damage and oxidative stress) changes, as well as more obvious behavioral (algal clearance and valve movement) and physiological (oxygen

consumption) responses. This allows a more complete picture of how noise affects the biology of these animals to be generated.

2. METHODS: Permits and Ethical Approval: The work conducted required no specific permits but was conducted following the ethical guidelines of Edinburgh Napier University.

2.1. Animals and Husbandry: Individual *M. edulis* were manually collected at low tide two weeks prior to noise exposure (12 October 2015, 9 November 2015, 1 March 2016, 10 October 2016, and 27 October 2016) from Fisherrow Sands, Musselburgh, UK (55.94° N, 3.07° W). Following collection, the animals were transported to the St Abbs Marine Station (St Abbs, Berwickshire, UK) for noise exposure and sampling for biochemical experiments, or to the AquaLab at Edinburgh Napier University for behavioral and physiological experiments. Full details of husbandry are available in the electronic supplementary material.

2.2. Sound Recordings and Playback: Ship noise playbacks produced by Wale *et al*. (2013) were used in all experiments and presented to the animals using a similar set-up to this study, a full description of which can be found in the supplementary material.

Playbacks were presented at a sound level representing exposure to ship noise at approximately 200-300 m from the source (Erbe *et al*., 2012; McKenna *et al*., 2013) and for periods that would be experienced in regularly used shipping lanes. Received sound pressure levels at the position of the exposed mussels in the 670 L tank (DNA integrity and oxidative stress) peaked at 150-155 dB re 1 μPa^2Hz^{-1} for ship noise playbacks and 85-95 dB re 1 μPa^2Hz^{-1} for control conditions (Fig. 1A), as measured in PAMGuide (Merchant *et al*., 2015). Particle acceleration peaked at 160-165 dB re 1 $(\mu ms^{-2})^2Hz^{-1}$ for ship noise playbacks, and 140-148 dB re 1 $(\mu ms^{-2})^2Hz^{-1}$ for control conditions (Fig. 1B), as measured in paPAM (Nedelec *et al*., 2016). In the 120 L tanks

(algal filtration, oxygen consumption, and valve movement) the noise peaked at 140-145 dB re 1 μPa^2Hz^{-1} for ship noise and 85-100 dB re 1 μPa^2Hz^{-1} for ambient tank noise (Fig. 1A). Particle acceleration peaked at 165-175 dB re 1 $(\mu ms^{-2})^2Hz^{-1}$ for ship noise playbacks, and 150-155 dB re 1 $(\mu ms^{-2})^2Hz^{-1}$ for control conditions (Fig. 1B).

2.3. DNA Integrity: For each of the two experimental runs, following acclimation, the mussels were suspended on a tray (30 x 15 cm) midwater in a 675 L natural seawater tank in the same system as used for the holding tanks. The tray was vibrationally insulated from the tank walls by suspending it with nylon twine into the center of the exposure tank level with the subsurface speaker. Each treatment (noise and control) was run with two replicate groups of six mussels. The mussels were given 24 h to acclimate to the experimental tanks followed by exposure to either ship noise playback or silence playback as a control for six hours.

Following exposure, haemocytes and gills cells were isolated as per Hartl *et al*. (2010) and stored at 4ºC in osmotically corrected Hanks Balanced Salt Solution (Coughlan *et al*., 2002). Comet Assay analysis was performed on all samples within 24 hrs of collection following the procedure of Coughlan *et al*. (2002) and modified by Al-Shaeri *et al*. (2013). Prepared slides were viewed under an epifluorescence microscope (Zeiss Axioplan, Carl Zeiss Microscopy, Oberkochen, Germany), using Comet Assay IV software (Perceptive Instruments, Bury Saint Edmunds, UK). DNA damage is expressed as % tail DNA. To remove any potential bias, all samples were given a six-digit code prior to laboratory work, these codes were not revealed to the assays operator until all results were generated.

2.4. Oxidative Stress: Gill samples for oxidative stress assays were collected at the same time and from the same animals as those for the Comet Assay and flash frozen in liquid nitrogen. Samples were stored at -80 ºC until further analysis. In all assays the prepared microplates were read using a Spectramax M5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Due to sample restrictions the Glutathione and Glutathione Peroxidase assays were conducted solely on gills collected during the November exposure. Glutathione (GSH) and Thiobarbituric acid reactive substances (TBARS) assays were performed according to Smith *et al*. (2007). Glutathione Peroxidase (GPx) assays were completed using the BioVision Glutathione Peroxidase Activity Colorimetric Assay Kit (Catalog #K762-100). For superoxide dismutase (SOD) assays, the Sigma-Aldrich SOD determination Kit (19160) was used.

2.5. Oxygen Consumption: Following acclimation to the laboratory system (see above), individual mussels were placed into a custom built transparent acrylic respiration chamber (170 mm long and 85 mm diameter, Jemitech Technische Komponenten, Germany; Fig. S2) manually set, through a movable lid, to hold 200 ml of natural seawater, and placed in the center of a 120 L exposure tank. Mussels were given 23 h of acclimation to the respiration chamber, followed by exposure to either ship noise playback or silence playback as a control for one hour. During that time, the changing oxygen saturation inside the respiration chambers was measured every second with a computer-controlled setup using a Fibox 3 trace v3 fibre-optic trace oxygen meter (Presens – Precision Sensing, Regensburg, Germany) and a laptop (Acer E5-571 series, Acer inc., New Taipei City, Taiwan). Readings were adjusted against a blank for bacterial respiration and calculated per gram of mussel tissue. Two animals for each treatment and their matching blank chambers were measured each day over a five-day period. Mussels were measured individually and only used once. An alternating system of exposure (noise, control, noise, control) was

130 employed and this order reversed each day. Oxygen consumption was calculated using equations
131 adapted from Presens (2006), a full description of which can be found in the supplementary
132 material.

133 Oxygen consumption was plotted over time so that any sudden changes in consumption
134 could be easily seen and analyzed. It also prevented any changes from skewing the final result if
135 only total consumption rate was analyzed during the one hour exposure.

136 **2.6. Algal Filtration Rate**: A group of 25 similarly sized adult mussels (mean length 57.9 mm for
137 noise exposure, 58.2 mm for control animals) were placed in a 10 L (300 x 200 x 200 mm) tank,
138 which itself stood inside a 120 L exposure tank containing the noise source. The 10 L tank was
139 raised off the floor of the 120 L exposure tank and acoustically isolated from any transmitted
140 vibrations using neoprene matting. Both tanks contained natural filtered seawater from the aquaria
141 system and remained separate with no water transfer occurring. Inside the 10 L tank the mussels
142 were held on a raised mesh platform, allowing them to filter algae whilst preventing the build-up
143 of pseudofaeces, which, if resuspended, could have skewed the overall results. Animals were
144 starved for 48 h prior to noise exposure to remove any algae currently being digested, creating a
145 level feeding state across all animals. After starvation, the 10 L tank was inoculated with ≈ 3,000
146 cells ml-1 dried *Tetraselmis suecica* (ZMSystems, Hampshire, UK Riisgård *et al*. (1981). Mussels
147 were exposed to ship noise playback or silence playback as a control for three h. Five replicate 1
148 ml water samples were taken from the center of the tank midwater after 0, 90, and 180 min of
149 exposure. The tank water was vigorously stirred (a glass rod was moved across the width and
150 length of the tank) for 10 s to resuspend any settled algae and ensure that the samples taken were
151 representative of the effects of noise on the mussels' filtration, rather than an effect on the algal
152 settlement. Any turbulence created in this process was allowed to disperse prior to sample

153 collection. A total of five tanks were used for both the noise and control treatments, with one noise
154 and one control exposure taking place each day for five days. Each animal was used only once.

