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Manatū Ahu Matua

Climate change exacerbates marine pollution impacts

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By Julien Vignier, Olivier Champeau, Anne Rolton Vignier, Louis Tremblay

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Executive Summary

The Greenshell™ mussel (GSM) is a commercially, ecologically and culturally significant species to New Zealand. Summer die-offs, spat retention issues and a severe decline in GSM spat settlement have been reported in the Marlborough Sounds. The causes of these effects are unknown but preliminary evidence indicates that pressure from anthropogenic activities could contribute. The aims of this study were to (1) test the toxicity of water and sediment extracts on early life stages of the GSM to identify potential sources of stressors affecting GSM recruitment, and (2) investigate the effects of temperature on the toxicity of these stressors to help understand the potential impact of climate change and pollution on GSM.

Four study sites were selected across a gradient of impacts on spat recruitment. Water, sediment and tissue samples were collected at all sites for characterising contaminant load and toxicity testing. Passive sampler devices (PSDs), including diffusive gradients in thin films (DGT) were used to capture organic chemicals and trace metals from the water column, respectively. The toxicity of metals, field-collected sediments and extracts was tested on early life stages of the GSM using the sperm viability, embryo-larval development, and spat survival assays.

The GSM embryo-larval development showed toxicity for all the sediment preparations where aluminium and iron were the most prevalent metals. Increasing temperatures (21°C) had a compounding effect on sediment toxicity, on both embryos and spat. Some of the PSD extracts caused spermiotoxicity but the causative agents have not been identified as further chemical analysis is required. The approach using embryo-larval development and spermiotoxicity assays are effective to investigate the effects of complex mixtures, at environmentally relevant levels, combined with climate-change condition scenarios on native species. The studies confirmed that discharges of run-off-associated sediments, in conjunction with rising seawater temperature, impacted the fitness of the different GSM life stages tested, suggesting that climate-driven stressors may largely contribute to the negative effects on population recruitment in coastal waters.

1 Introduction

The Greenshell™ mussel (GSM) / kūtai, *Perna canaliculus*, is a commercially, ecologically and culturally significant species to Aotearoa New Zealand (NZ). Unfortunately, summer die-offs, spat retention issues and a severe decline in GSM spat settlement have been reported in NZ's main aquaculture region, the Marlborough Sounds. These factors are impacting seed supply (Atalah et al. 2017) and thus limiting the industry's ability to expand and achieve the New Zealand Government's strategic aquaculture target of NZ$3 billion in sales by 2030 (New Zealand Government 2019).

The causes of these effects are unknown; however, preliminary evidence indicates that deteriorating water quality and particularly the presence of trace metals, pesticides and / or emerging organic contaminants, which originate from land-use activities, are contributing factors to the lack of settling larvae and unusual mortality patterns (McDougall et al. 2022; Rolton et al. 2022; Greenhough et al. 2023). At the same time, rising temperatures and an intensification of El Niño Southern Oscillations and storm events are causing rapid and extreme changes to coastal environments (Fasullo et al. 2018; Oliver et al. 2021; Swales et al. 2021). These climate-associated stressors (marine heatwaves, high sedimentation load and freshwater inputs, pH changes) may exacerbate any effects from anthropogenic inputs (Vasquez et al. 2022). A better understanding of these complex interactions in the Marlborough Sounds is essential to prevent GSM recruitment declines occurring in other regions of NZ, such as Northland, which provides the vast majority of NZ's GSM spat (65% of the industry's spat originate from one location, Te Oneroa-a-Tōhē / Ninety Mile Beach) (Aquaculture New Zealand 2020).

The performance of animals across a thermal range can be described by a curve, with an optimal temperature and decreasing performance at temperatures above and below optimum. The specific temperature at which the performance of an animal starts decreasing ('turning worse') is defined as the 'pejus temperature' (Pörtner and Farrell 2008).Our lab-based research showed that exposure of GSM adults to a sustained temperature of 24 °C results in impaired fitness, whereas exposure to 26 °C appears to be rapidly lethal (Ericson et al. 2022; Venter et al. 2023). For marine bivalves, planktonic early life stages such as sperm, embryos and larvae are more sensitive to stressors (either pollutants or abiotic stressors) than adult stages (Davis and Calabrese 1964; His et al. 1999; Vignier et al. 2019). For an accurate ecotoxicological assessment of chemical contaminants, it is crucial to determine adverse effects at different levels of biological organisation (i.e. multiple life stages and endpoints; Figure 1) and test these contaminants at environmentally relevant concentrations. Our research has already shown that the developing embryos and larvae of GSM are particularly sensitive to changes in water chemistry (McDougall et al. 2020b; French et al. 2021). It is therefore expected that elevated temperature will exacerbate the toxic effects of pollution on GSM as previously shown with another species of mussel (Freitas et al. 2019a).

