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Effects of pile driving sound playbacks and cadmium co-exposure on the early life stage development of the Norway lobster, *Nephrops norvegicus*

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ABSTRACT

There is an urgent need to understand how organisms respond to multiple, potentially interacting drivers in today's world. The effects of the pollutants anthropogenic sound (pile driving sound playbacks) and waterborne cadmium were investigated across multiple levels of biology in larval and juvenile Norway lobster, *Nephrops norvegicus* under controlled laboratory conditions. The combination of pile driving playbacks (170 $dB_{pk\text{-}pk}$ re 1 μPa) and cadmium combined synergistically at concentrations >9.62 $\mu g_{[Cd]}\ L^{-1}$ resulting in increased larval mortality, with sound playbacks otherwise being antagonistic to cadmium toxicity. Exposure to 63.52 $\mu g_{[Cd]}\ L^{-1}$ caused significant delays in larval development, dropping to 6.48 $\mu g_{[Cd]}\ L^{-1}$ in the presence of piling playbacks. Pre-exposure to the combination of piling playbacks and 6.48 $\mu g_{[Cd]}\ L^{-1}$ led to significant differences in the swimming behaviour of the first juvenile stage. Biomarker analysis suggested oxidative stress as the mechanism resultant deleterious effects, with cellular metallothionein (MT) being the predominant protective mechanism.

1. Introduction

Many marine environments and the species therein are facing unprecedented pressure resultant of anthropogenic activities. To date, many studies have considered the effects of individual drivers, however in reality environments are complex with multiple drivers co-occurring or interacting (Boyd et al., 2018; Griffen et al., 2016). The need to better understand the impacts and implications of multiple drivers is emphasised by inclusion as an objective of the United Nations Decade of Ocean Science for Sustainable Development (Ryabinin et al., 2019). Though climate change is arguably at the forefront of people's minds when discussing environmental drivers in our oceans, there are many other drivers including pollution in the form of chemical loading (Bocchetti et al., 2008; Matlock et al., 2002; Rider et al., 2014), and sound pollution caused by shipping, marine construction and other anthropogenic activities (Solan et al., 2016; Lancaster et al., 2021; Lucke et al., 2009; Nikolich et al., 2021; Richardson et al., 2013; Tyack, 2008). Whilst many studies have evidenced impacts of chemical pollutants on marine life, and a growing body of work addressing the biological impacts of sound pollution exists (Wale et al., 2021), few studies have investigated these drivers in combination, despite their potential co-occurrence.

To assess whether interaction between anthropogenic sound and chemical pollution occurs, the toxicity of waterborne cadmium was assessed in combination with simulated pile driving sound achieved via playback of *in situ* recordings. Pile driving was selected as a sound driver given the expected prevalence of offshore construction in the coming years, particularly in the energy sector (Gourvenec et al., 2022). Cadmium was selected given its common use as a reference toxicant, but also due to its legacy prevalence in marine sediments. The specific combination of pile driving sound and cadmium exposure was considered environmentally plausible as sediment disturbances during construction activities can result in dissolution of sediment-associated minerals and chemicals (Eggleton and Thomas, 2004; Gutiérrez-Galindo et al., 2010).

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Pile driving, or piling, is the process of driving supports known as 'piles' deep into bedrock to provide solid structural foundation for construction. This often involves percussively hammering metal piles through the ground, the acoustic profile of which varies depending on the installation specifics, with source sound pressure levels exceeding 250 dB re 1 μPa @ 1 m recorded for installations in UK waters (Nedwell et al., 2007). Based upon recordings of piling derived in the North Sea and specific sound propagation modelling, it has been estimated that pile driving sound exposure levels remain at 168 dB re 1 μPa^2 s even at 2 km distance from the source (Bolle et al., 2012; Hazelwood and Macey, 2021). Piling also produces impulsive sounds, so in addition to typically producing high amplitudes of sound pressure and particle motion, these shifts in pressure and particle motion occur extremely rapidly (Hastie et al., 2019).

Cadmium is a heavy metal predominantly produced as a by-product of zinc refinement (Shiel et al., 2010) and is widely used in industry, including use in batteries, pigments, and as an alloying material (Hasanuzzaman and Fujita, 2013). Cadmium is also a well-established and highly potent environmental toxicant with known carcinogenic and teratogenic properties (Witeska et al., 2014), the effects of which have been widely researched across a variety of taxa (Bohra et al., 2015; Marettová et al., 2015; Okocha and Adedeji, 2011). Although environmental discharge is now regulated in much of the world, cadmium of historic origin present in sediments remains a persistent contaminant (Ei Tun et al., 2009; Kühn et al., 1992), especially in estuarine and marine settings where it is likely to deposit given its physicochemical properties (Jiann and Ho, 2014; Stephenson et al., 1996). Given a plausible co-occurrence of sound-producing construction activity and heavy metal enrichment of waters, the aim of this study was to evaluate the potential interactive effects of the combination of pile driving sound playbacks and cadmium exposure. The study focused on early life stage organisms given these are often more sensitive to stressors than mature counterparts (Braunbeck et al., 2014). Hence, early life stage exposures can lead to population 'bottlenecks' (Pineda et al., 2010).

The model species chosen for this study, the Norway lobster, *Nephrops norvegicus* (henceforth *Nephrops*), is a decapod crustacean common to the shallow-water regions of the North-East Atlantic and North Sea regions (Fisheries Global Information System (FAO-FIGIS), 2016). *Nephrops* has a biphasic life-cycle — undergoing planktonic larval development, followed thereafter by a benthic existence excavating and occupying burrows in muddy sediments. Adult female *Nephrops* produce a clutch of eggs annually, typically fertilised during summer months. The egg clutch is carried on the female's pleopods for around nine months before hatching in the following spring. Newly hatched pelagic Zoea larvae (Zoea I) are dispersed into the water column, and over a six-week period undergo two moults (to stages Zoea II and Zoea III) before metamorphosing to the first juvenile stage and commencing a benthic existence (Powell and Eriksson, 2013).

The conservation status of *Nephrops* is currently considered as 'least concern' by the International Union for Conservation of Nature (IUCN); nonetheless the species faces a variety of pressures as the result of human activities. *Nephrops* are highly sought after across NE Europe for their commercial value, representing the third largest target-species fishery in Scotland by mass, valued at £86 m for 2019 (The Scottish Government, 2020). In addition to fishing pressure, the North Sea habitat range of *Nephrops* overlaps with a region of high utilisation for energy production, with a high density of existing and/or planned fixed structures including windfarms and fossil fuel platforms, the construction of which usually requires some degree of pile driving.

This study aimed to ascertain (i) whether anthropogenic sound playbacks and/or chemical pollutants affect the early life stages of *Nephrops*, (ii) whether these two drivers combine and interact, and (iii) the potential mechanism contributing to any such interactions.

2. Methods

The study comprised two complementary experiments conducted in a controlled laboratory setting. Experiment 1 focused on impacts of individual and combined sound and cadmium exposure on mortality, growth and development of *Nephrops* larvae and behavioural fitness of the first *Nephrops* juvenile stage. Experiment 2 addressed a potential mechanistic link to the phenomenological observations from Experiment 1, focusing on quantification of oxidative stress biomarkers.

2.1. Animal husbandry

Berried female *Nephrops* were procured from DR Colin & Sons Ltd. of Eyemouth, Berwickshire, UK during July 2018 (Experiment 1) and June 2019 (Experiment 2). All *Nephrops* were trawl-caught, landed, sorted, and held in refrigerated seawater before being transported to the St Abbs Marine Station, Berwickshire, UK on the same day they were caught. Healthy-appearing berried females with eggs in an advanced stage of development were selected and placed individually in 15 l conical upwelling hoppers (30 cm top-diameter, 15 cm bottom-diameter, 30 cm depth) with 1.5 mm mesh-covered outflows to enable retention of larvae of known maternity upon hatching. Hoppers contained segments of PVC pipes to provide shelter and were covered by 75% shade netting to reduce light intensity. The females were fed cooked blue mussel (*Mytilus edulis*) *ad libitum*. Flow-through conditions were maintained using raw, ambient-temperature seawater. On a weekly basis, each berried female was carefully transferred to a freshly cleaned hopper. Hoppers were visually inspected each morning for presence of newly hatched larvae. If present, these were collected into a 2-litre beaker, and subsequently transferred individually to experimental conditions using a 10 ml pipette. Larvae were maintained in UV sterilised seawater dosed to a designated cadmium concentration (detailed below). Water temperature was maintained at 12 ± 1 °C, and 75% shade netting used to reduce light intensity and minimise disturbance. Vessels were cleaned and received a 95% water change twice weekly, at which time larvae were fed *ad libitum* with *Artemia* sp. nauplii.

2.2. Experimental system

Two identical exposure systems, each facilitating a different sound treatment, were set up to allow concurrent co-exposure of sound and cadmium under controlled conditions. Each system comprised a 750-litre fibreglass tank (internal dimensions (LWD): 152 cm × 94 cm × 60 cm) acting as a water bath, containing a Clark Synthesis Diluvio AQ339 Aquasonic underwater speaker (frequency response: 20–17,000 Hz) suspended centrally above the tank floor and orientated upwards towards the water surface, such that the speaker cone was 14 cm above the floor of the tank, and a minimum of 40 cm from the base of the closest exposure vessels (Fig. S1). Speaker suspension was employed to minimise extraneous vibration transfer between the speaker body directly into the tank superstructure and to reduce the potential for additional, diffuse sound pressure and/or particle motion sources. Each speaker was coupled to a Samson Audio Servo 300 Power Amplifier with signal input facilitated via a laptop computer and M-Audio M-Track QUAD Audio Interface. Each fibreglass tank was filled with raw seawater (34 ppt salinity) maintained at 12 ± 1 °C using a Teco TK-2000 heater-chiller unit fed by an Eheim Universal 3400 pump operating at a flow rate of 775 l/h, forming a static recirculating system. Both the pump and heater-chiller unit were externally isolated from the fibreglass tank to prevent transfer of additional sound and vibration, and all necessary contact points between in/outflow piping dampened by 4 mm-thick rubber sheeting placed between their interfaces. Water levels within the fibreglass tank were maintained where necessary by addition of deionised water, accounting for losses to evaporation and maintaining consistent seawater density (and associated sound propagation) conditions. Each fibreglass tank contained a table positioned centrally above

the speaker and isolated from the tank floor using anti-vibrational rubber sheeting, providing a surface onto which exposure vessels could be placed. Table height maximised the distance of the exposure vessels from the speaker to reduce the impacts of potential sound cone and near-field sound effects, whilst ensuring the working volume of the exposure vessels remained submerged. This resulted in a water-based interface for sound propagation, and effective temperature regulation of the exposure vessels.