155 Algal cells were counted using a Sedgewick-Rafter counting cell. Each 1 mm x 1 mm
156 square was converted into an xy coordinate containing 1 µl of sample. 5 random squares per ml
157 sample were imaged in cellSens (Olympus, Southend on Sea, UK) and coded to remove bias when
158 the number of individual algal cells were manually counted. These readings were further converted
159 to filtration rate per g of mussel wet weight and, with data for live biomass per m^2 of mussel reef
160 extrapolated to obtain an estimated filtration rate reduction if the laboratory results were translated
161 to the field. Reef biomass was calculated through photographic analysis of 250 cm^2 quadrats.
162 Photos were taken for five quadrats, randomly placed within a 5 m radius of a marker pole (yacht
163 turning pole) in the area that the mussels were collected. From these quadrats, 10 individual
164 mussels were blindly selected and removed from the quadrat. Their length was then measured from
165 posterior to anterior tips of the shell and a cubic relationship fitted (Fig. S3) which was used to
166 convert mean mussel length to mean mussel weight. Total biomass was calculated by manually
167 counting the top layer of mussels in each quadrat (to restrict the number of potentially empty shells)
168 and multiplying this by the mean weight to gain biomass per m^2 of reef in the collection area. This
169 extrapolation assumes constant environmental conditions.

170 **2.7. Valve Movement**: Individual mussels were placed on a custom-built stand with their valve
171 opening pointing towards a GoPro Hero 4 Silver camera (GoPro Inc, San Mateo, CA, USA). The
172 stand was placed centrally inside the same 120 L tank used for the algal filtration rate and oxygen
173 consumption experiments. The mussels were acclimated to the experimental set-up for 24 h, after
174 which they were exposed to either ship noise playbacks or silence playback as a control for one h.
175 Valve movements were filmed throughout the exposure. To remove bias, video files were coded

until fully analyzed, and observed without sound. The resulting footage was manually analyzed for valve gape to the nearest mm between valves (mean generated from readings at five min intervals, 13 total readings over the one h of exposure), and valve opening time to the nearest s (presented as cumulative opening time). Any animal that remained closed from the start of the exposure for the entire exposure length was removed from the analysis to prevent skewing the results by zero inflation. A total of 10 mussels were filmed for each treatment, with two mussels filmed for each treatment each day, for five consecutive days. Each animal was used only once.

2.8. Statistical Analysis: Statistical analyses were performed in R version 3.3.1 (The R foundation for Statistical Computing). Data were tested for normality and heterogeneity of variance around the mean, normality was shown for all biochemical data and oxygen consumption without transformation, and algal filtration data with log transformation. Non-normality was identified for valve gape and opening time, and normality was shown for valve gape over time. Full explanation of employed statistical analysis can be found in the supplementary material.

Significance indicators for all experiments * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.0001$.

3. RESULTS AND DISCUSSION: Comet Assay analysis revealed that animals exposed in the first run of the experiment showed significantly more DNA damage in both the gills and haemolymph than those tested in the second run (two-way ANOVA, $F_{2,35} = 22.65$ $P < 0.001$ (Gill), $F_{2,35} = 7.36$ $P = 0.002$ (Haemo) Fig. 2A). In both runs mussels exposed to six hours of ship noise playback demonstrated significantly higher single strand breaks in the DNA of both haemocytes and gill epithelial cells compared to those exposed to a silent control (two-way ANOVA, $F_{1,35} = 573.40$ $P < 0.001$ (Gill), $F_{1,35} = 346.82$ $P < 0.001$ (Haemo) Fig. 2A). Approximately 25 - 33% tail

DNA occurred in noise exposed cells, six times higher than in control cells with only 5% damage. Additional biochemical tests were undertaken to identify causes of the observed DNA damage.

To detect whether noise causes a build-up of reactive oxygen species (ROS) that can trigger DNA damage (Alves de Almeida *et al*., 2007), we measured the presence of four oxidative stress endpoints, SOD, GSH, GPx, and TBARS. The SOD, GPx, and GSH assays did not identify significant oxidative stress (two-way ANOVA, $F_{1,42} = 0.062$ $P = 0.80$ (SOD), two sample t-test $t_{20.425} = 0.74$ $P = 0.47$ (GSH), $t_{17.256} = 0.79$ $P = 0.44$ (GPx), Fig. 2B to D). TBARS assays however revealed a significant 39% increase in malondialdehyde (two-way ANOVA $F_{51,37} = 4.93$ $P = 0.013$, Fig. 4E), indicating lipid peroxidation in the gill epithelia of noise exposed specimens, consistent with the observed DNA damage.

Changes in oxygen consumption and thus metabolic rate of mussels, in response to a one-hour exposure of ship noise playback, are indicated by a significant interaction between treatment and time (mixed-model ANOVA, ($F_{1, 218} = 4.90$, $P = 0.028$, Fig. 3A). Noise-exposed mussels consumed significantly less oxygen over time (linear regression slope, b ($mgL^{-1}g^{-1}h^{-1}$) = 0.00017, SE = 0.00001) than the control animals (linear regression slope, b ($mgL^{-1}g^{-1}h^{-1}$) = 0.00021, SE = 0.00002), with an overall reduction in oxygen consumption of 19%.

To investigate whether the filtration rate changes in response to noise, the water of the noise and control treatment tanks was supplemented with known algal cell quantities and subsamples counted at 90-minute intervals during a three-hour exposure. Noise exposed mussels consumed significantly less algal cells over the three-hour period than those in control conditions. The interaction between treatment and time was highly significant (mixed-model ANOVA, $F_{1, 138} = 41.96$, $P < 0.0001$, Fig. 3B). Mean cell count decreased significantly over time in the control

220 treatment (b = -0.483, SE = 0.047), whereas there was no such decline in the noise treatment (b =
221 -0.077, SE = 0.06). This difference corresponds to an 84% reduction in algal filtration rate in
222 response to noise. Extrapolating the observed reduced filtration rate to the density of mussels from
223 the reef where the experimental animals were sourced yields an estimated reduction of 247.1 ±
224 13.5 million algal cells per L of surrounding water removed every h for each square meter of
225 established mussel reef, assuming constant environmental conditions.

226 To investigate whether the observed reduction in algal clearance rate and oxygen
227 consumption could be attributed to a change in valve movement, mussels were filmed during a
228 one-hour exposure of either ship noise playback, or a silent control, and their valve gape (the
229 distance between each valve) and cumulative valve opening time recorded. Since the number of
230 animals that remained closed throughout the exposure did not differ between treatments ($\chi 2$ =
231 0.9524. P = 0.329114), suggesting that consistent valve closure was not related to noise, these
232 mussels were removed from further analysis (n=4 in noise and n=2 in control treatment). Valve
233 gape was significantly increased by 144% in noise exposed animals relative to control animals
234 (Wilcoxon rank sum test, W = 7, P = 0.033, Fig. 3C), while cumulative valve opening time did not
235 differ between the two treatments (Wilcoxon rank-sum test, W = 24 P = 1, Fig. 3D).
236
237 This study is the first to investigate DNA damage in response to noise in any marine
238 species. It is also, to the best of our knowledge, the first to use oxidative stress endpoints as
239 biomarkers of the effects of underwater noise in marine organisms. Such sub-cellular damage can
240 be a direct result of exposure to high intensity low frequency noise (Solé *et al*., 2013a, 2013b).
241 However, here this is unlikely due to the comparatively low (realistic) exposure level (150-155 dB
242 re 1 μPa^2Hz^{-1}). In our study it is more likely that Malondialdehyde, the end product of lipid

peroxidation and the endpoint of the TBARS assay, and the DNA damage found in the gill tissue of the mussels, occurred as a result of exposure to noise related metabolic stress and related oxidative radicals (Marnett, 1999; Barzilai and Yamamoto, 2004). The oxygen radicals could then have moved from the gills into the haemolymph, via the mussels open circulatory system (Yonge, 1976), causing the observed damage in haemocytes. Links between oxidative stress and DNA damage are well known (Alves de Almeida *et al*., 2007), and both are common biochemical markers for stress. As shown here, the application of established ecotoxicological techniques, i.e. the Comet Assay and oxidative stress assays can greatly benefit the field of noise research. When investigating species that produce little visually obvious responses to anthropogenic noise, these assays are key to revealing (cryptic) effects of noise and thus enable a better understanding of how this emerging but often overlooked stressor affects animals without complex behaviors.

Elevated stress in noise-exposed mussels was further indicated by reduced oxygen consumption, despite increased valve gape. This seemingly converse reaction is more akin to a shock response (Bracha, 2004; Gladwin *et al*., 2016) than that of a general stress response. The exposed mussels seem to have been startled by the onset of noise, and attempted to conserve energy, and therewith reduced oxygen demand through relaxation of the adductor muscles, causing the observed opening of the valves (Livingstone, 2013).