Despite the commercial and ecological significance of GSM, most studies that have investigated the effects of contaminants in NZ have used blue mussel embryos (*Mytilus galloprovincialis*); however, this species may not respond in the same way as GSM embryos (Markich 2021). As part of our MBIE-funded research (Shellfish Aquaculture SSIF platform, CAWX1801), we have developed lab-based assays to study the toxicity of contaminants on the early life stages (sperm, embryos and larvae) of GSM and compared its sensitivity with the NZ and international standards for blue mussel species (Rolton et al. 2022). Using two reference contaminants to compare effects between mussel species (triclosan, an organic antimicrobial agent found in many consumer products and zinc, an essential metal ubiquitous in the environment and used extensively in industries), we showed that developing embryos of GSM (i) had a comparable sensitivity to triclosan as blue mussels embryos (Rolton et al. 2022), but (ii) were about five to six times more sensitive to zinc ($LC_{50\text{-}48h}$ of 32 $\pm$ 0.6 for GSM embryos and 177 $\pm$ 29 μg/L for blue mussel embryos, unpublished data).

*Figure 1. Life cycle of the Greenshell™ mussel (*Perna canaliculus*) showing the multiple life stages (red arrows) investigated during this project. Image credit: Eden Cartwright.*

Bioavailability plays an important role in the uptake of toxicants in aquatic invertebrates and is influenced by the binding affinity of these chemicals to substrates such as sediments (Means et al. 1980; Rainbow 2002). Passive sampler devices (PSDs), including diffusive gradients in thin films (DGT) for determining metal species in water, are simple and inexpensive tools used to characterise *in situ* bioavailable contaminant concentrations in surface waters (Poulier et al. 2014; Zhang and Davison 2015; Morin et al. 2018; Koppel et al. 2020; French et al. 2021). Because measurements are time integrated, the use of PSDs and DGTs, in contrast to traditional grab water sampling, enable the determination of contaminants over extended sampling periods and offer the capability to detect trace concentrations of micropollutants; thus PSDs and DGTs are well suited for the assessment of contaminant load and bioavailability (ASTM 2021). Field measurements identified that levels of trace metals (including zinc) shown to be toxic to GSM embryos are commonly detected in water samples from the Marlborough Sounds. Furthermore, our research indicates that sensitivity to contaminants is magnified when embryos of blue mussels are co-exposed to a 'high' temperature (23 °C, unpublished data). These early findings highlight the need to take into consideration temperature interaction when predicting the toxic response of all life stages of GSM to contaminants and other stressors.

External sediment loading to estuaries (or siltation) is increasing in many coastal systems because of land-use activities, particularly those related to forestry and agriculture (Hawks et al. 2022). In recent years, siltation events have intensified due to more extreme weather causing further land erosion and sediment run-offs, which can have deleterious effects on marine life, including mussels (Poirier et al. 2021; Swales et al. 2021). Sediment elutriate (i.e. sediment supernatant) toxicity tests are commonly used to assess contaminated field-collected sediment, and bioassays have been developed using echinoderms, bivalves and fish (Geffard et al. 2002; Volpi Ghirardini et al. 2005; Brown-Peterson et al. 2015; Boulais et al. 2018).

The key objectives of this project were to (1) identify the potential sources and fate of anthropogenic contaminants, (2) improve our understanding of GSM's susceptibility to stressors under climate-induced conditions, and (3) establish toxicity thresholds of ecologically relevant contaminants to ultimately assist regulators and industry as well as inform decision-making on environmental policies and land-use management.

2 Material and methods

2.1 Field sampling

2.1.1 Study area and site selection

Based on historical data on GSM wild-caught spat collected by the Marine Farming Association in the Marlborough Sounds over 60 years (Figure 1; https://cawthron.shinyapps.io/BMOP/), four study sites were selected (Figure 3): one site located in the Kenepuru Sound with moderate spat recruitment (Site 1: Skiddaw Bay, 41°12'3.03"S; 173°54'50.82"E), two sites with poor recruitment (Site 2: Beatrix Bay, 41°2'1.28"S; 174°2'1.12"E; Site 3: Garne Bay, 41°1'9.20"S; 173°48'10.10"E) and a fourth site located at the Cawthron Aquaculture Park in Glenduan, Nelson (Figure 3), which was used as a reference site with intensive land-based hatchery / nursery facilities producing GSM spat.