2.3. Sound exposures

Exposure to ambient and pile driving sounds was simulated using playbacks of field-derived sound recordings made available by Rick Bruintjes (Defra, UK), Sophie Nedelec (University of Exeter, UK) and Irene Voellmy (University of Berne, CH). Piling playback tracks were compiled from multiple recorded strikes of a 1.2 m diameter monopole being driven approximately 25 m into the seabed in a water depth of 6.5 m, recorded at distances between 87 and 200 m from the sound source using a Hi Tech Inc. HTI-99HF hydrophone with inbuilt preamplifier (manufacturer calibrated sensitivity −204 dB re 1 V μPa^{-1}, 20–125,000 Hz frequency range) and a RTsys EASDA data logger using a 44.1 kHz sampling rate. Ambient playback tracks were compiled from comparable recordings made in the absence of any evident anthropogenic sound, taken using a HiTech HTI-96-MIN hydrophone with inbuilt preamplifier, and an Edirol R09-HR 24-Bit recorder (44.1 kHz sampling rate). All sound files for experimental playback were compiled in Audacity 2.2.2 and output as 24-bit WAV files. The ambient sound treatment comprised a 4:00 h looped ambient recording repeated continuously. The piling playback sound treatment comprised four pile driving tracks of varying length (1:00 h, 1:15 h, 1:30 h, 2:15 h) interspersed with four ambient tracks (3:30 h, 4:00 h, 4:30 h, 6:00 h duration). Pile driving and ambient tracks were alternated such that no single track was repeated within a 24-hour period, amounting to a pseudorandomised sound regime, whilst maintaining a known, consistent sound exposure each day. Received sound pressure level (SPL) in exposure vessels was targeted at 118 dB_{RMS} re 1 μPa in ambient playback phases to approximately match the noise-floor of the tanks, and at 170$_{pk\text{-}pk}$ re 1 μPa for piling playback phases. Received sound exposure levels were measured within each exposure vessel. Sound pressure was measured using a manufacturer-calibrated HiTech HTI-94-MIN hydrophone (sensitivity: −165 dB re 1 V μPa^{-1}) coupled with a calibrated Roland R-26 2-channel Portable Recorder. Particle motion (as three-dimensional magnitudinal acceleration) was measured using a calibrated custom-built triaxial accelerometer (STMicroelectronics LIS344ALH) potted within epoxy resin and suspended within the exposure vessels using 1 mm diameter elastic cord (Wale, 2017).

2.4. Cadmium exposures

All equipment and exposure vessels were acid washed using 2 M nitric acid prior to use to remove trace-metal contamination. Exposure vessels were then subsequently conditioned with a cadmium solution of their respective designated nominal concentration. In each experiment, larvae were exposed to one of four cadmium treatments with nominal waterborne cadmium ion concentrations (Cd^{2+}) of 0 μg L^{-1}, 1 μg L^{-1}, 10 μg L^{-1}, and 100 μg L^{-1} — henceforth referred to as Control$_{[Cd]}$, Low$_{[Cd]}$, Medium$_{[Cd]}$ and High$_{[Cd]}$ respectively. All chemical exposures were conducted under semi-static renewal conditions, with twice-weekly (Experiment 1) and daily (Experiment 2) 95% water changes and full cadmium renewal. In each experiment, replication was fulfilled using conspecific larvae originating from a single berried female. Detailed cadmium dosing regimens for both Experiment 1 and Experiment 2 can be found in the supplementary materials (Table S1).

Time-averaged waterborne Cd^{2+} concentrations were quantified from paired water samples taken immediately after dosing and immediately preceding water changes pooled from each replicate and preserved by acidification to pH < 2 using addition analytical grade nitric acid. Cadmium quantification was conducted by GEOMAR Helmholtz Centre for Ocean Research, Germany using solid-phase extraction ICP-MS.

2.5. Experiment 1: phenomenological observations

For Experiment 1 a total of 160 *Nephrops* Zoea I larvae were evenly distributed between treatment groups, resulting in 20 independent replicates (i.e. larvae) per treatment. Larvae were allocated over a two-day period due to timing of hatching, with 80 larvae evenly allocated across treatment groups on each of the two days. Larvae were maintained individually in 330 ml BPA-free, food-grade virgin polypropylene plastic cups containing 250 ml of cadmium-dosed UV sterilised seawater. Treatment replicates were arranged in a 14 × 6 Latin-square array to account for environmental factors and sound gradient effects (Fig. S2).

Larvae were concurrently exposed to sound and chemical treatments for the duration of their planktonic development (Zoea I, II, III), and for an additional three days following metamorphosis to the benthic juvenile.

2.5.1. Mortality

All larvae were observed daily. In the event of a mortality, this was noted and the carcass collected, labelled, and frozen in a −20 °C freezer for later biometric analysis.

2.5.2. Development

In the event of a successful moult, the day of moulting was recorded relative to the date each individual larva initially hatched.

2.5.3. Behavioural fitness

Nephrops that successfully transitioned to juveniles were subjected to a behavioural fitness assessment three days post metamorphosis. Their tail-flick escape response was assessed using methods similar to those described in Kellie et al. (2001) and (Bolger (2022). The fitness assessment was conducted in a circular 'arena' (19 cm diameter, height 12 cm), filled with 250 ml of fresh, temperature acclimated UV sterilised seawater. The contents of individual exposure vessels, juvenile *Nephrops* included, were carefully poured into the arena. Following a five-minute acclimation period, the first-stage juvenile *Nephrops* were provoked by vertically lowering a small plastic rod onto the arena floor directly in front of them (0.5 cm from the rostrum) at a speed of approximately 10 cm s^{-1}. For each provocation, the presence/absence of an induced escape response was recorded on video at 720p resolution and 60 fps using a DSLR camera suspended above the arena for later analysis using ImageJ Fiji (Schindelin et al., 2012). Where a tail-flick response was provoked, the total number of tail flicks within that response was counted, along with the distance the larva had travelled and its average swimming speed of each component tail flick within that response. This generated a hierarchical data set detailing each aspect of the swimming dynamics across all provocations. Fitness assessment and reviewing of the video files were both conducted blind to reduce potential bias.

2.5.4. Biometrics

Following completion of the behavioural fitness experiment, carapace length (mm) of first-stage juveniles (rostrum to anterior carapace) was measured using digital calipers and whole organism wet- and dry-weight (mg) (dried at 60 °C to constant mass) determined using a gravimetric balance.

2.6. Experiment 2: biomarker assays

For Experiment 2 a total of 672 *Nephrops* Zoea I larvae were evenly distributed across seven replicates of each treatment. Each replicate constituted a 1000 ml borosilicate glass beaker, containing 800 ml of

cadmium-dosed UV sterilised seawater, and 12 larvae. Communal allocation of larvae within replicates was required to provide sufficient tissue quantities to facilitate biomarker analyses. Full allocation was conducted over a 12-day period over which the larvae hatched, with specimens hatching on any given day being evenly distributed across all treatments. Exposure vessels were randomly allocated to one of 16 positions (arranged in a 4 × 4 square within the central portion of the exposure system (Fig. S3) where sound levels were most consistent, and randomly reallocated to one of these 16 positions following each water change to minimise the influence of any environmental variation.

Following the 5-day exposure period, surviving larvae from each replicate were removed from the vessels, gently dried with absorbent tissue, and all individuals within a replicate collected into a single cryovial before being flash-frozen in liquid nitrogen and stored at −80 °C. Replicate whole-organism samples were homogenised in 800 μl Tris-HCl (50 mM, 0.15 M KCl, pH 7.4) buffer solution using a motorised pestle, and spun at 10,000 RPM for 3 min in an Eppendorf Mini Spin centrifuge. The resulting supernatant was split into aliquots for each of the oxidative stress assays, and re-frozen at −80 °C until required. Quantitative assays were normalised against total protein content (Bradford, 1976). Superoxide dismutase (SOD) inhibition was quantified using the Sigma-Aldrich SOD Determination Kit (19160). Catalase (CAT) activity was quantified using the Cayman Chemical Catalase Assay Kit (707002). Glutathione (GSH) concentration was determined according to methods outlined by (Smith et al., 2007) adapted from (Owens and Belcher, 1965). Glutathione peroxidase (GpX) was quantified using the Cayman Chemical Glutathione Peroxidase Assay Kit (703102). Thiobarbituric acid reactive substances (TBARS) were quantified following the protocol of Al-Shaeri et al. (2013) adapted from Smith et al. (2007). Metallothioenein (MT) was quantified in accordance with the methods derived from Viarengo et al. (1997) and Cenov et al. (2018) assuming 18 Cys residues per metallothionein residue (Cenov et al., 2018; Zhu et al., 1994). Detailed methods for all biomarker assays can be found in the supplementary materials.