In addition to organismal level effects, which may influence mussel growth, survival and reproductive success, the observed decline in algal clearance rate indicates that noise can also reduce mussel ecological performance. *M. edulis* clears particulates from the surrounding water and deposits them on the seafloor in the form of faeces and pseudofaeces (Garrido *et al*., 2012). A reduction in the overall filtration rate caused by noise would thus have important carry-over effects by reducing the role of mussels in benthic-pelagic coupling. Our extrapolations were made using

data generated from a fixed volume of water with a known algal content, and as such the experienced environmental differences may change with variance in environmental conditions.

Given the wide distribution of mussels in areas where they may be exposed to noise, impacts do not appear sufficient to result in extirpation from high noise areas, but this does not preclude habituation, or the existence of cryptic effects, such as suboptimal growth. Removal of noise from the environment has been shown to improve the condition of *Crangon crangon* through reduced oxygen consumption and ammonia excretion, along with increased growth and reproduction (Regnault and Lagardere, 1983). A similar effect could be seen in *M. edulis* if noise was removed from the areas surrounding their assemblages. *M. edulis* used in this study were intertidal and the noise levels they would experience in their natural environment vary with tidal inundation. As such, the likelihood of habituation to anthropogenic noise is reduced, with regular non-continuous exposure to noise resulting in a persistant negative effect on marine organisms (Bolger *et al*., 2018; Harding *et al*., 2018) as they are unable to build up a tolerance to this stimulus.

Using a mechanistic multi-method approach for investigating the effects of noise on *M. edulis* allowed the characterization of individual (and sometimes cryptic) effects, underlying drivers, and interactions. This integrated approach to noise research can be used as a model for other invertebrate species and faunal groups (Kunc *et al*., 2016; Sabet *et al*., 2012) and inform the development of effective methods for assessing and monitoring noise impacts. Our study also shows that noise needs to be considered as a potentially confounding factor in any laboratory trials aiming to determine the effects of other stressors, such as chemical pollutants, where laboratory noise could affect the generated results. Likewise, field monitoring programs for pollutants, e.g. the NOAA Mussel Watch Program (Kimbrough *et al*., 2008), should regard noise as a potential (co)contaminant, and take potential noise exposure into account.

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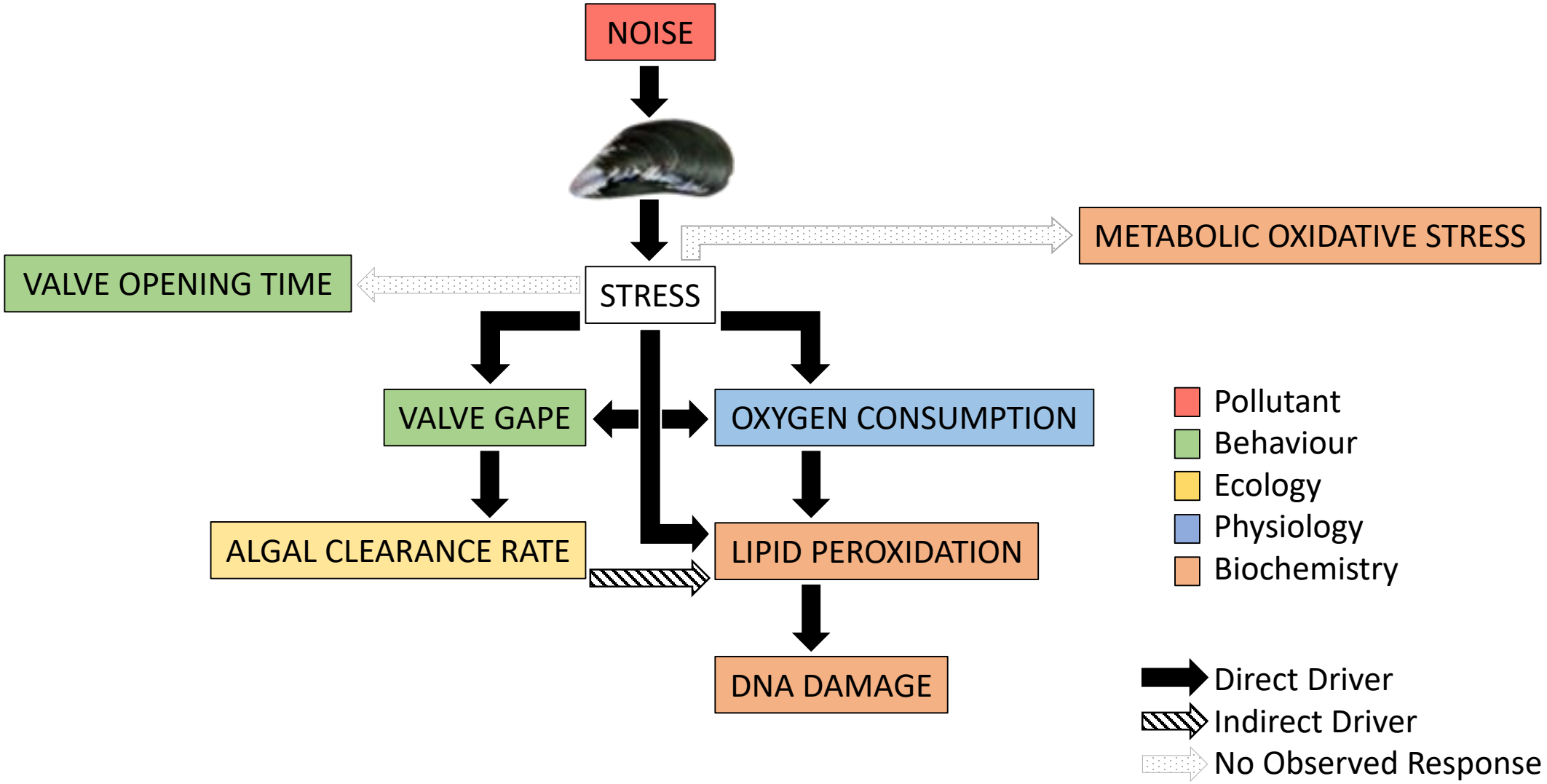

NOISE
METABOLIC OXIDATIVE STRESS
VALVE OPENING TIME
STRESS
VALVE GAPE
OXYGEN CONSUMPTION
ALGAL CLEARANCE RATE
LIPID PEROXIDATION
DNA DAMAGE
Pollutant
Behaviour
Ecology
Physiology
Biochemistry
Direct Driver
Indirect Driver
No Observed Response