Water (via PSDs) and mussel tissue samples were collected from the four sites. An additional site in Waiona Bay was used to collect sediment samples, along with the three other sites in the Marlborough Sounds; however, no GSM spat recruitment data are available for the Waiona Bay site due to the absence of mussel farming.

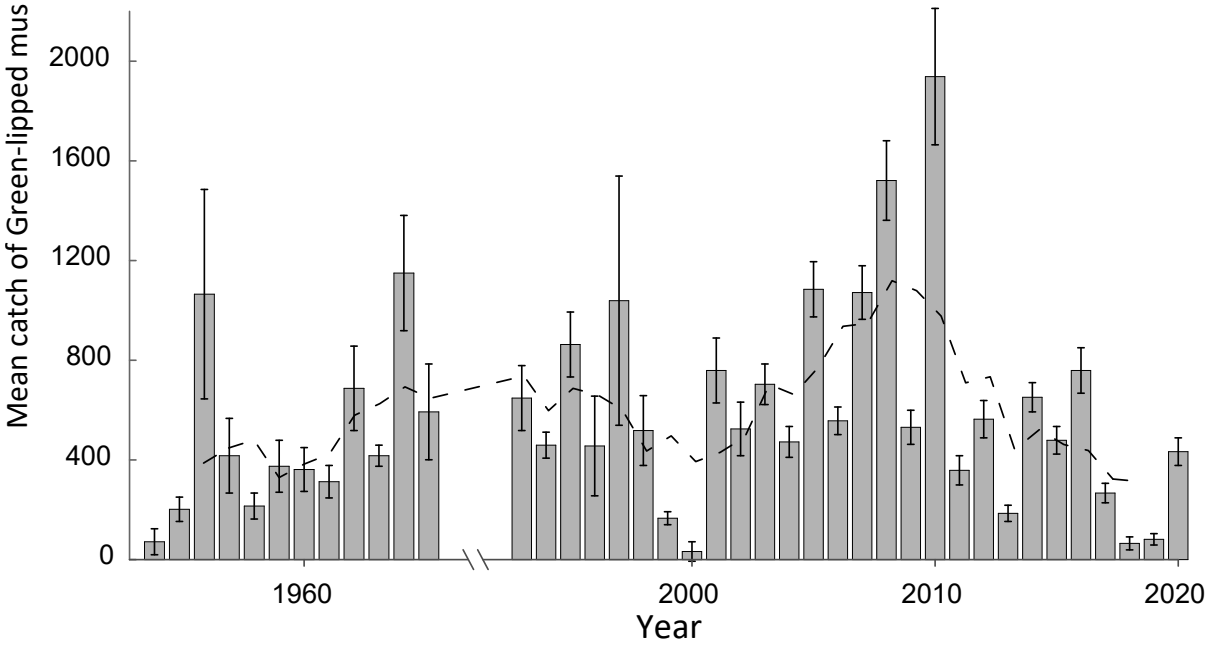


*Figure 2. Annual mean catches of Greenshell™ (*Perna canaliculus*) mussel spat (± S.E.) per 30 cm sampling rope in Pelorus Sound / Te Hoiere and Kenepuru Sound. The trend line indicates the 3-year rolling mean of spat catches and clearly shows changes since 2010. Note the gap between 1986 and 1992 when spat recruitment monitoring was paused. Data provided by NZ Marine Farming Association.*