2.7. Treatment characterisation and quantification

Sound analyses were conducted using the Signal Processing Toolbox in MATLAB R2021b (The MathWorks Inc, 2021). Sound pressure was analysed over a broadband frequency range of 1–24,000 Hz from 10 s recordings. Sound particle motion was analysed over a 50–3000 Hz range using a 3rd-order Butterworth bandpass filter. Ambient sound treatments were characterised as continuous sounds, and therefore quantified as root-mean-square (RMS) sound pressure. Piling playback treatments were characterised as impulsive sounds, and thus primarily quantified using peak-to-peak sound pressure, with RMS being calculated for comparative purposes only.

2.8. Statistical analyses

All statistical analyses were performed using the R version 4.1.0 (R Core Team, 2021). Analyses were selected based on underlying analytical assumptions and data conformity. Where multiple comparisons were undertaken, statistical significance ($\alpha = 0.05$) was conveyed according to direct pairwise comparisons (p), with secondary values controlling for false discovery rate (p_{FDR}) using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) providing robustness of interpretation.

2.8.1. Mortality

Total larval mortality was modelled using logistic regression. Model significance was reported as deviance compared to the null model. Treatment mortality rates were also compared using Kaplan-Meier survival analysis and *post-hoc* log-rank Mantel-Cox test.

2.8.2. Developmental rate

Developmental duration of *Nephrops* larvae was assessed by the timing of the transition (through moulting) between each zoeal stage up to-and-inclusive of the first juvenile stage. Day of transition was compared using Kruskal-Wallis test, with *post-hoc* analysis via Dunn's test.

2.8.3. Biometrics

Carapace length of both larvae and juveniles was compared using a two-way ANOVA with cadmium concentration and sound treatment as factors. Dry tissue weight was compared using a Kruskal-Wallis test across discrete treatment groups.

2.8.4. Behavioural fitness

Behavioural fitness of the first-stage juveniles was analysed using a hurdle model approach. Firstly, it was assessed whether the simulated threat provoked an escape response, and secondly the dynamics (average speed, distance travelled, duration, number of responses, flick per response) of resulting tail-flick escape responses. The total number of induced escape responses and non-responses to provocation, and the proportional response rate for juveniles were analysed using a Kruskal-Wallis test. For elicited escape responses, principal component analysis (PCA) was conducted combining data duration, distance travelled, and the average swimming speed for each tail flick within each response. For both the primary and secondary components of the PCA, variation in scores between treatments was analysed using a Kruskal-Wallis test with *post-hoc* analysis via a Dunn's test.

2.8.5. Oxidative stress

Biomarkers for GPx, GSH and SOD were each analysed using a two-way ANOVA. CAT, TBARS and MT were each analysed using a Kruskal-Wallis test, with *post-hoc* analysis via a Dunn's test. Collective biomarker responses were assessed using PCA, with scores for both the primary and secondary components being analysed seperately using a two-way ANOVA with *post-hoc* analysis via Dunn's test.

3. Results

3.1. Sound exposure

Measured SPLs in all sound treatments were in line with nominal target exposures and broadly consistent between the two experiment set-ups (Table 1).

Ambient playback SPLs were mostly consistent regardless of location within the experimental system, but a sound cone effect was present during the piling playback consistent with proximity to the speaker (Fig. S4). There were also some discrepancies in the sound frequency distribution between the piling sound as recorded *in situ* and recreated via experiment playbacks (Fig. S5). The marginally higher sound levels during piling playback phases of Experiment 2 compared to Experiment 1 were a consequence of exposure vessels being more confined within the sound cone. However, the 24-hour cumulative sound exposure levels (SEL_{cum}) in each sound treatment were highly comparable between experiments. Differences in ambient playback sound levels were likely a consequence of variation in background laboratory noise between the times of measurement. Power spectral analysis confirmed that the received sound levels during piling playback phases were consistently greater than ambient playback treatments across all calculated frequencies for both pressure and particle motion (Fig. 1).

3.2. Cadmium exposure

Time-averaged Cd^{2+} concentrations of experimental media were approximately 65% of nominal dosage across both experiments (Table 2). Paired water samples showed no consistent evidence of Cd^{2+} depletion in the media between dosing and subsequent water change.

Table 1
Received sound levels in exposure vessels for Experiment 1 and Experiment 2. Sound pressure measurements were conducted across a 1–24,000 Hz frequency range. Particle motion measurements were conducted across a 50–3000 Hz frequency range using a third-order Butterworth bandpass filter.

Pressure						
Expt.	Sound playback treatment	Mean (±SD) received sound SPL (dB re 1 μPa)		SEL (dB re 1 μPa^2 s)		
		pk-pk	RMS	SELss	SEL	SELcum
Expt. 1	Ambient	–	118.0 ± 0.3	–	156.5 ± 0.3	195.8 ± 0.3
	Piling	169.2 ± 1.9	136.8 ± 1.7	185.8 ± 1.7	175.4 ± 1.7	217.0 ± 1.7
Expt. 2	Ambient	–	115.9 ± 0.2	–	154.5 ± 0.2	193.8 ± 0.2
	Piling	171.1 ± 1.3	141.2 ± 1.5	184.9 ± 3.1	175.0 ± 2.7	216.6 ± 2.7
Particle motion						
Expt.	Sound playback treatment	PM (dB re 1 μm s^{-2})		SEL (dB re 1 (μm s^{-2})^2 s)		
		pk	RMS	SELss	SEL	SELcum
Expt. 1	Ambient	–	56.6 ± 0.2		106.4 ± 0.2	139.8 ± 0.2
	Piling	82.7 ± 3.4	62.7 ± 2.2	118.8 ± 3.3	109.1 ± 2.2	154.1 ± 3.3
Expt. 2	Ambient	–	56.7 ± 0.2	–	106.6 ± 0.2	139.9 ± 0.2
	Piling	84.6 ± 2.6	64.0 ± 2.0	120.0 ± 2.7	110.4 ± 2.0	155.9 ± 2.7

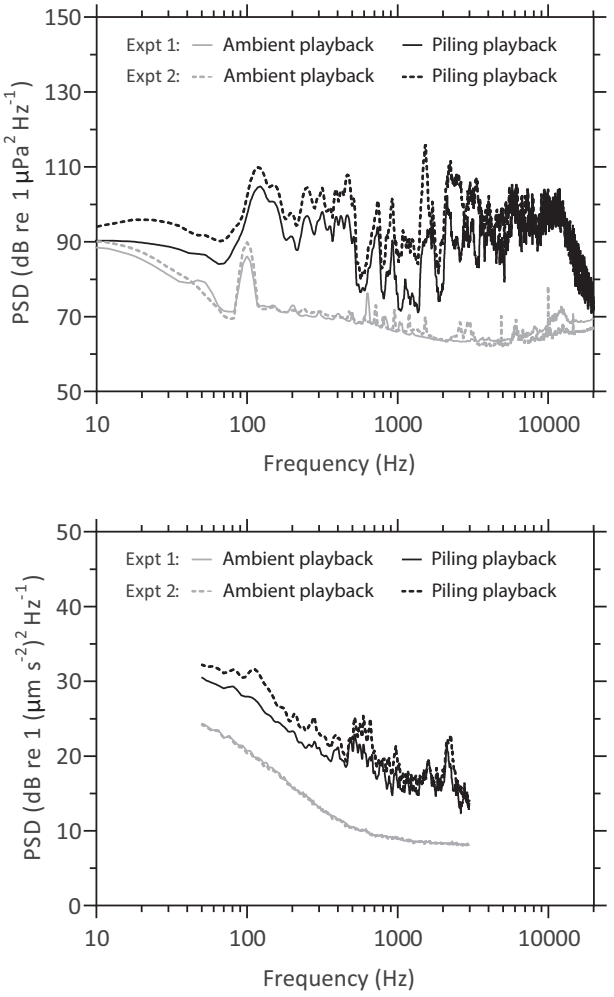


Fig. 1. Power spectral density of sound playback exposures. RMS power spectral density of received ambient- and piling playback in each experiment as recorded in exposure vessels. Top: sound pressure; 0.1 s Hann window and 50% overlap. Bottom: particle motion; 0.1 s Hamming window and 50% overlap.

Table 2
Mean ± SD time-averaged waterborne Cd^{2+} concentrations of experimental media. n = 6 samples (n = 3 paired samples) per average.

Cadmium treatment	Nominal Cd^{2+} concentration	Time-averaged Cd^{2+} concentration (μg L^{-1})	
		Expt 1	Expt 2
Control	0 μg L^{-1}	0.08 ± 0.02	0.07 ± 0.02
Low	1 μg L^{-1}	0.71 ± 0.11	0.71 ± 0.06
Medium	10 μg L^{-1}	6.48 ± 0.13	6.31 ± 0.14
High	100 μg L^{-1}	63.52 ± 2.55	62.47 ± 1.56

3.3. Experiment 1: phenomenological observations

3.3.1. Total larval mortality

Under ambient sound playbacks, larval mortality rates were 35%, 50%, 40% and 75% in the $Control_{[Cd]}$, $Low_{[Cd]}$, $Medium_{[Cd]}$ and $High_{[Cd]}$ treatments, respectively. This compared with mortality rates of 25%, 15%, 25% and 100% in the corresponding cadmium treatments under piling playbacks. Both sound treatment and cadmium concentration significantly affected larval mortality rates with a significant interaction also occurring (logistic regression: $\chi^2 = -31.748$, df = 3, $p < 0.001$) (Fig. 2 and Table S2).