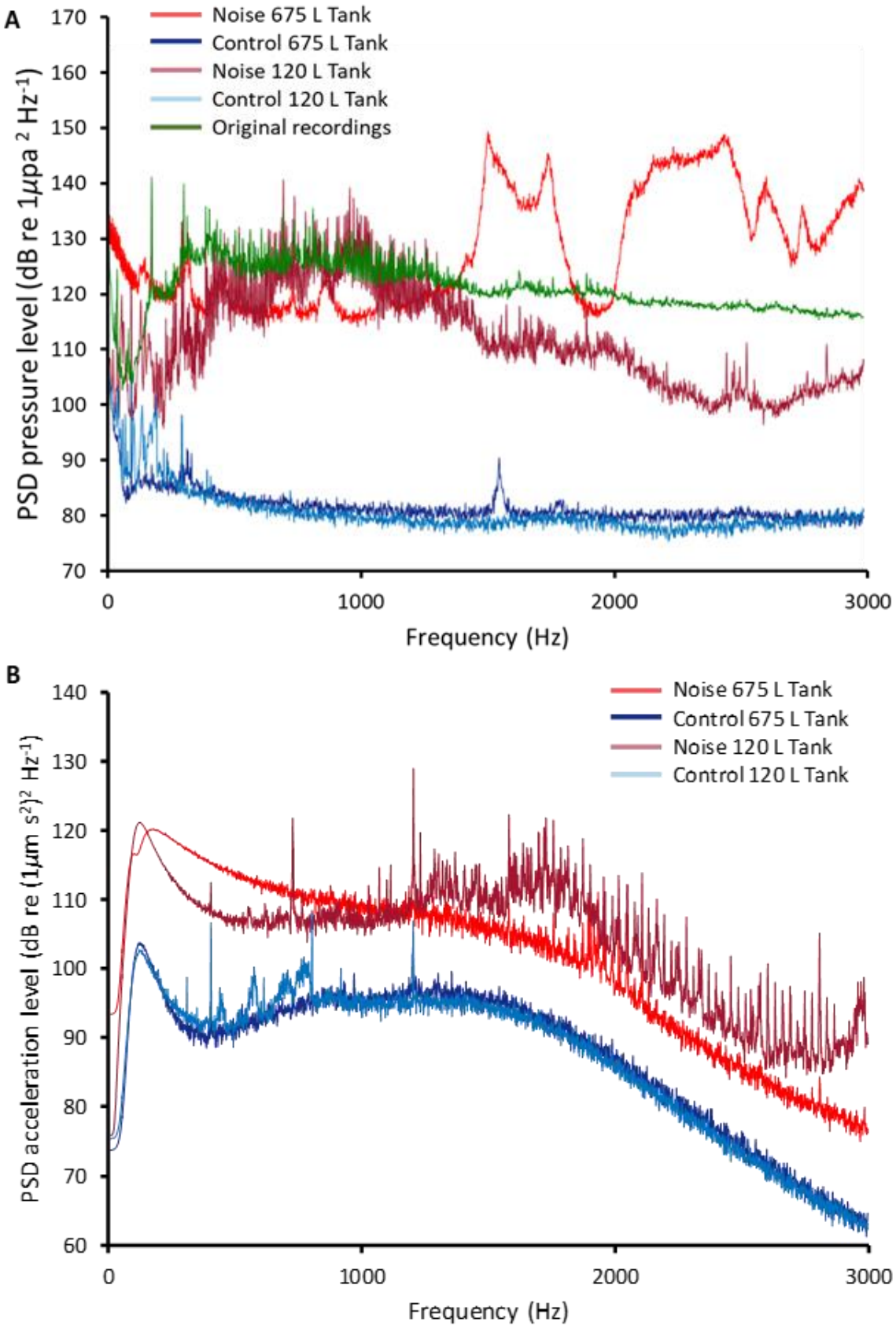
HIGHLIGHTS:

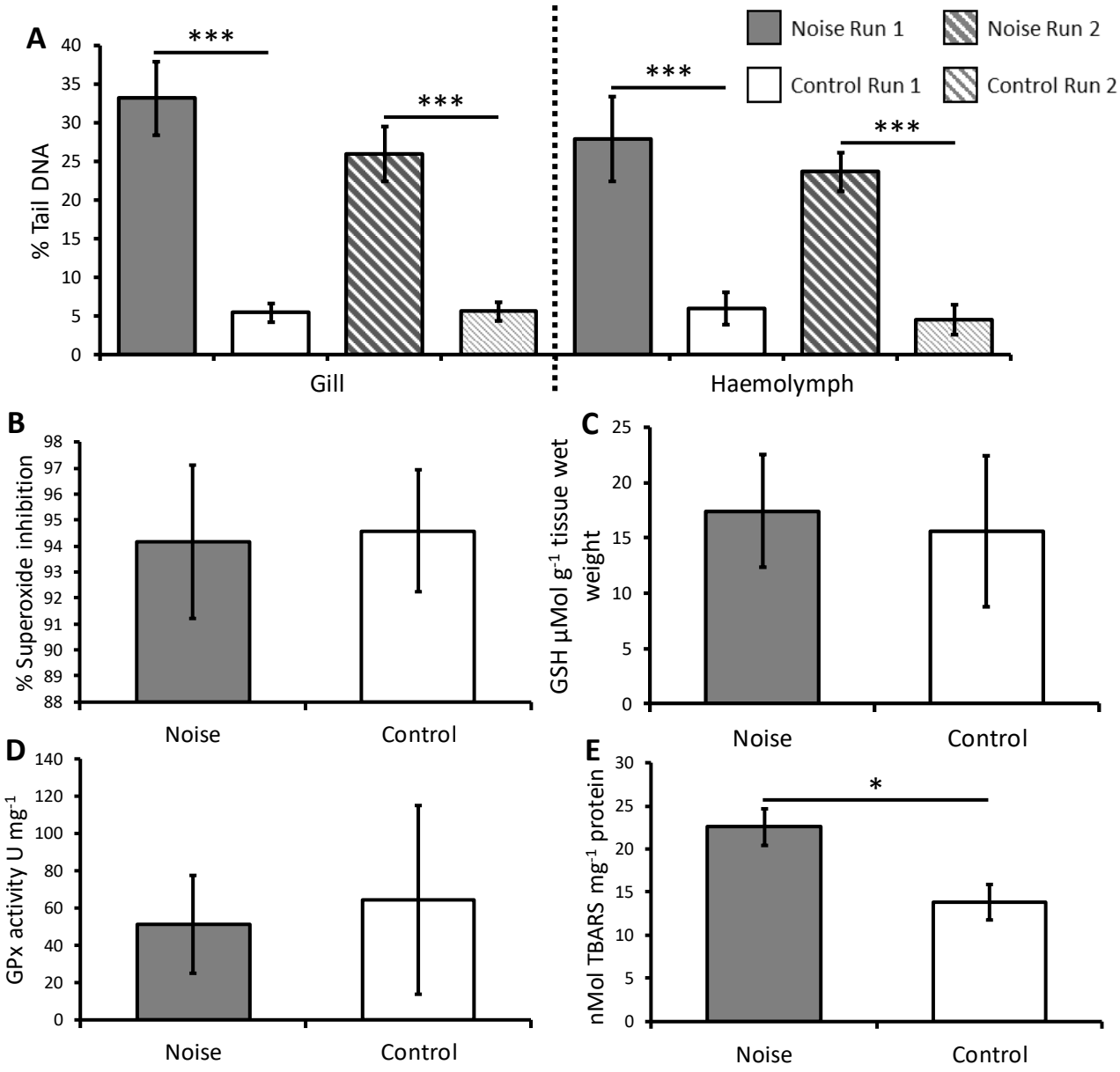
- Evidence of noise induced changes at multiple levels of biological organization
- DNA damage in mussel gills and haemolymph following anthropogenic noise playbacks
- Changes in oxygen consumption and filtration rate also evident
- Potential impact on ecological performance of biogenic reefs
- Noise should be considered a potential confounding factor in other stressor studies

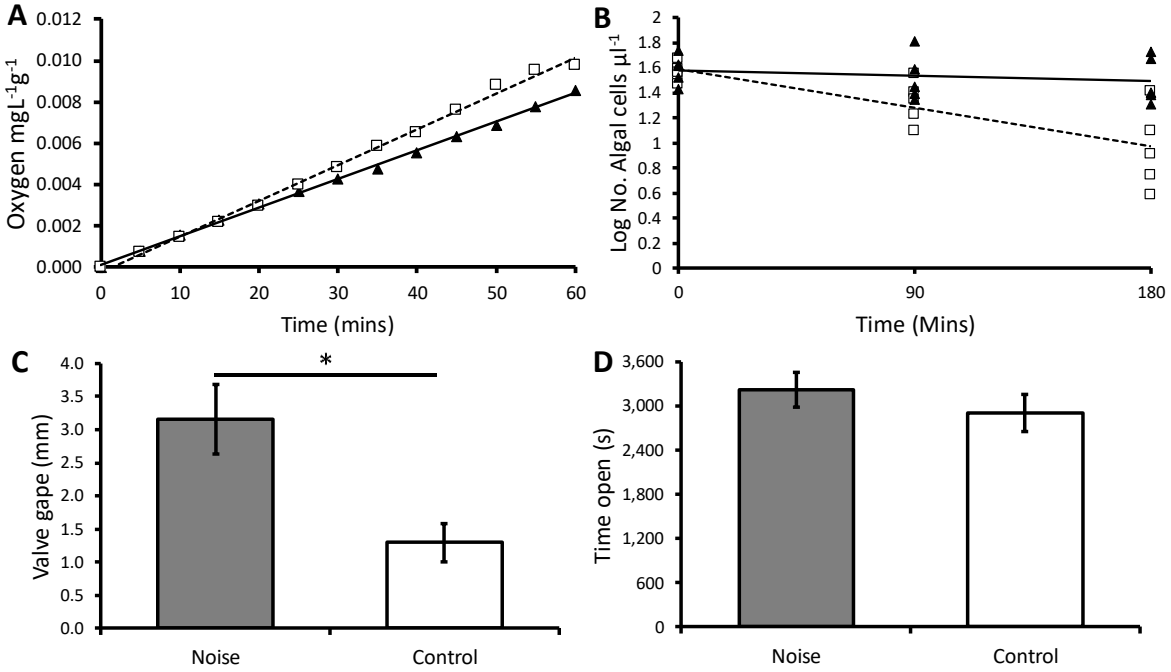
Figure 1. Analysis of acoustic stimuli and sound playback conditions. Mean power spectral density of 30 s of each sound condition of (**A**) acoustic pressure and (**B**) particle acceleration, for control and exposure conditions in both the 675 l (DNA integrity and oxidative stress) and 120 l (Oxygen consumption, algal filtration, and valve movement) tanks. In both the pressure and particle motion domains there was a clear difference between the noise exposure and the control conditions. Analysis performed in MATLAB R2015b (pressure) and MATLAB Compiler Runtime R2013a (particle acceleration). fft lengths = 48 kHz (pressure) and 44.1 kHz (particle acceleration), both resulting in 1 Hz bands.