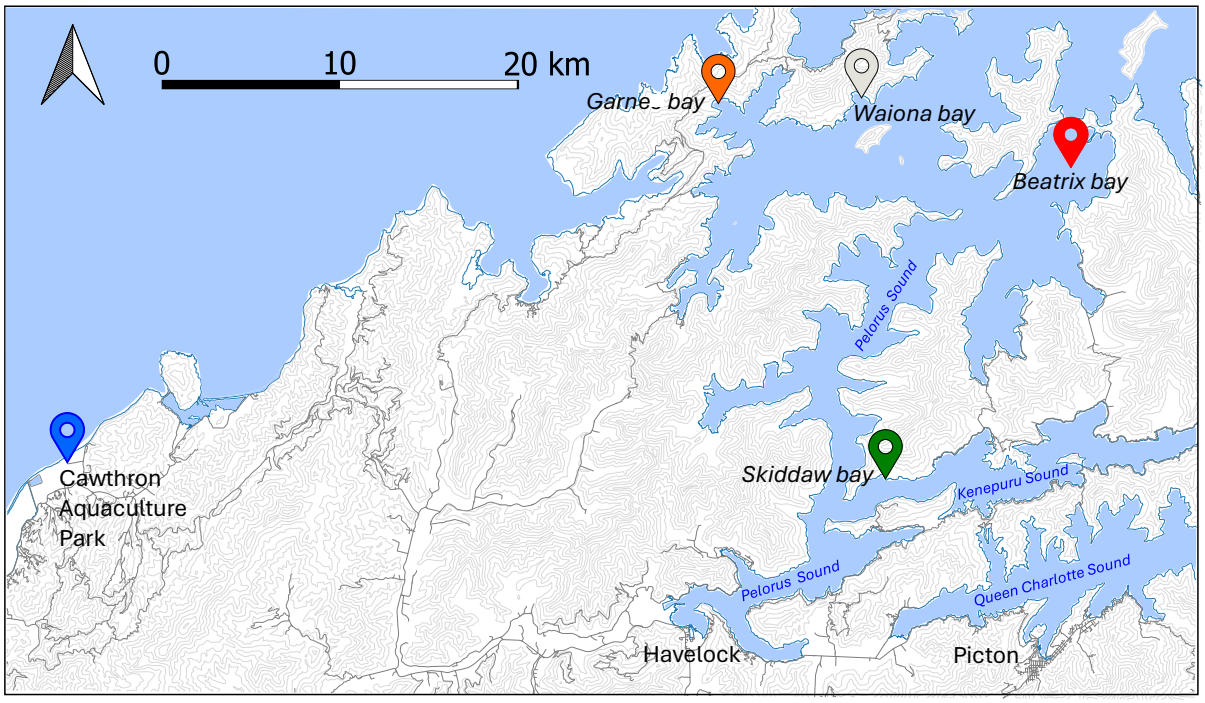


Figure 3. Study area (Pelorus Sound / Te Hoiere) and study sites in the Marlborough Sounds. Site 1: Skiddaw Bay; Site 2: Beatrix Bay; Site 3: Garne Bay; Site 4: Cawthron Aquaculture Park; Waiona Bay (control site used for further studies).

2.1.2 Collection of water samples via passive sampling devices

Passive sampling devices (PSDs) were chosen for this study because they accumulate chemicals over time, which provides a time-weighted average concentration and avoids short-term variability associated with grab sampling. PSDs were deployed *in situ* at the study sites between November 2021 and May 2023 – a period encompassing two natural reproductive seasons and peak settlements for green-lipped mussels. The PSDs comprised DGT for measuring the bioavailable (labile) concentrations of trace metals, and a Chemcatcher® device fitted with either Empore™ Anion-SR or Empore™ SDB-RPS for capturing a broad range of pesticides and organic compounds. The DGT samplers were purchased from DGT® Research Ltd (Lancaster, UK) and stored moist in deionised water (Milli-Q® 18 MΩ/cm) at 4 °C until deployment at sea. For the Chemcatcher® devices, stainless-steel housings were washed with Decon 90® followed by methanol and allowed to dry. Fixings (screws and nyloc nuts) were sonicated with methanol and Millipore water (18.2 MΩ) for 30 minutes and dried in an oven at 37 °C overnight. For deployment, the Empore™ discs were mounted in ring holders to facilitate uptake on both sides of the disc. Each set of PSDs included duplicate field blanks and a single laboratory blank for every deployment event for quality control. One day before field deployment, triplicate PSDs (i.e. three DGTs, three Anion-SR and three SDB-RPS) were carefully placed inside a plastic cage (Berley type) and secured by means of a nylon fishing line / cable ties and stored moist in Milli-Q® at 4 °C in a clean plastic bag. A temperature logger was also placed in each cage. Field blank DGT samplers and Chemcatcher® devices were also prepared but kept refrigerated in resealable bags during each deployment.

Figure 4. (A) Berley cage used to deploy passive samplers (B) out on the mussel longlines. Photo credits: Julien Vignier, Cawthron Institute.

Cages containing the PSDs were deployed at each site (Site 1 to Site 3) for 21 days at a depth of 4–8 m (Figure 4). Alternatively, PSDs from our control site (Site 4) were deployed at a hatchery and placed in a tank of running filtered seawater. At the end of the 21-day deployment, the DGT samplers and Chemcatcher® devices were recovered, rinsed with Milli-Q®, packaged at 4 °C (DGTs) or stored in resealable bags and frozen at -20 °C (Chemcatcher® devices) before being sent to the University of Waikato (Hamilton, NZ) and Plant and Food Research (Ruakura, Hamilton, NZ) for extraction and analytical chemistry.