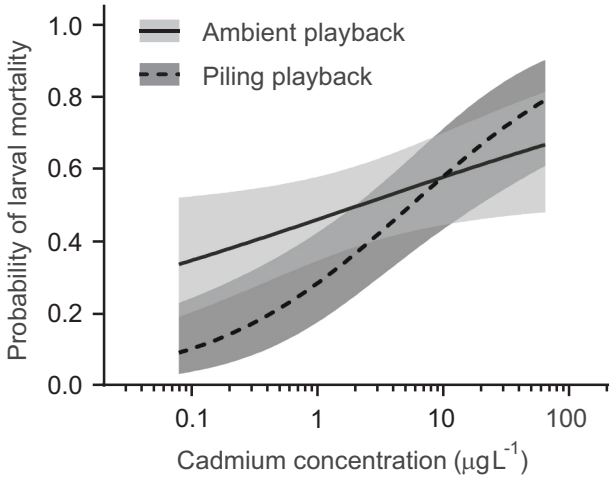


Fig. 2. Modelled probability of *N. norvegicus* larval mortality. Logistic regression model of predicted probability of larval mortality of *N. norvegicus* exposed to ambient and piling playbacks, and waterborne cadmium concentrations. Shaded areas represent 95% CI. Model fit using $N = 20$ observations for each sound treatment at each cadmium concentration of 0.08 μg L^{-1}, 0.71 μg L^{-1}, 6.48 μg L^{-1}, and 63.52 μg L^{-1}.

The modelled data demonstrate that survival was higher in the piling playback treatments at waterborne cadmium concentrations of <9.62 μg L^{-1} compared to equivalent concentrations in ambient playbacks but reduced at concentrations exceeding this. This corroborates comparisons of the raw count data (Fig. S6), evidencing a mechanistically antagonistic interaction between piling playback in the $Low_{[Cd]}$ and $Medium_{[Cd]}$ treatments, and a synergistic interaction in the $High_{[Cd]}$ treatment with respects to mortality.

3.3.2. Temporal patterns of mortality

There were significant differences in mortality curves (Fig. 3) between treatments (Kaplan-Meier: $\chi^2 = 49.2$, df = 7, $p < 0.001$). *Post-hoc* log-rank Mantel-Cox analysis determined lowest observed effect concentration (LOEC) of cadmium to be 63.52 μg L^{-1} in both ambient ($Z = 2.017$, df = 1, $p = 0.043$, $p_{FDR} = 0.100$) and piling ($Z = 4.464$, df = 1, $p < 0.001$, $p_{FDR} < 0.001$) playback treatments, and significant interactions with piling playbacks at cadmium concentrations of 0.71 μg L^{-1} ($Z = -2.310$, df = 1, $p = 0.002$, $p_{FDR} = 0.051$), and 63.52 μg L^{-1} ($Z = 2.632$, df = 1, $p = 0.005$, $p_{FDR} = 0.015$). No other treatment groups differed significantly (Table S3).

3.3.3. Developmental duration

The timing of transition from Zoea I to Zoea II was unaffected by exposure treatments, but significant differences in timing were observed in the transitions to Zoea III ($\chi^2 = 22.342$, df = 7, $p = 0.002$) and juvenile ($\chi^2 = 15.129$, df = 6, $p = 0.019$) (Fig. 4). *Post-hoc* analysis (Table S4) showed that under ambient sound playback conditions, $High_{[Cd]}$ exposures caused significant delays in transition to Zoea III (Dunn's test, $Z = 2.616_{(14,\ 12)}$, $p = 0.009$, $p_{FDR} = 0.042$), however these did not persist with regards to transition to juvenile. Conversely, larvae exposed to piling playbacks transitioned to Zoea III significantly earlier in the $Low_{[Cd]}$ treatment relative to $Control_{[Cd]}$ ($Z = -2.630_{(16,\ 17)}$, $p = 0.009$, $p_{FDR} = 0.042$), with this trend persisting through to metamorphosis ($Z = -2.744_{(15,\ 15)}$, $p = 0.006$, $p_{FDR} = 0.050$). Larvae in the $Medium_{[Cd]}$ treatment also showed consistently earlier development when exposed to piling playbacks relative to ambient playbacks at transition to both Zoea III ($Z = -2.837_{(14,\ 17)}$, $p = 0.005$, $p_{FDR} = 0.042$) and juvenile ($Z = -2.823_{(12,\ 15)}$, $p = 0.005$, $p_{FDR} = 0.050$).

3.3.4. Behavioural fitness of the first stage juveniles

When considered independently, no statistical differences were observed in the total number of provoked escape responses, the number of non-responses to provocation, or the relative proportion of responses

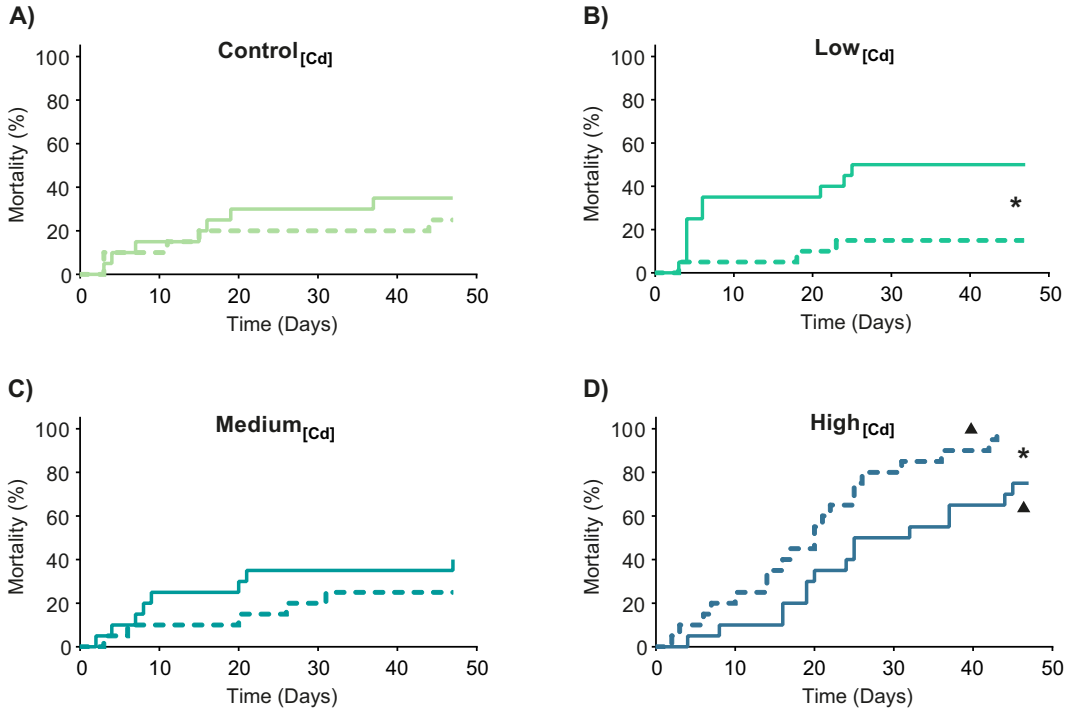
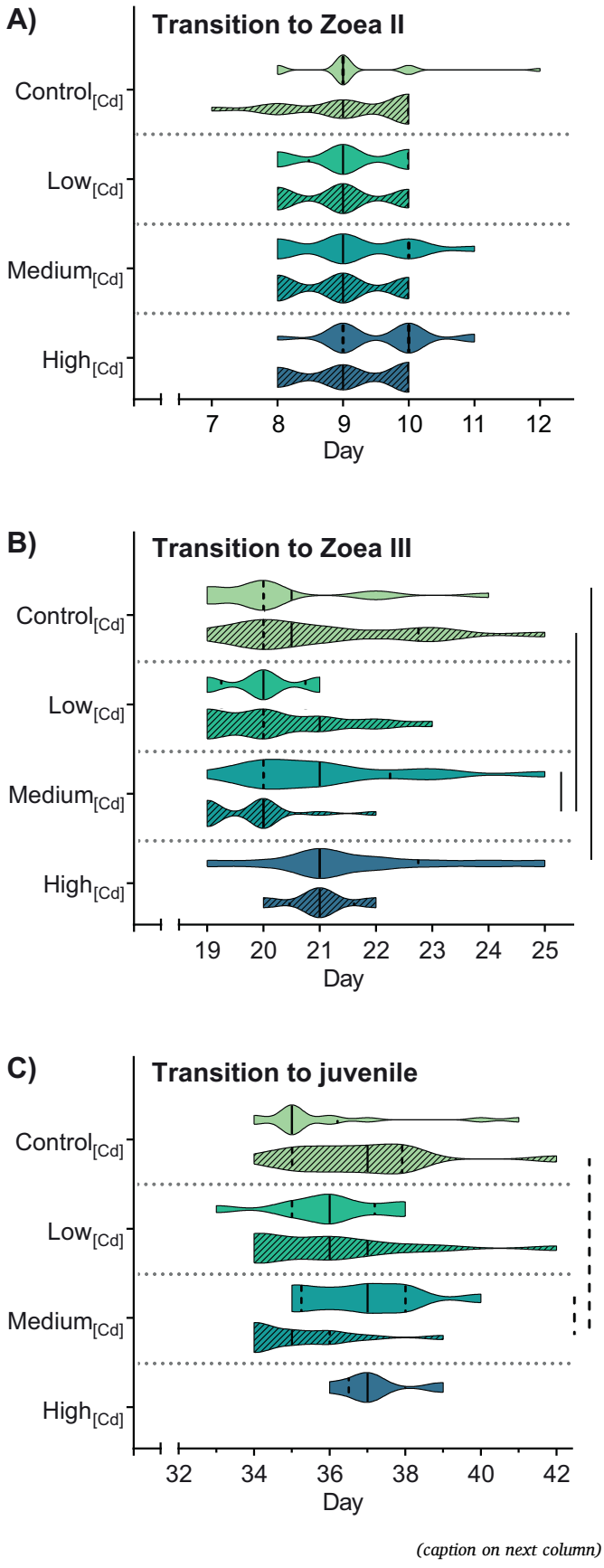


Fig. 3. Mortality curves of *N. norvegicus* larvae. Kaplan-Meier plots of cumulative mortality between sound treatments in each cadmium treatment: A) $Control_{[Cd]}$; B) $Low_{[Cd]}$; C) $Medium_{[Cd]}$; D) $High_{[Cd]}$. $Control_{[Cd]}$, $Low_{[Cd]}$, $Medium_{[Cd]}$, and $High_{[Cd]}$ represent Cd^{2+} ion concentrations of 0.08 μg L^{-1}, 0.71 μg L^{-1}, 6.48 μg L^{-1}, and 63.52 μg L^{-1} respectively. Solid lines = ambient playback sound treatment, dashed lines = piling playback sound treatment. *Significant difference ($p < 0.05$) between sound treatments; ▲significant difference ($p < 0.05$) between cadmium treatment and $Control_{[Cd]}$ in respective sound treatment.