Figure 2. Effects of ship-noise playbacks on the biochemistry of *Mytilus edulis*. (**A**) Mean ± Stdev percentage tail DNA of gill and haemolymph*** (n = 9 for noise run 1, n = 10 for all other treatments and times). (**B**) Mean ± Stdev % SOD inhibition in gills (n = 21 for both treatments). (**C**) Mean ± Stdev GSH µMol g^{-1} tissue wet weight (n = 12 for both treatments). (**D**) Mean ± Stdev GPx activity U mg^{-1} (n = 12 control, n = 9 noise). (**E**) Mean ± Stdev nMol TBARS mg^{-1} protein in gills* (n = 21 for both treatments).

Figure 3. Effects of ship-noise playbacks on the behavior and physiology of *Mytilus edulis*. (**A**) Oxygen consumed (mg L^{-1}) per g of *M. edulis* tissue* over 1 h of noise or control exposure (n = 10 for both treatments). (**B**) Consumed algal cells μl^{-1} seawater*** (n = 5 for both treatments). (**C**) Mean ± Stdev valve gape* (n = 6 for noise, n = 8 for control). (**D**) Mean ± Stdev seconds with valve open (n = 6 for noise, n = 8 for control).


A
Noise 675 L Tank
Control 675 L Tank
Noise 120 L Tank
Control 120 L Tank
Original recordings
PSD pressure level (dB re 1μpa ² Hz-1)
170
160
150
140
130
120
110
100
90
80
70
0
1000
2000
3000
Frequency (Hz)
B
Noise 675 L Tank
Control 675 L Tank
Noise 120 L Tank
Control 120 L Tank
PSD acceleration level (dB re (1μm s-2)2 Hz-1)
140
130
120
110
100
90
80
70
60
0
1000
2000
3000
Frequency (Hz)


A
% Tail DNA
Noise Run 1
Noise Run 2
Control Run 1
Control Run 2
***
***
***
***
Gill
Haemolymph
B
% Superoxide inhibition
Noise
Control
C
GSH µMol g-1 tissue wet weight
Noise
Control
D
GPx activity U mg-1
Noise
Control
E
nMol TBARS mg-1 protein
*
Noise
Control


A
Oxygen mgL-1g-1
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0
10
20
30
40
50
60
Time (mins)
B
Log No. Algal cells µl-1
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
90
180
Time (Mins)
C
Valve gape (mm)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Noise
Control
D
Time open (s)
3,600
3,000
2,400
1,800
1,200
600
0
Noise
Control

From DNA to Ecological Performance: Effects of Anthropogenic Noise on a Reef-Building Mussel

Matthew A. Wale, Robert A. Briers, Mark G. J. Hartl, David Bryson, Karen Diele

Supplementary Information

1. INDEPTH METHODS

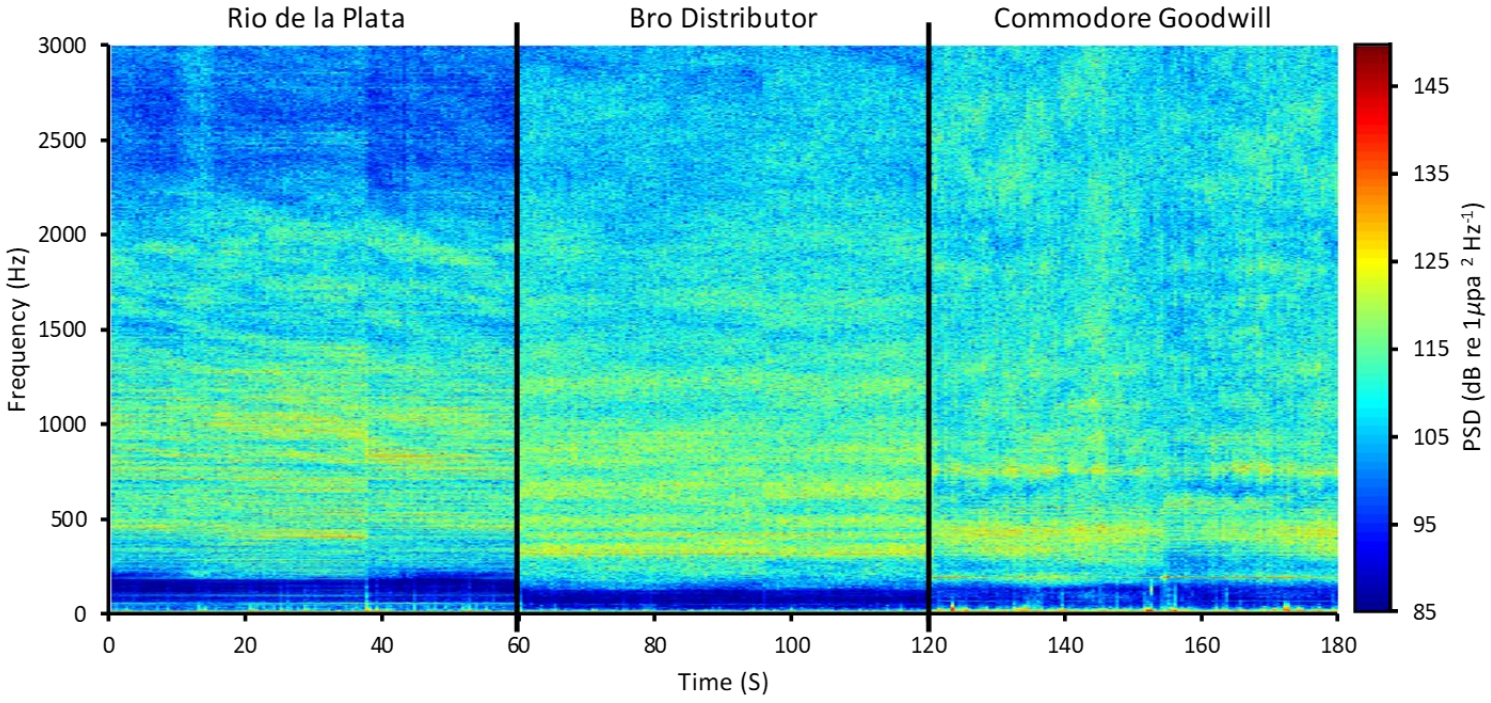
1.1. Animals and Husbandry: Individual *Mytilus edulis* were manually collected at low tide two weeks prior to noise exposure (12 October 2015, 9 November 2015, 1 March 2016, 10 October 2016, and 27 October 2016) from Fisherrow Sands, Musselburgh, UK (55.94° N, 3.07° W). The area experiences anthropogenic noise caused through frequent irregular watercraft and traffic of larger ships further afield. The mussel beds themselves are intertidal, and as such regularly receive natural noise from waves and tidal movements. Following collection, the animals were transported to the St Abbs Marine Station (St Abbs, Berwickshire, UK) for noise exposure and sampling for biochemical experiments or to the AquaLab at Edinburgh Napier University for behavioral and physiological experiments. Once on site the animals were cleaned of all macroscopic epibiota, predominately consisting of *Elminius modestus* and *Semibalanus balanoides* barnacles, by carefully scrapping the edge of an oyster knife along the shell of the mussel.