2.1.3 Collection of mussel tissue samples

To characterise the bioaccumulated fraction of metals in mussel tissue, 15 crop-sized adult mussels (mean lengths of 90 mm [± 10]; mean live weight of 60 g [± 20]) were collected at each study site in November 2021, February 2022, May 2022, September 2022, March 2023 and May 2023. Samples of gill / mantle (≥ 1 g ww) were dissected from each individual using a ceramic scalpel and plastic tweezers, rinsed with Milli-Q®, dried to remove excess water, placed into a cryo-vial, flash frozen in liquid nitrogen and stored at -80 °C. All samples were then freeze-dried and stored at -20 °C for subsequent inductively coupled plasma-mass spectrometry (ICP-MS) analyses.

2.1.4 Collection of sediments samples

Sediments were collected on 25 January 2023. Conditions were clear with little wind, with only 2 mm of rain recorded in the 10 days before sampling (source NIWA National Climate Database). Sediments were collected with a van Veen grab sampler (Figure 5A) at the locations indicated in Figure 3: Garne Bay (S 41.013724°, E 173.79788°), Waiona Bay (S 40.99626°, E 173.91291°, depth 22 m), Beatrix Bay (S 41.02721°, E 174.04375°, depth 30 m) and Skiddaw Bay (S 41.20143°, E 173.90008°, depth 14 m). Three or four grabs per site were used to obtain the required amount for the experiments. Sediments (Figure 5B to 5E) were hand-mixed before being immediately sub-sampled in either 10 L buckets or resealable bags. Sampling was carried out to allow for two full sets: one for experiments and one as a backup. Sediment containers were kept frozen at -20 °C until use.

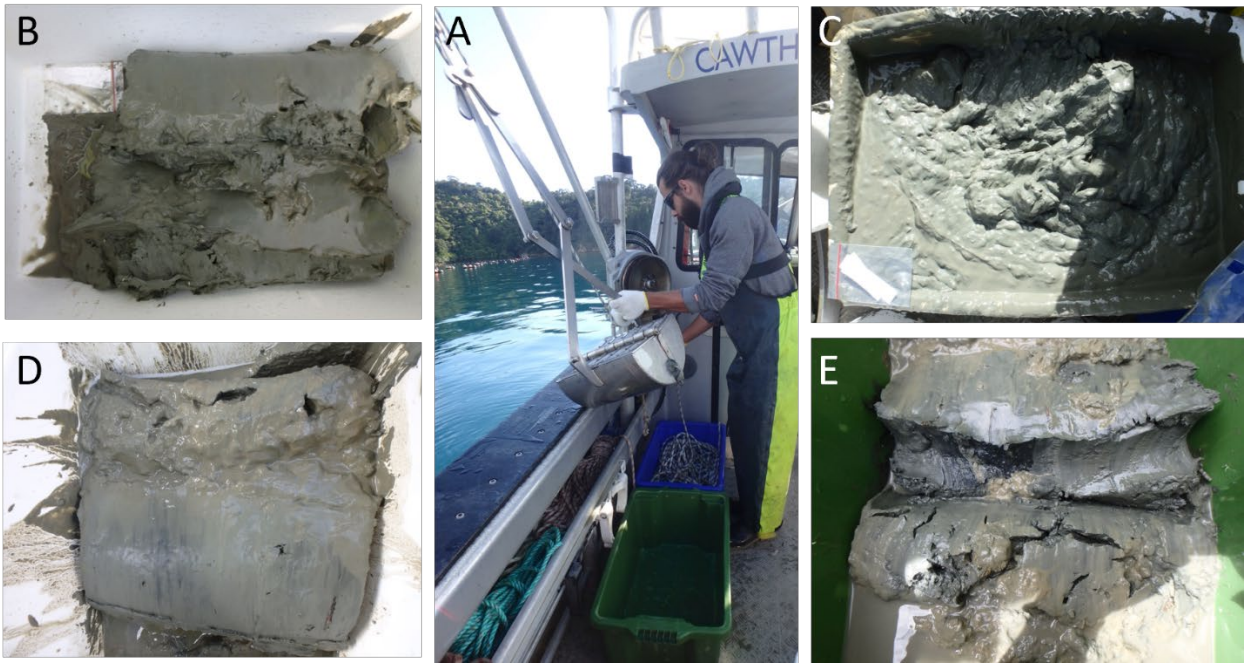


Figure 5. (A) Sediment collection with a van Veen grab sampler from (B) Garne Bay, (C) Waiona Bay, (D) Beatrix Bay and (E) Skiddaw Bay. Photo credits: Lucy Bizzozero, Ifremer Centre de Nantes.