(caption on next column)

Fig. 4. Timing of *N. norvegicus* moulting. Violin plot showing timing of transition moults between zoeal stages of *N. norvegicus* larvae as measured from day of hatching. A) Zoea I to Zoea II; B) Zoea II to Zoea III; C) Zoea III to juvenile. $Control_{[Cd]}$, $Low_{[Cd]}$, $Medium_{[Cd]}$, and $High_{[Cd]}$ represent Cd^{2+} ion concentrations of 0.08 μg L^{-1}, 0.71 μg L^{-1}, 6.48 μg L^{-1}, and 63.52 μg L^{-1} respectively. Absent violin in $High_{[Cd]}$ piling playback treatment is a consequence of no larvae surviving to metamorphosis. Solid and hatched plots represent ambient and piling playback sound treatments respectively. Solid and dashed vertical bars within plots represent median and quartile values respectively. Vertical markers beside violins denote significant differences between groups (Dunn's test, dashed lines $p < 0.05$, solid lines corrected $p_{FDR} < 0.05$).

(Fig. S7 and Table S5). Analysis of tail-flick escape response dynamics by PCA (Fig. 5) implied the presence of two key axes of response behaviour. The primary principal component (PC1), representing 48.3% of variance within the data (Table 3), broadly corresponds to an axis of physical swimming dynamics, contrasting swimming speed against swimming duration and total distance travelled. The secondary principal component encompasses a further 23.1% of data variance, seemingly forming an axis contrasting the total number of responses against their magnitude (number of tail-flicks). Comparison of PCA scores by Kruskal-Wallis evidenced significant differences in both PC1 ($\chi^2 = 349.91$, df = 6, $p < 0.001$) and PC2 scores ($\chi^2 = 114.08$, df = 6, $p < 0.001$) between experimental treatments.

Under ambient sound playback conditions, *post-hoc* analysis of PCA scores (Table S6) for behavioural response dynamics found the LOEC of cadmium to be 6.31 μg L^{-1}, which led to a noticeable and significant shift towards prioritising swimming distance and duration at the expense of speed, with individual responses comprising fewer tail-flicks. Similarly, juveniles from the $Low_{[Cd]}$ ambient playback treatment also demonstrated fewer tail-flicks per individual response, but did not display any specific response in relation to speed or distance. Piling playback in the $Control_{[Cd]}$ treatment also led to a significant shift in juvenile behaviour towards more responses of fewer tail-flicks, maximising total distance and duration. Disparities in escape responses were also seen upon co-exposure to cadmium and piling playback. Whilst the general trend of elevated cadmium resulting in escape responses of lesser magnitude and greater frequency persisted, under ambient playback conditions this paired with juveniles covering an overall greater distance, whereas those exposed to piling playbacks had faster swimming speeds at the expense of distance and duration.

3.3.5. Biometrics

Neither carapace length (two-way ANOVA: sound: $F = 0.228_{(1, 80)}$, $p = 0.634$, cadmium: $F = 0.241_{(3, 80)}$, $p = 0.868$; sound × cadmium: $F = 0.699_{(2, 80)}$, $p = 0.500$) nor dry tissue weight (Kruskal Wallis test: $\chi^2 = 4.609$, df = 6, $p = 0.565$) of juvenile *Nephrops* differed significantly between treatment groups.

3.4. Experiment 2: biomarker assays

3.4.1. Oxidative stress biomarkers

One replicate sample from the $Low_{[Cd]}$ ambient playback treatment exhibited spurious results across multiple biomarkers and was thus censored from all analysis as an outlier. Biomarker quantities were highly variable (Fig. 6), with only MT varying significantly between treatment groups (Kruskal-Wallis test, $\chi^2 = 14.565$, df = 7, $p = 0.032$), with significantly lower quantities present in larvae exposed to $Medium_{[Cd]}$ piling playback treatments relative to both their respective $Control_{[Cd]}$ treatments (Dunn's test, $Z = -2.718_{(7, 7)}$, $p = 0.007$, $p_{FDR} = 0.092$) and larvae experiencing ambient playback ($Z = -3.669_{(7, 7)}$, $p < 0.001$, $p_{FDR} = 0.007$). Full details of statistical outputs can be found in Table S7 and Table S8.

Principal component scores were consistent with trends of the individual results (Fig. 7). The primary principal component (PC1) accounted for 37.0% of variance within the data, primarily aligning with

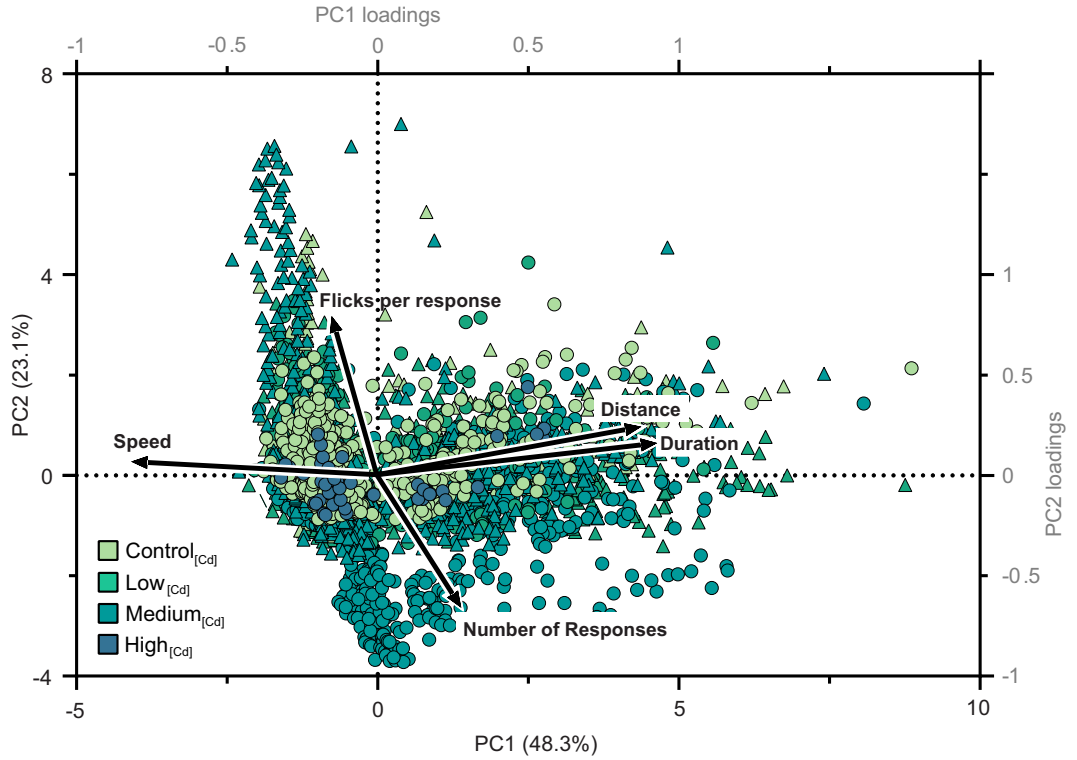


Fig. 5. Juvenile *N. norvegicus* escape behaviour dynamics. Biplot of loadings and scores generated by principal component analysis of escape behaviour dynamics (number of responses, flicks per response, duration of escape response, speed of escape response, distance travelled during escape response. $Control_{[Cd]}$, $Low_{[Cd]}$, $Medium_{[Cd]}$, and $High_{[Cd]}$ represent Cd^{2+} ion concentrations of 0.08 μg L^{-1}, 0.71 μg L^{-1}, 6.48 μg L^{-1}, 63.52 μg L^{-1} respectively. Circles = ambient playback sound treatment; triangles = piling playback sound treatment.

Table 3
Eigenvalues and proportion of variance PCA of juvenile *N. norvegicus* tail-flick escape response.

Principal component	Eigenvalue	Proportion of variance
PC1	2.415	48.31%
PC2	1.156	23.12%
PC3	0.820	16.40%
PC4	0.467	9.33%
PC5	0.142	2.84%

GPx and CAT activities, whilst the secondary principal component (PC2), representing a further 21.1% of data variance, seemingly aligned primarily to MT. When contrasted using ANOVA, PC1 scores did not differ significantly between treatment groups (Table S9), whereas PC2 scores were significantly driven by both cadmium ($F_{[1,46]} = 6.104$, $p = 0.017$) and an interaction between sound and cadmium ($F_{[3,46]} = 5.830$, $p = 0.002$). Further *post-hoc* analysis (Table S10) attributed these differences to the $Medium_{[Cd]}$ treatments, with differences occurring in the $Medium_{[Cd]}$ piling playback treatment relative to $Control_{[Cd]}$ piling playback treatment ($Z_{[7,\ 7)} = 2.361$, $p = 0.018$, $p_{FDR} = 0.102$) and between the sound treatments ($Z_{[7,\ 7)} = 3.890$, $p < 0.001$, $p_{FDR} = 0.003$), mirroring statistical significance attributed to the individual biomarkers and reinforcing assertions of PC2 being driven by MT.

4. Discussion

4.1. Sound exposures

Speaker playbacks and acoustic properties of tanks are unlikely to enable realistic recreation of sounds as experienced in the ocean. All presented sound metrics reflect values as recorded within the exposure vessels at each occupied location within the exposure system. As such, values are fully representative of the received sound levels within the waterbody of the exposure vessels, accounting for inherent transmission losses and tank acoustic effects.