At St Abbs Marine Station the mussels were housed in groups of 40 animals suspended in mesh bags inside a 675 L (1040 x 1000 x 650 mm) holding tank with flow-through natural seawater. Both the holding tank and the experimental tanks (same volume and dimensions as holding tanks) were fitted with a subsurface inflow pipe to prevent noise from falling water or collision with the tank floor, minimizing ambient sound levels. The tanks were isolated from

the surrounding surfaces with neoprene rubber to minimize vibration transmission. The flow rate of this system varied (192-384 L per hour) over the course of acclimation depending on tides, due to the raw seawater draw design of the Marine Station. Water parameters were monitored throughout acclimation (temperature, salinity, and pH measured by WTW Multi 3430 {Xylem Analytics - WTW, Weiheim, Germany} chemical parameters measured with Salifert Profi-Test kits {Salifert, Holland}. Salinity and temperature in the tanks matched the surrounding coastal waters and therefore varied daily depending on flow rate (10 - 14 ^{o}C, salinity 32 - 35 ppt; NO_2^-: < 0.3 mg L^{-1}; NO_3^-: 0 mg L^{-1}; NH_4^+: ≤ 0.25 mg L^{-1}; pH: 7.8 - 7.9). At Edinburgh Napier University, the mussels were housed in 120 L (788 x 528 x 306 mm) flow-through tanks within a closed recirculation system of natural seawater (12 - 13 ^{o}C, salinity 32 - 35 ppt; NO_2^-: < 0.3 mg L^{-1}; NO_3^-: 0 mg L^{-1}; NH_4^+: ≤ 0.25 mg L^{-1}; pH: 7.8 - 7.9). The holding tanks were kept in an insulated temperature-controlled room, with soundproofing to remove noise from other laboratories. The holding tanks were separated from adjacent surfaces by anti-vibrational matting to prevent the transfer of vibration from any surrounding activity. All animals were acclimated in holding tanks for two weeks prior to the onset of the experiments and allowed an additional 24 h acclimation to the experimental set-up prior to any noise exposure.

1.2. Sound Recordings and Playback: Ship noise playbacks produced by Wale *et al*. (2013) were used in all experiments. The tracks were compiled in Audacity® 2.0.5 and included a 30 s fade in, 6.5 min of ship noise and a 30 s fade out for each of the recorded vessels. A random selection of these tracks was compiled to create a six h playback track of continuous ship passes (Fig. S1). Experimental tracks were played back as WAV files. The set-up consisted of an Mp3 player (SanDisk Sansa clip+ 8GB, frequency range 10-20,000 Hz, Western Digital Technologies Inc., Irvine, CA, U.S.A); amplifier (Pioneer A-10-K, 50W, frequency response: 20-20,000 Hz, Pioneer Corporation, Tokyo, Japan); and Clark Synthesis AQ339 underwater

speaker (effective frequency range 20-17,000 Hz, Clark Synthesis Inc., Littleton, CO, U.S.A). To ensure consistency between sound levels of replicate tanks in each treatment, tracks were re-recorded in the center of the experimental tank (HiTech HTI-94-SSQ with inbuilt preamplifier, Roland R-26 24-bit recorder – calibrated with the methods above and PAMGuide; (*30*)) and modified (uniform amplification or attenuation) until reaching the desired exposure pressures. Particle acceleration was measured using a custom-built calibrated sensor (Wale *et al*., in prep), consisting of a STMicroelectronics LIS344ALH triaxial accelerometer (STMicroelectronics, Geneva, Switzerland) potted in clear epoxy resin and suspended via 1.0mm diameter elastic cord to two interlocking 3D printed nylon rings. The accelerometer was linked to a Roland R-26 24-bit recorder for recording. Particle acceleration was recorded separately for all three axes and combined internally within paPAM (*31*) during the analysis



process.

Figure S1. Spectral analysis of acoustic stimuli. Power spectral density of 1 min of each ship pass used in the generation of the playback tracks for all exposures in both the 675 L and 120 L tanks. Original recordings taken at 200-300 m from the passing ships. Analysis performed in

MATLAB R2015b, Hann window with 1 s length and 50 % overlap, fft length = 48 kHz resulting in 1 Hz bands.

Playbacks were presented at a sound level representing exposure to ship noise at approximately 200-300 m from the source (Erbe *et al*., 2012; McKenna *et al*., 2013) and for periods that would be experienced in regularly used shipping lanes. Received sound pressure levels at the position of the exposed mussels in the 670 L tank (DNA integrity and oxidative stress) peaked at 150-155 dB re 1 μPa^2Hz^{-1} for ship noise playbacks and 85-95 dB re 1 μPa^2Hz^{-1} for control conditions (Fig. 1A), as measured in PAMGuide (*30*). Particle acceleration peaked at 160-165 dB re 1 $(\mu ms^{-2})^2Hz^{-1}$ for ship noise playbacks, and 140-148 dB re 1 $(\mu ms^{-2})^2Hz^{-1}$ for control conditions (Fig. 1B), as measured in paPAM (*31*). In the 120 L tanks (algal filtration, oxygen consumption, and valve movement) the noise peaked at 140-145 dB re 1 μPa^2Hz^{-1} for ship noise and 85-100 dB re 1 μPa^2Hz^{-1} for ambient tank noise (Fig. 1A). Particle acceleration peaked at 165-175 dB re 1 $(\mu ms^{-2})^2Hz^{-1}$ for ship noise playbacks, and 150-155 dB re 1 $(\mu ms^{-2})^2Hz^{-1}$ for control conditions (Fig. 1B).

For all experiments a control was created using a track of silence presented in the same way as the ship noise tracks. These silent controls consisted of six h of silence generated in Audacity 2.0.5 and played through the sound equipment to control for potential electromagnetic effects, and any internal noise generated by the equipment. Acute (<12 h) noise exposure was presented. Acute exposure was presented in all experiments and chosen so the initial reaction to noise, and any negatives associated with it, could be identified.

1.3. DNA Integrity: For each of the two experimental runs, following acclimation, the mussels were suspended on a tray (30 x 15 cm) midwater in a 675 L natural seawater tank in the same system as used for the holding tanks. The tray was vibrationally insulated from the tank walls

by suspending it with nylon twine into the center of the exposure tank level with the subsurface speaker. Each treatment (noise and control) was run with two replicate groups of six mussels. A size deviation of no more than 5 mm between the largest and smallest animal was maintained across treatments (size range and mean [mm], Run 1: N1, 48.2 – 54.4, 51.5; N2, 48.0 – 52.6, 50.3; C1, 49.6 – 55.1, 52.4; C2, 49.2 – 50.5, 49.7; Run 2: N1, 41.4 – 52.7, 48.3; N2, 47.7 – 53.7, 49.1, C1, 47.1 – 51.2, 48.8, C2, 48.3 – 53.6, 50.3). The mussels were given 24 h to acclimate to the experimental tanks followed by exposure to either ship noise playback or silence playback as a control for six hours.

Following exposure, haemocytes and gills cells were isolated as per Hartl *et al*. (2010) and stored at 4ºC in osmotically corrected Hanks Balanced Salt Solution (Coughlan *et al*., 2002). Comet Assay analysis was performed on all samples within 24 hrs of collection following the procedure of Coughlan *et al*. (2002) and modified by Al-Shaeri *et al*. (2013). Prepared slides were viewed under an epifluorescence microscope (Zeiss Axioplan, Carl Zeiss Microscopy, Oberkochen, Germany), using Comet Assay IV software (Perceptive Instruments, Bury Saint Edmunds, UK). DNA damage is expressed as % tail DNA. To remove any potential bias, all samples were given a six-digit code prior to laboratory work, these codes were not revealed to the assays operator until all results were generated.