2.2 Analytical chemistry

2.2.1 Extraction and analysis of metals from DGTs

The Chelex® binding resin was retrieved from the DGT housing for elution (French et al. 2021). Briefly, the binding resin was retrieved from the DGT housing and placed in 1 mL of 1 M HNO_3 for ≥ 24 hours. The eluents were diluted to a final concentration of 2% HNO_3 prior to analysis via ICP-MS. Metal concentrations within the DGT eluents were determined by ICP-MS using an Agilent 8900 ICP-MS (Agilent Technologies, Santa Clara, California, USA) controlled by MassHunter Workstation (version 4.5) at the University of Waikato, Hamilton, NZ. The ICP-MS was optimised daily to maximum sensitivity, ensuring oxides and doubly charged ions were less than 2%. Concentrations of aluminium (Al), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), cadmium (Cd), mercury (Hg) and lead (Pb) were determined using helium (He) mode to remove potential interferences. A five-point calibration curve, consisting of concentrations between 0.1 µg/L and 500 µg/L, was prepared for all trace elements using stock standard IV71-A (Inorganic Ventures, Christiansburg, VA, USA). A separate calibration curve, consisting of concentrations between 100 µg/L and 10,000 µg/L, was prepared for phosphorus (P) and iron using single-element standards (Inorganic Ventures, Christiansburg, VA, USA). Check standards were analysed every 20 samples and recalibration was performed every 100 samples. Blank samples were analysed every 10 samples to ensure minimal carryover. An online internal standard containing 45Sc, 72Ge, 103Rh, 193Ir and 205Tl was used to monitor and correct for instrumental drift and matrix effects. Instrument detection limits measured in µg/L were 0.22 (Al), 0.012 (Cr), 0.0046 (Mn), 0.13 (Fe), 0.0012 (Co), 0.0034 (Ni), 0.014 (Cu), 0.42 (Zn), 0.024 (As), 0.049 (Cd), 0.0057 (Hg) and 0.0055 (Pb). Final DGT concentrations were determined using the following equation:

$$C_{DGT} = \frac{M\Delta G}{DtA} \quad (1)$$

where M is the accumulated mass (mg); ΔG is the thickness of the diffusive gel (0.8 mm) plus the thickness of the filter membrane (cm); D is the diffusion coefficient of metal in the gel (cm^2/s); t is the deployment time (s); and A is the exposure area (cm^2).

Within this study, the effect from the diffusive boundary layer was presumed negligible due to the flow rate used (Warnken et al. 2006). Site-specific profiling of a suite of 12 trace metals was determined for each deployment and expressed in µg/L.

2.2.2 Extraction of organic compounds from passive sampler devices and preparation for bioassays

Duplicated Chemcatcher® membranes of each type (Anion-SR and SDB-RPS) were carefully handled using stainless-steel tweezers, taking care not to contaminate the discs. Discs were transferred into sintered glass vials (20 mL), with 10 mL of acetone added, and the vials were capped and shaken for 30 minutes at 150 RPM. The acetone was decanted into a 250 mL round-bottom flask. The process was repeated with 1:1 acetone:methanol (10 mL) and methanol (10 mL). Each sample was concentrated by rotary evaporation to approximately 2 mL and passed through a small bed of sodium sulfate / cellite and dissolved into a final volume of 0.6 mL of 100% dimethyl sulfoxide (DMSO). Samples were subsequently transferred into two aliquots of 0.3 mL for later analysis by bioassay, while the remaining replicate was archived / stored at -20 °C. Replicated solvent controls (100% DMSO) were also prepared.

2.2.3 Metal analyses from mussel tissue

A weighed sample of freeze-dried mussel tissue (≥ 100 mg dry weight) was placed in a 100 mL Teflon® tube. One mL of H_2O_2 and 3 mL of concentrated HNO_3 (69%) were added to each tube; screw-on caps were used to seal the tubes before 70 minutes of digestion in a microwave digestion system (Milestone Srl, Sorisole, Italy) at a temperature of 180 °C and 1,800 W microwave power. Thirty-six mL of type 1 deionised water (ASTM 2011) was then added to each tube. Each resulting solution was analysed with ICP-MS (7700x, Agilent, Santa Clara, CA) and the concentrations of aluminium (Al), sulfur (S), vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), silver (Ag), cadmium (Cd), mercury (Hg), lead (Pb) and uranium (U) were back-calculated for the original samples. The ICP-MS was operated in He mode to reduce polyatomic interferences. Calibration standards were prepared in a matrix-matched solution from 1,000 ppm single-element standards (Peak Performance, CPI International, Santa Rosa, CA) as well as a blank matrix-matched solution containing no single-element standards. An online internal standard (20 ppb Sc, Y & Tb) was used to monitor and correct for instrument drift and matrix effects.