Currently, only Germany imposes legal limits on sound produced by pile driving, with SPL limits 190 $dB_{pk\text{-}pk}$ re 1 μPa and 160 dB_{SELss} re 1 μPa^2 s as measured at 750 m from the piling source (Müller et al., 2019). Whilst $SPL_{pk\text{-}pk}$ levels in this study were well below the 190 $dB_{pk\text{-}pk}$ re 1 μPa limits, accompanying SELss levels far exceed the limits. A comparison of the relative power spectral density (PSD) of sound pressure between the piling as recorded *in situ* and as received as playback (Fig. S5) clearly demonstrates that playbacks result in lesser proportion of sound at frequencies <2000 Hz and an increase in the proportion above this. The larger proportion of sound at frequencies exceeding 2000 Hz (Fig. S5) in the current study have also inflated SELss and SEL values compared to those of natural environments. Sound levels in the sub-3000 Hz range in the current study are however comparable with similar studies (Nedelec et al., 2014, 2017). Despite some inevitable discrepancies between *in situ* piling sound and experimental playbacks, some frequency dependent features were nonetheless maintained between the *in situ* and playback signals in the lower frequency domain, and discrepancies in the upper frequency domain may be irrelevant in the context of *Nephrops* sensory capability.

There is clear evidence that some decapod larvae can utilise sound as an orientation and settlement cue, and hence are auditorily capable (Radford et al., 2008; Stanley et al., 2010). To date, it is unknown whether the sensory capabilities of *Nephrops* larvae are comparable with those of mature specimens. Assessments of behavioural responses of mature specimens suggest that *Nephrops* is only responsive to the particle motion aspect of sound, with a displacement response threshold of 0.888 μm independent of the assessed frequency range of 20–200 Hz (Goodall et al., 1990), but an audiogram has not yet been established for the species. Mature *Nephrops* nonetheless have an array of mechanoreceptive structures associated with both physical orientation and sound

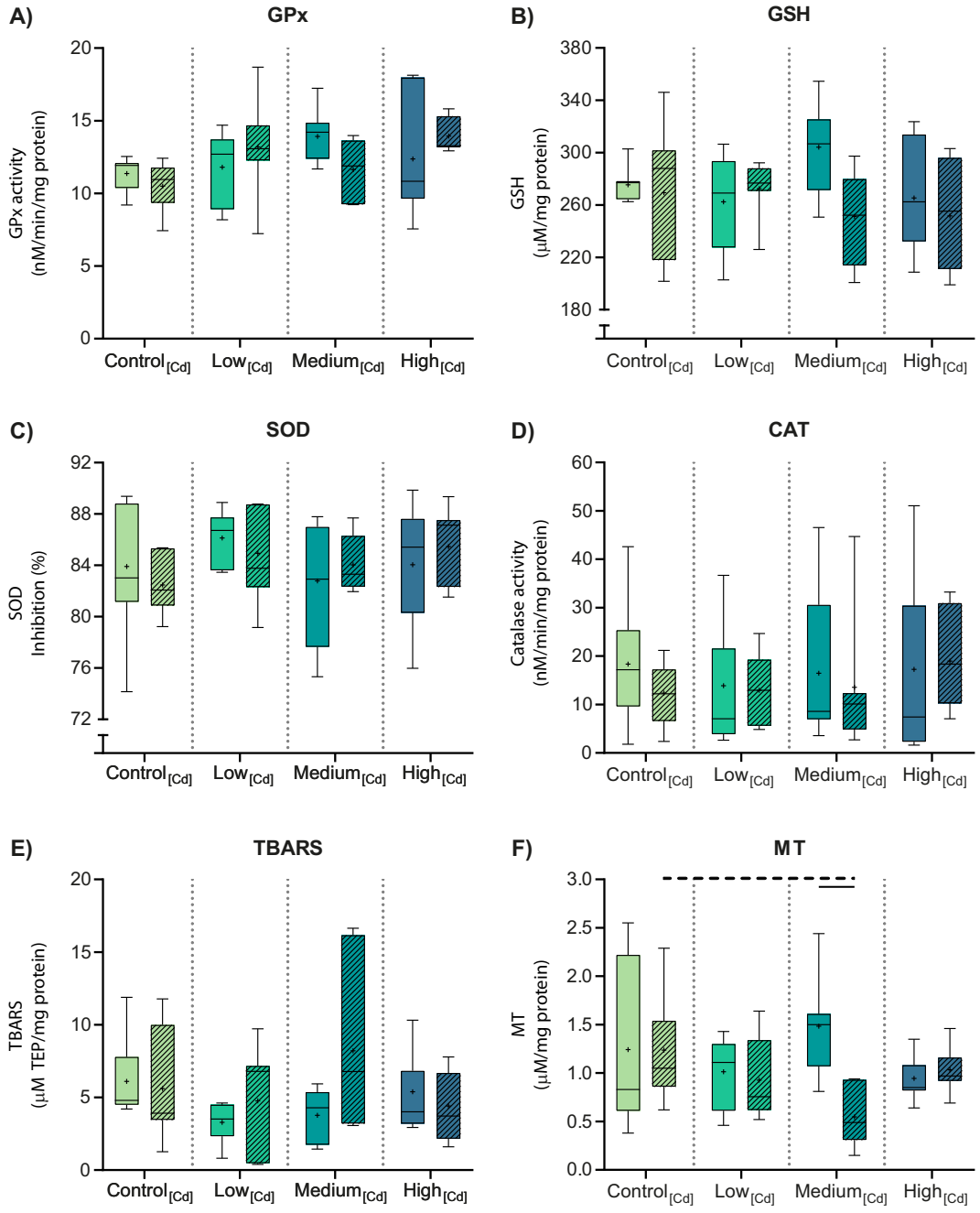


Fig. 6. Larval *N. norvegicus* oxidative stress biomarkers. Box-and-whisker plots of biomarker responses for A) GPx; B) GHS; C) SOD; D) CAT; E) TBARS; F) MT. Plots shows range, interquartile range, and median. Mean denoted by +. Solid and hatched bars represent ambient and piling playback sound treatments respectively. $Control_{[Cd]}$, $Low_{[Cd]}$, $Medium_{[Cd]}$, and $High_{[Cd]}$ represent Cd^{2+} ion concentrations of 0.07 μg L^{-1}, 0.71 μg L^{-1}, 6.31 μg L^{-1}, and 62.47 μg L^{-1} respectively. Horizontal markers above plots denote significant differences between groups (Dunn's test, dashed line $p < 0.05$, solid line $p_{FDR} < 0.05$).

reception, including cuticular setae widely distributed across their exoskeleton and statocysts located in the basal portion of the sensory antennules (Katoh et al., 2013).

Given the uncertainties in the sound perception capabilities of *Nephrops* larvae, and the discrepancies in tank-based sound studies, it is impossible to adequately assess the realism of *in situ* vs playback sounds as experienced by the organisms, nor was this the principal aim of the study. Nonetheless, both sound pressure and particle motion in the present study were consistently higher during piling playback than in the ambient playback sound treatment regardless of sound frequency. At a minimum, the study should therefore be considered in the context of exposure to additional sound sharing characteristics to that resultant of pile driving.

4.2. Cadmium exposures

Quantified cadmium concentrations were approximately 65% of nominal concentrations. Such discrepancies are expected given losses associated with adsorption and cross-reactivity with other chemical species. These losses do not impact the study as quantified values were used for all modelling and metric purposes. Waterborne cadmium concentrations in the $Control_{[Cd]}$ were consistent with background levels of

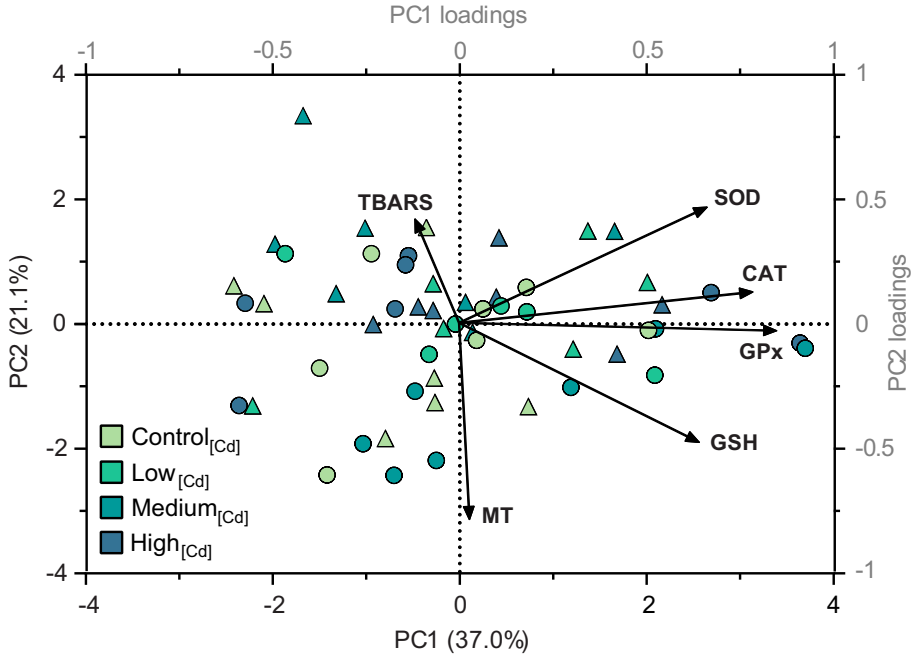


Fig. 7. Oxidative stress Principal Component Analysis. Biplot of loadings and scores generated by principal component analysis of oxidative stress biomarkers. $Control_{[Cd]}$, $Low_{[Cd]}$, $Medium_{[Cd]}$, and $High_{[Cd]}$ represent Cd^{2+} ion concentrations of 0.07 μg L^{-1}, 0.71 μg L^{-1}, 6.31 μg L^{-1}, 62.47 μg L^{-1} respectively. Circles = ambient playback sound treatment; triangles = piling playback sound treatment.