1.4. Oxidative Stress: Gill samples for oxidative stress assays were collected at the same time and from the same animals as those for the Comet Assay and flash frozen in liquid nitrogen. Samples were stored at -80 ºC until further analysis. In all assays the prepared microplates were read using a Spectramax M5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Due to sample restrictions the Glutathione and Glutathione Peroxidase assays were conducted solely on gills collected during the November exposure. Glutathione (GSH) and Thiobarbituric acid reactive substances (TBARS) assays were performed according to Smith *et al*. (2007). Glutathione Peroxidase (GPx) assays were completed using the BioVision

Glutathione Peroxidase Activity Colorimetric Assay Kit (Catalog #K762-100). For superoxide dismutase (SOD) assays, the Sigma-Aldrich SOD determination Kit (19160) was used.

1.5. Oxygen Consumption: Following acclimation to the laboratory system (see above), individual mussels were placed into a custom built transparent acrylic respiration chamber (170 mm long and 85 mm diameter, Jemitech Technische Komponenten, Germany; Fig. S2) manually set, through a movable lid, to hold 200 ml of natural seawater, and placed in the center of a 120 L exposure tank. Mussels were acclimated to the respiration chambers for 23 h during which the water was actively pumped from the surrounding tank through the respiration chamber at 150 L h^{-1}. During the 23 h acclimation and subsequent exposure, the chambers were covered by a felt sleeve to eliminate any potential visual stimuli from the surrounding laboratory. At the onset of the exposures the water flow through the respiration chambers was stopped and mussels exposed to either ship noise playback or silence playback as a control for one hour. During that time, the changing oxygen saturation inside the respiration chambers was measured every second with a computer-controlled setup using a Fibox 3 trace v3 fibre-optic trace oxygen meter (Presens – Precision Sensing, Regensburg, Germany) and a laptop (Acer E5-571 series, Acer inc., New Taipei City, Taiwan). Readings were adjusted against a blank for bacterial respiration and calculated per gram of mussel tissue. A total of two animals for each treatment and their matching blank chambers were measured each day over a five-day period. Mussels were measured individually and only used once. An alternating system of exposure (noise, control, noise, control) was employed and this order reversed each day.

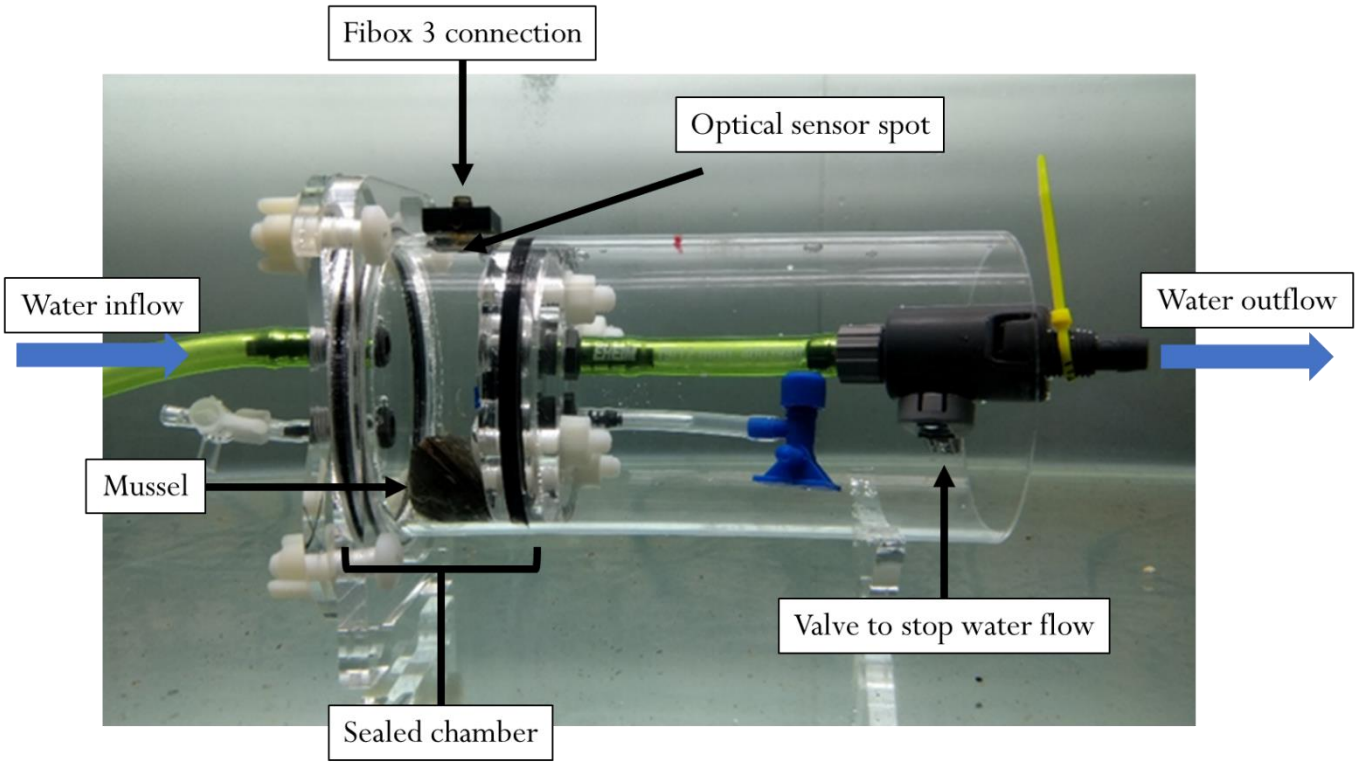


Figure S2. Oxygen consumption set-up. Custom built size-adjustable respiration chamber, sealed area calibrated to hold 200ml of water when empty. Tank dimensions: 788 x 528 x 306 mm, speaker placed 120 mm from the tank wall and 70 mm from the tank floor. Respiration chamber placed 250 mm from the speaker. Noise levels measured directly behind the sealed chamber holding the mussel without the end acrylic attached.

Following noise exposure, the mussels' wet weight to the nearest centigram, water displacement (ml), length from posterior to anterior shell tip, and width across the widest area of the shell (mm) were recorded. The mean length of tested mussels was 51.4 mm for noise exposure, and 50.4 mm for control animals, and did not significantly differ (two-sample *t*-test: $t_{16.96} = 0.4275$, $P = 0.67$). The temperature (12 – 13 ºC, recorded via the Fibox 3 trace) and ambient air pressure (hPa, recorded using Met office data (Met Office, 2016)) were recorded for each exposure period, for use in the below oxygen consumption calculations. Oxygen consumption was calculated using equations adapted from Presens (2006).

Oxygen saturation readings (%O_2) were converted to mg L^{-1} using the following equations:

$$C_{O_2}\left[mg\,L^{-1}\right] = \frac{P_{atm} - P_W(T)}{P_N} \cdot \frac{\%O_2}{100} \cdot 0.2095 \cdot \alpha(T) \cdot 1000 \cdot \frac{M(O_2)}{V_m} \quad \text{(I)}$$

P_{atm}: Atmospheric pressure at time of measurement

T = Temperature in Kelvin of water at time of measurement

$P_W(T)$: vapor pressure of water at T

P_N: Standard pressure (1013 hPa)

0.2095: Volume content of oxygen in air

$\alpha(T)$: Bunsen absorption coefficient at T; given in $cm^2(0_2)\ cm^{-3}$

$M(O_2)$: Molecular mass of oxygen (32 g mol^{-1})

V_M: Molar volume of oxygen (22.414 L mol^{-1})

$$P_W(T) = \exp\left[A - \frac{B}{T} - C \cdot \ln T\right] \quad \text{(II)}$$

A = 52.57

B = 6690.9

C = 4.681

$$\alpha(T) = \alpha + b \cdot \theta + c \cdot \theta^2 + d \cdot \theta^3 + e \cdot \theta^4 \quad \text{(III)}$$

a = 48.998

b = -1.335

c = $2.755*10^{-2}$

Θ = Temperature in ^{o}C of water at time of measurement

d = $-3.220*10^{-4}$

e = $1.598*10^{-6}$

Oxygen consumption was then calculated per g of mussel tissue using the following equations:

$$O_2\ Consumption\left[mg\ L^{-1}\right] = \frac{Initial_{O_2} - Final_{O_2}}{Water\ volume} \quad \text{(IV)}$$

$$O_2\ Consumption\left[mg\ L^{-1}\ g^{-1}\right] = \frac{Mussel_{O_2} - Blank_{O_2}}{Mussel\ weight} \quad \text{(V)}$$

Oxygen consumption was plotted over time so that any sudden changes in consumption could be easily seen and analyzed. It also prevented any changes from skewing the final result if only total consumption rate was analyzed during the one hour exposure.