2.2.4 Metal analyses from sediment, elutriate and water samples

Sediment and elutriate samples were analysed to determine the total recoverable concentrations of trace metals (Al, Co, Fe, Mn, Mo, Ag, As, Ca, Cu, Pb, Hg, Ni and Zn), with the determination of dry matter and total organic carbon. The raw elutriates (100%) were analysed for Al, Ca, Cr, Co, Cu, Fe, Pb, Mn, Ni and Zn, whereas diluted elutriates were only analysed for metals that were above detection limits (Al, Cr, Fe, Pb, Mn and Zn). Some elutriate samples were analysed for total organic carbon and dissolved metals. The total concentration of metals was analysed for metal-spiked seawater. All samples were kept refrigerated in the dark until being sent to Hill Laboratories for chemical analysis.

2.3 Toxicity testing

2.3.1 Preparation of sediment elutriates

Sediment samples were mixed with seawater at a ratio of 1:4 sediment:dilution water or 250 g raw sediment per litre (ASTM 2014). The dilution water was either reconstituted (for embryo exposures) or natural filtered seawater (FSW; for spat exposures) depending on the assay. Sediment and seawater were mixed for 1 hour in the dark at 4 °C. The mix was then centrifuged for 3 minutes at 3,000 g to allow the finest particles to settle. The pellet was discarded and the supernatant aerated to reach an oxygen saturation > 95%. The supernatant, or elutriate, was then diluted with the corresponding dilution seawater.

2.3.2 Test animals, gametes and embryos preparation

Multiple batches of sexually mature GSM (n = 35–60, shell length of 90–120 mm) of wild origin were collected from a commercial farm in the Marlborough Sounds, NZ. After collection, mussels were cleaned to remove external debris and transported on ice to the hatchery located at the Cawthron Aquaculture Park in Nelson, NZ. The mussels were maintained 'dry' in a chilly bin overnight to avoid spontaneous spawning.

Spawning was induced using a thermal cycling procedure (Adams et al. 2009). Once strong and sustained gamete emission was achieved, spawning mussels were rinsed and removed from the spawning tank. Females were placed in individual containers with approximately 500 mL of 0.3 µm-filtered, ultraviolet-treated (100 mJ cm^{-2}) and carbon-filtered seawater (FSW) at 10 °C. They were allowed to spawn for approximately 10 minutes before oocytes were discarded to avoid collecting any pre-fertilised embryos. Oocytes were then

collected in FSW and stored at 5 °C prior to experiments. A sub-sample of oocytes from each female was microscopically examined for pre-fertilisation (i.e. presence of polar bodies or cell cleavage) and 'clean' oocytes from up to three females were combined into one replicate. Three to five separate oocyte replicates were created. These pooled oocytes were then gently rinsed on a 70 µm Nitex® mesh with FSW to remove any gonadal debris before being suspended in FSW in a sterile 1 L glass beaker. Egg density was then determined for each pool / replicate by taking a 50 µL aliquot from a known dilution of concentrated eggs.

Spawning males were placed anterior-up in 50 mL plastic containers and left to spawn 'dry' (i.e. no seawater was added). Once collected, sperm from individual males were stored at 5 °C. A sub-sample of sperm from each male was examined under 400× magnification to verify motility before 'good' sperm from three males were combined to create three to five separate replicates. Sperm were gently rinsed through a 70 µm Nitex® mesh to remove gonadal debris, and concentrations were determined for each pool / replicate using a Neubauer® haemocytometer. Sperm were then stored at 5 °C until use for subsequent experiments or fertilisation.

Gametes were then used to make four or five pools of three individuals for male and female gametes, with each pool representing a replicate. Eggs were aged for 45 minutes in FSW previously treated with ethylenediaminetetraacetic acid (EDTA; 12µM). Each pool of eggs was then fertilised with one pool of male gametes, at a ratio of 500 spermatozoids per egg for 45 minutes, to allow for the formation of polar bodies before use. Density of embryos was adjusted to 10,000 embryos/mL, and 40 µL of the suspension was distributed in the test chambers, which contained 10 mL of either control reconstituted seawater or the test solution, to reach a final density in each test chamber of about 40 embryos/mL. Embryos were left to develop into swimming larvae for 48 hours in the dark. For each experiment two controls were carried out: a reconstituted seawater control and a natural FSW (12 µM EDTA) control.