British coastal waters (Neff, 2002). $Low_{[Cd]}$ and $Medium_{[Cd]}$ treatment concentrations were consistent with those reported of coastal regions featuring discrete and or point-sources of cadmium (Abe, 2007; Delly et al., 2021). $High_{[Cd]}$ treatment concentrations are considered environmentally unrealistic, but provide useful context for modelling and risk assessment purposes.

Uptake of cadmium by the *Nephrops* larvae is thought to have predominantly occurred from the waterborne fraction via the cuticle, which is poorly calcified and permeable in larval-stage *Nephrops* (Eriksson and Baden, 1997). Absorption via the gills, a known uptake site of toxic metals in aquatic crustaceans (Henry et al., 2012), is unlikely in this instance given that gills are absent or rudimentary in larval-stage *Nephrops* (Spicer and Eriksson, 2003). Direct ingestion of cadmium bioaccumulated within their *Artemia* prey is also considered to have been minor given the frequency of water changes and *Artemia* replacement. Bioaccumulation of cadmium within the *Nephrops* larvae was not assessed due to tissue availability being considered insufficient to meet limits of detection of available analytical methods.

4.3. Mortality

Modelled data show dose-dependent increases in mortality rate with regards to cadmium in both sound treatments. Mortality in the $Control_{[Cd]}$ treatment was lower in larvae exposed to piling playbacks, but increased at a greater rate with increasing cadmium concentration compared to treatments experiencing ambient playbacks, leading to a switch in predominating mortality. Notably, whilst overall mortality below the 9.62 $μg_{[Cd]}$ L^{-1} equivalence concentration was lower for larvae exposed to piling playback, they showed greater sensitivity to elevating cadmium relative to larvae in the ambient playback sound treatment. This context is important, as it suggests survival of larvae in cadmium-contaminated water may be enhanced by anthropogenic sound exposure, whilst larvae commonly exposed ro anthropogenic sound in otherwise more pristine environments may be more susceptible to chemical pollution events.

Observed mortality rates across experimental treatments are hypothesised to reflect differences in oxidative stress responses between treatments (discussed in detail below). Results suggest that the $Low_{[Cd]}$ concentration was sufficiently high to cause toxicity, but not to invoke a suitably strong antioxidant response. Piling playbacks meanwhile triggered a larger oxidative stress response, which consequently offered additional protection from the cadmium. Correspondingly, the switch in the driver interaction from initially antagonistic to synergistic, modelled to occur above 9.62 $μg_{[Cd]}$ $L_{,}^{-1}$ equates to the concentration above which the cadmium toxicity protection afforded by the oxidative responses to piling playback is seemingly overwhelmed, with piling-induced oxidative stress not only ceasing to be beneficial but actually increasing the burden.

Scrutiny of the raw mortality data (Fig. S6) shows that Zoea I larvae reared in ambient playback sound conditions and exposed to $Low_{[Cd]}$ were significantly more susceptible to cadmium toxicity than those exposed to piling playbacks. This pattern was also mirrored, albeit to a lesser extent, in the $Medium_{[Cd]}$ treatment. Furthermore, larval mortalities during the study coincided with moulting, suggesting that the process of moulting increases susceptibility to acute metal toxicity; possibly reflecting the stressful and energetically intensive undertaking of moulting itself (Bacqué-Cazenave et al., 2019; Chang and Mykles, 2011). Whilst tissue limitations of the *Nephrops* larvae and exuviae precluded a similar analysis of metal distribution in the present study, the commonality of metal distributions between the investigations of Bergey and Weis (2007) and Perugini et al. (2014) suggests that rather than being selectively sequestered into the exuviae prior to moulting, cadmium may actually have been reabsorbed into the soft tissues along with calcium, as also seen in the grass shrimp *Palaemonetes pugio* (Keteles and Fleeger, 2001). If this was the case, it would suggest that chronic exposure led to continued accumulation of cadmium in *Nephrops* with limited ability to depurate. Permeability to, and absorption of cadmium is also likely greater in recently moulted individuals as seen in the shore crab *Carcinus maenas* (Bondgaard and Bjerregaard, 2005). Correspondingly, it may be that the process of moulting effectively results in short-term concentration of cadmium in soft tissues, which are known to accumulate metals in *Nephrops*. Perugini et al. (2014) found that approximately 85% of the total cadmium loading within *Nephrops* was distributed in the 'brown meat' (including the gills and

hepatopancreas), with the remainder distributed between the exoskeleton, legs, and claws. These observations are consistent with the role of the hepatopancreas in the detoxification of metals in decapod crustaceans (Ahearn et al., 2004), and evidence of the hepatopancreas being the primary location of cadmium accumulation even in the case of waterborne exposures (Canli et al., 1997).

4.4. Developmental and biometrics

Following metamorphosis, carapace length and wet-weight of juveniles were consistent across treatment groups, demonstrating little variation.

Piling playbacks experienced by *Nephrops* larvae in the absence of cadmium caused developmental delays of two days compared to the ambient playback treatment. This delay was not statistically significant, however this is believed to be a Type II error resulting from two notable outliers in the ambient playback sound Control$_{[Cd]}$ group. This assertion is supported by results demonstrating significant delays in timings of *Nephrops* metamorphosis in response to ship sound playbacks (Bolger, 2022) and developmental delays observed in scallops (*Pecten novaezelandiae*) exposed to seismic pulse playbacks (de Soto et al., 2013).

Delays in development may result, at least in part, from differences in metabolic rates and/or energy budgeting. Metal exposures can induce dose-dependent increases in metabolic rate and developmental duration in several taxa including crustaceans (Lyla and Khan, 2010; Vernberg et al., 1974). Such variations in developmental rate in response to environmental conditions and stressors are not uncommon. Metabolic consequences have been evidenced in response to osmotic stress (Curtis and McGaw, 2010), and temperature is well known to impact metabolism and energy assimilation of invertebrates, influencing their growth and development (Chiasson et al., 2015; Dickey-Collas et al., 2000; Han and Li, 2018; Xiao et al., 2014).

Metals such as cadmium can also have teratogenic and endocrine-disrupting effects (Takiguchi and Yoshihara, 2006), adding another facet to potential impacts where complex, hormonally driven processes such as crustacean moulting are concerned. Moulting in crustaceans is predominantly mediated by the negative regulation of moult-inhibiting hormone (MIH) produced by the X-organ, which inhibits the release of ecdysteroid hormones produced by the Y-organ (Devaraj and Natarajan, 2006). Cadmium at concentrations of 3–900 μg L^{-1} elevated the levels of ecdysteroid in embryos of the amphipod *Gammarus fossarum* (Abidi et al., 2016), suggesting low concentrations of cadmium could alter the balance of these hormones mediating growth and development. Disruption of ecdysteroid receptors in the Y-organ can also disrupt moulting (Zou, 2005), which may explain observations of moult inhibition in the crab *Chasmagnathus granulata* induced by cadmium despite consistent ecdysteroid levels (Rodríguez Moreno et al., 2003). Nevertheless, in the current study, endocrine-related effects of cadmium on growth and development are not thought to be the primary mechanism driving the observed differences in developmental rate. Firstly, post-moult feedback between MIH and crustacean hyperglycemic hormone levels is proposed to effectively reset moult cycles after each moult-phase (Techa and Chung, 2015), which would explain inconsistencies in intermoult periods at different developmental stages. This also suggests acute exposure to endocrine disruptors may have limited impacts on continued development of larvae as MIH is rapidly cleared from haemolymph, and therefore episodic releases of the hormone are unlikely to contribute to a critical threshold (Chung and Webster, 2005). Secondly, only modest deviations in moult timing were observed in the current study (around 5% compared to controls). In comparison, targeted inhibition of MIH in the prawn *Fenneropenaeus indicus* reduced intermoult period to around 50% of that of controls (Devaraj and Natarajan, 2006).

Although the mechanisms controlling the moult process itself are relatively well studied (Chang and Mykles, 2011), a consensus on triggers and timing of ecdysis events is apparently lacking, adding to the difficulties in identifying the mechanism driving the observations of this study. It is accepted that multiple factors relating to bioenergetics such as food availability, temperature, behaviour, as well as endogenous aspects such as 'biological clocks' impact reproduction, growth, and development (Sardà, 1991). Despite this, under consistent and favourable conditions, other crustacean larvae have been observed to have similarly consistent and predictable biometrics following ecdysis, albeit at variable ages (Anger, 1998). This, and the timing of ecdysis in *Nephrops* larvae in the present study, suggest a certain plasticity that would be beneficial — enabling energetic prioritisation to body condition preceding the energetically intense moult process under sub-optimal conditions, but also expediting development in more favourable conditions. It is important to state that even the relatively minor developmental delays observed represent a protracted period in the planktonic phase of *Nephrops* life-cycle, and could therefore have consequences on larval dispersion and susceptibility to predation pressures. Resultantly, developmental delays could prove ecologically significant despite lacking statistical significance.

4.5. Behavioural fitness

Differences in behavioural responses to simulated threats were observed in first stage juvenile *Nephrops* following experimental exposures. Principal component analysis evidenced that the provoked tail-flick responses can be considered in terms of two axes of variation: an axis that is considered to represent the 'sprint-marathon' continuum, and a second interpreted as reflecting whether individual reactions comprise a greater number of responses of lesser magnitude, or fewer responses of greater magnitude.