1.6. Algal Filtration Rate: A group of 25 similarly sized mussels were placed in a 10 L (300 x 200 x 200 mm) tank, which itself stood inside a 120 L exposure tank containing the noise source. The mean length of tested mussels was 57.9 mm for noise exposure, and 58.2 mm for control animals, and did not significantly differ (two-sample *t*-test: $t_{16.96} = 0.464$, $P = 0.832$). The 10 L tank was raised off the floor of the 120 L exposure tank using an inverted plastic tray drilled through to allow the escape of air bubbles when submerging, and acoustically isolated from any transmitted vibrations using neoprene matting. Both tanks contained natural filtered seawater from the aquaria system and remained separate with no water transfer occurring. Inside the 10 L tank the mussels were held on a raised mesh platform, allowing them to filter algae whilst preventing the build-up of pseudofaeces, which, if resuspended, could have skewed the overall results. Animals were starved for 48 h prior to noise exposure to remove any algae currently being digested creating a level feeding state across all animals. After starvation, the 10 L tank was inoculated with ≈ 3,000 cells ml-1 dried *Tetraselmis suecica* (ZMSystems, Hampshire, UK) (Riisgård *et al*., 1981). Mussels were exposed to ship noise playback or silence playback as a control for three h. Five replicate 1 ml water samples were taken from the center of the tank midwater after 0, 90, and 180 min of exposure. The tank water was vigorously stirred (a glass rod was moved across the width and length of the tank) for 10 s to resuspend any settled algae and ensure that the samples taken were representative of the effects of noise on the mussels' filtration, rather than an effect on the algal settlement. Any turbulence created in this process was allowed to disperse prior to sample collection. A total of five tanks were used for both the noise and control treatments, with one noise and one control exposure taking place each day for five days. Each animal was used only once.

Algal cells were counted using a Sedgewick-Rafter counting cell. Each 1 mm x 1 mm square was converted into an xy coordinate containing 1 µl of sample. 5 random squares per ml sample were imaged in cellSens (Olympus, Southend on Sea, UK) and coded to remove

bias when the number of individual algal cells were manually counted. These readings were further converted to filtration rate per g of mussel wet weight and, with data for live biomass per m^2 of mussel reef extrapolated to obtain an estimated filtration rate reduction if the laboratory results were translated to the field. Reef biomass was calculated through photographic analysis of 250 cm^2 quadrats. Photos were taken for five quadrats, placed within a 5 m radius of a marker pole (yacht turning pole) in the area that the mussels were collected. From these quadrats, 10 individual mussels were blindly selected and removed from the quadrat. Their length was then measured from posterior to anterior tips of the shell. Their length was then measured from posterior to anterior tips of the shell and a cubic relationship fitted (Fig. S3) which was used to convert mean mussel length to mean mussel weight. Total biomass was calculated by manually counting the top layer of mussels in each quadrat (to restrict the number of potentially empty shells) and multiplying this by the mean weight to gain biomass per m^2 of reef in the collection area. This extrapolation assumes constant environmental conditions.

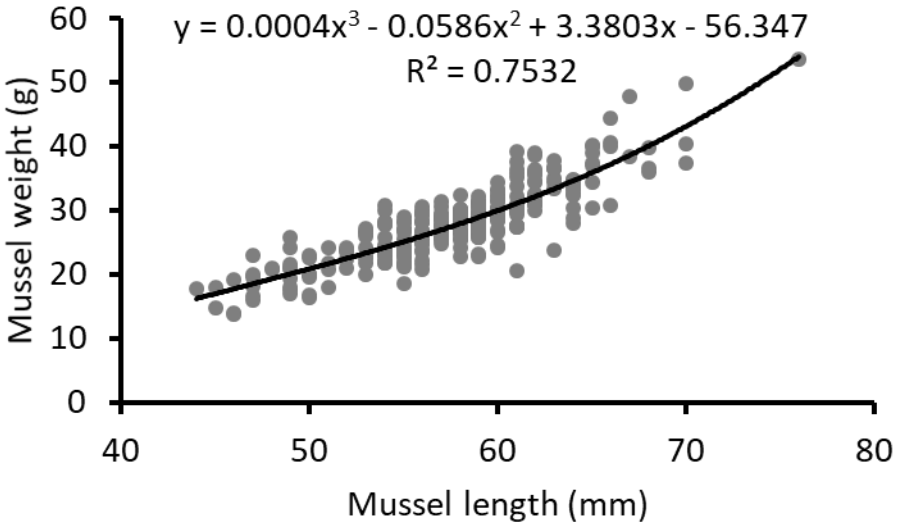


Figure S3. Mussel size calculation. Regression on scatter plot of average *M. edulis* size generated from the size metrics collected throughout all experiments.

1.7. Valve Movement: Individual mussels were placed on a custom-built stand with their valve opening pointing towards a GoPro Hero 4 Silver camera (GoPro Inc, San Mateo, CA, USA). The stand was placed centrally inside the same 120 L tank used for the algal filtration rate and oxygen consumption experiments. The mussels were acclimated to the experimental set-up for 24 h, after which they were exposed to either ship noise playbacks or silence playback as a control for one h. Valve movements were filmed throughout the exposure. To remove bias, video files were coded until fully analyzed, and observed without sound. The resulting footage was manually analyzed for valve gape to the nearest mm between valves (mean generated from readings at five min intervals, 13 total readings over the one h of exposure), and valve opening time to the nearest s (presented as cumulative opening time). Any animal that remained closed from the start of the exposure for the entire exposure length was removed from the analysis to prevent skewing the results by zero inflation. A total of 10 mussels were filmed for each treatment, with two mussels filmed for each treatment each day, for five consecutive days. Each animal was used only once.

1.8. Statistical Analysis: Statistical analyses were performed in R version 3.3.1 (The R foundation for Statistical Computing). Data were tested for normality and heterogeneity of variance around the mean, normality was shown for all biochemical data and oxygen consumption without transformation, and algal filtration data with log transformation. Non-normality was identified for valve gape and opening time, and normality was shown for valve gape over time.

Two sample t-tests were used to compare the DNA damage between treatment tanks testing the % tail DNA (dependent variable) against treatment (independent variable). Two-way ANOVAs were employed to test the effects of both the run (independent variable) and treatment (independent variable), with tank as a nested variable, on % tail DNA (dependent variable) for both gills and haemolymph. Additionally, two-way ANOVAs were performed to

test the effects of run (independent variable) and treatment (independent variable) on both superoxide dismutase inhibition (dependent variable) and the presence of thiobarbituric acid reactive substances (dependent variable). Two sample t-tests were used to test the effect of treatment (independent variable) on both glutathione concentrations (dependent variable) and glutathione peroxidase activity (dependent variable).

A repeated-measure mixed-model ANOVA was used to assess the effect of the interaction between treatment (independent variable) and time (independent variable) on oxygen consumption in the form of O_2 saturation (dependent variable). The individual mussel was considered as a random effect in the model. A similar repeated-measure mixed-model ANOVA was used to assess the effect of the interaction between treatment (independent variable) and time (independent variable) on algal clearance in the form of log transformed algal cell count (dependent variable). The exposure tank was considered a random effect in the model. Time was considered as a fixed effect as time points were decided in advance and were consistent throughout all exposures. Wilcoxon rank sum tests were used to assess the effect of treatment (independent variable) on the valve gape (dependent variable) and valve opening time (dependent variable). A mixed-model ANOVA was run to assess the effect of the interaction between treatment (independent variable) and time (independent variable) on valve gape (dependent variable). Time was considered a random effect within this model.

Significance indicators for all experiments * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.0001$.

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