Maximal swimming speed of the juveniles appeared to be highly constrained and showed little variation, undoubtedly reflecting the consistency in the size of the juveniles assessed, given swimming speed is constrained by maximal displacement of water and therefore correlates with carapace size (Newland et al., 1988). Total distance travelled was highly correlated with the duration of the tail-flick response, both of which were negatively correlated to swimming speed. Therefore, a reduction in swimming speed must result from a reduction in muscular contraction power. It is however uncertain whether these differences between 'sprinters' and 'marathoners' are mediated by physiological limitations in musculature condition, energy partitioning, behaviour choices, or a combination of these factors. Irrespective of the causal drivers, significant differences in the composition of tail-flick responses were evident between treatments. Both response axes demonstrated a LOEC of 6.48 μg$_{[Cd]}$ L^{-1} in both ambient- and piling playback treatments. Likewise, significant interactions between piling playback and cadmium were seen at all concentrations, though there was little consistency in terms of magnitudes and/or directionality of effects. In the context of swimming dynamics, it is difficult to assess the ecological implications of how such differences are likely to impact the long-term survival prospects of individual *Nephrops* given this would be context- and threat-specific. For example, those prioritising speed may have an advantage against ambush predators, but at the detriment of resilience against more persistent foraging predators, and vice versa.

The ability to perceive and respond to potential threats, primarily predators, is of undoubted importance to long-term survival, as even slight changes in response rate to perceived threats could have serious consequences. Observations on response rates were consistent with the experimental exposures potentially having had energetic consequences during development. However, where responses were provoked, PCA analysis implied no correlation between swimming dynamics (speed and distance), and response dynamics (number of responses and tail-flicks per response), implying responses may better reflect a continuum of either behavioural boldness or corresponding reaction to threat, rather than being indicative of energetic budgeting.

Variation in response dynamics could also theoretically reflect differences between non/reflex-reactions, however this seems less likely

given different nervous pathways are responsible for initiation and continuation of tail-flick responses (Faulkes, 2009). Tail-flick responses are a low-latency reflex reaction mediated by medial giant (MG) and lateral giant (LG) nerves in response to mechanosensory stimulation of the anterior cephalothorax and abdomen respectively (Jackson and MacMillan, 2000). Whilst a similar non-reflex swimming response with higher latency mediated by non-giant nervous pathways has previously been characterised in *Nephrops* (Newland et al., 1988), only the MG pathway is considered of relevance to the assessment methods used here. Crayfish (*Procambarus clarkia*) exposed to predators showed survival rates of 50% when MG mediated responses were triggered, compared to just 20% for non-reflex responses due to the difference in response latency (Herberholz et al., 2004). As such, if piling playback has impacted on the ability of the MG pathway to function in the current study, this would also likely have a considerably detrimental impact on long-term survival potential.

Alternatively, observations may reflect a reduction in sensitivity to vibratory stimulus given high-intensity impulsive sound has been observed to cause mechanical damage to statocysts in both lobsters and scallops (Day et al., 2016, 2017, 2019, 2020). Reduction in synaptic transmission between sensory and motor neurons resultant of habituation to stimuli could also be a factor (Zucker, 1972), as could cross-modal sensory reduction in response to sound and vibration, postulated by Roberts and Laidre (2019).

4.6. Oxidative stress biomarkers

TBARS is a group of biomarkers indicative of lipid peroxidation (LPO), being the final product of other similar LPO biomarkers including malondialdehyde (MDA) (Camejo et al., 1998; Tsikas, 2017), and the only biomarker assessed that is directly indicative of pathology. The PCA analysis indicated that TBARS were predominantly mediated against by MT and GSH, whilst, SOD, GPx, and CAT were not strongly correlated with reducing pathology of cadmium and sound exposure. Glutathione (GSH) directly scavenges free radicals by acting as a hydrogen ion donator (Fanucchi, 2014), suggesting not only were free radicals the predominant cause of LPO, but that direct scavenging of these free radicals was the primary method for regulating against such ROS. This is further supported by the consistently high degree of SOD inhibition rates observed between treatments given that SOD catalyses the conversion of superoxide ions to hydrogen peroxide, which is then further catalysed into water and oxygen by both GPx and CAT (Fanucchi, 2014). The weak correlation shared between TBARS and both GPx and CAT further suggests that the presence of hydrogen peroxide influences LPO, albeit to a much lesser extent. This is consistent with the role of hydrogen peroxide as a non-radical oxidising agent (Phaniendra et al., 2015), and with observations by Badisa et al. (2007) who demonstrated that presence of cadmium can further potentiate the production of radicals from hydrogen peroxide. It is also possible that the weak correlation of TBARS to other biomarkers is indicative of a mechanism of cadmium toxicity unrelated to LPO.

Mitochondrial respiration inherently generates ROS as a by-product of ATP synthesis (Andreyev et al., 2015). Consequently, several highly conserved cellular antioxidant defences have evolved for mediating against oxidative stress, with commonly identified mechanisms in crustacea including the antioxidant GSH, and the enzymes GPx, CAT, and SOD (Fanjul-Moles and Gonsebatt, 2011). Where substantial and/or prolonged exposure to oxidative stress results in antioxidant capacity being exceeded, oxidative damage will occur. Cadmium exposure results in the production of free radical ROS — directly driving oxidative stress (Singh et al., 2017). Such exposures in crustacea can result in multifaceted effects including causing gill damage, altering metabolic activity, inducing differences in gene expression, and cause cellular apoptosis and necrosis (Torreblanca et al., 1989; Wang et al., 2013).

To date, the study of Charifi et al. (2018) is the only one having assessed oxidative stress in relation to co-exposure to anthropogenic noise and cadmium stress in a marine invertebrate, the Pacific oyster (*Magallana gigas*). Waterborne concentrations of approximately 14 $\mu g_{[Cd]}\ L^{-1}$ led to a significant positive correlation in expression of genes associated with SOD and GPx, whilst genes for CAT and MT were also positively correlated, though not significantly. However, when *M. gigas* was also exposed to ship noise of 150 dB_{RMS} re 1 μPa, no significant correlations in gene expression were identifiable. Blue mussels (*Mytilus edulis*) exposed to ship noise peaking at 150–155 dB re 1 $\mu Pa^2\ Hz^{-1}$ exhibited significantly elevated TBARS in comparison to the control group, yet SOD, GSH and GPx were unaffected (Wale et al., 2019). The present study did not observe similar increases in quantity of TBARS in response to sound, potentially due to the significantly lower RMS sound levels, but could equally reflect differences in species/taxa specific responses. Juvenile *Nephrops* that had been exposed to peak ship noise of 122 dB re 1 $\mu Pa^2\ Hz^{-1}$ throughout their larval development, showed no significant difference in SOD, GSH, GPx, CAT or TBARS (Bolger, 2022). This may evidence sound-specific differences in biological response, i.e. between 'continuous' ship noise versus 'impulsive' pile driving noise, but more likely reflects differences between life-stages. Even temporally close life stages can exhibit considerably different physiological responses, with disentanglement of differences resultant of natural development being fraught with difficulty (Rato et al., 2017; Styf et al., 2013). Differences in life-stage responses also likely contribute to the lack of a consistent trend between the oxidative stress data and mortality data in the present study. This will be further compounded by survivorship bias inherent of the oxidative stress protocols which precluded the assessment of organisms which had died during exposures. Regardless, oxidative stress being a driver of larval mortality supports many of the observed trends in the data of the present study. The mortality data show that piling playback had an antagonistic effect to total larval mortality in the presence of cadmium at concentrations $\leq$6.48 μg L^{-1}. Given that the current data suggest that piling playback alone results in limited stress, this antagonistic effect is consistent with assertions that exposure to low-level stress promotes an antioxidant response resulting in enhanced defence capacity (Niki et al., 2005). Similar observations in *Nephrops* embryos have been noted in response to ocean acidification, where oxidative stress was significantly higher in control groups compared to acidified treatments (Styf et al., 2013).

Biomarker assay results not only support the assertions advanced above in relation to life-stage specific physiological responses, but also suggest that oxidative defences in response to sound exposure either develop earlier than those of metals such as cadmium, or more likely, that responses to the piling playbacks during this developmental stage were more acute than those to cadmium.

5. Conclusion

Exposure to piling playbacks and cadmium caused a wide range of physiological effects on larval *Nephrops*, with the drivers each having individual effects, but also demonstrating various interactions when co-occuring. The multifaceted nature of these effects makes direct assessment of risk and harm of these drivers on the species difficult to judge. In some scenarios, exposure to piling playbacks could be considered beneficial, promoting larval survival and growth rates in cadmium-contaminated waters, however the opposite is also true for more pristine environments. Extrapolation between laboratory-based findings and real-world environmental impacts should be approached with caution, especially given the known discrepancies in the characteristics of *in situ* sounds vs experimental playbacks. Nonetheless, evidence of synergism leading towards net-negative impacts on larval survival at environmentally plausible cadmium concentrations of 9.62 μg L^{-1} highlights that consideration should be given to how the combination of metal pollutants and sound exposure modifies the risks posed as compared to each driver occurring in isolation. Future studies directly addressing current uncertainties regarding exposure dynamics, including if and how sound perception directly affects heavy metal

kinetics in decapod crustaceans, would contribute greatly to the understanding of this driver combination.

Ultimately, the results of this study simultaneously support both the adage "*what doesn't kill you makes you stronger*", and that of "*the straw that broke the camel's back*" — highlighting the need for more integrative and case-specific consideration of anthropogenic impacts in ecological contexts.

CRediT authorship contribution statement

C.A. Stenton: Conceptualization, Methodology, Formal analysis, Data curation, Investigation, Writing – original draft, Visualization. **E.L. Bolger:** Investigation, Methodology. **M. Michenot:** Investigation. **J.A. Dodd:** Formal analysis. **M.A. Wale:** Investigation. **R.A. Briers:** Supervision, Writing – review & editing. **M.G.J. Hartl:** Conceptualization, Supervision, Writing – review & editing. **K. Diele:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary materials

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2022.113667.

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