Physico-chemical parameters of the seawater used for testing (dissolved oxygen concentration [mg/L], saturation [%] and salinity [PSU]) were measured with a Hach Q40d hand-held multi-meter and an Orion Star™ A211 pH meter from ThermoFisher Scientific™ (for the pH). At the beginning of the experiments, salinity, oxygen saturation and pH of the reconstituted seawater was 34.0 $\pm$ 0.2 PSU, 98.4 $\pm$ 3.0% and 8.5 $\pm$ 0.1, respectively, while FSW with EDTA was at 34.3 $\pm$ 0.8 PSU, 100.8 $\pm$ 1.1% and 8.3 $\pm$ 0.1, respectively.

At the end of the exposure period, larvae were fixed in 10% formalin. Larvae were assessed for their survival depending on their shape (Figure 6). Larvae that developed to the D-stage were considered normal, while any mussels presenting morphological defects (e.g. D-larvae with either a convex hinge, indented shell margins, incomplete shells, a protruded velum, or an extrusion of mantle) or arrested development (e.g. embryos or trocophores) were considered abnormal (His et al. 1999). Mean percent of normal D-larvae (or D-yield) was determined for each treatment based on the total number of larvae (normal, abnormal and dead) enumerated in each replicate. Tests were considered valid if at least a 70% D-yields was achieved in the control (ASTM 2021).

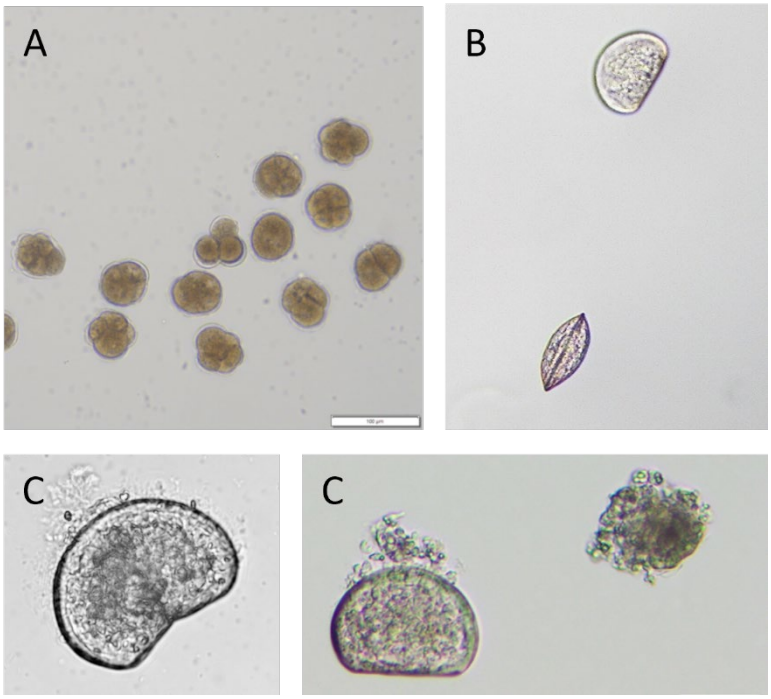


Figure 6. (A) Greenshell™ mussel fertilised eggs showing cell divisions after 180 minutes post-fertilisation. (B) Normal (lateral and frontal view) and (C) abnormal D-veliger larvae after 48 hours post-fertilisation. Photo credits: Cawthron Institute.

2.3.3 Embryo-larval development assay with a combination of temperature and metal or sediment elutriates

Impact of temperature on embryo-larval development

The impact of temperature was assessed by subjecting mussel embryos to a broad range of temperatures. The range was obtained with an aluminium block (Figure 7) connected on each side to a recirculatory thermoregulated water bath providing the lowest and highest temperatures (Sewell and Young 1999). The coldest and the warmest temperatures diffuse through the block to produce a gradient of temperatures. Test chambers containing 10 mL of reconstituted seawater and embryos were fitted into the holes in the block, representing 10 different temperatures (columns) with a gradient from 16 °C to 26 °C (approximately 1 °C per column), with five replicates (rows) per temperature tested. The experiment was carried out twice, once at the end of summer (end of February) and once at the beginning of winter (end of June) (Figure 8), to assess any seasonal changes. These experiments allowed us to determine which target temperatures would then be used in combination with metals of interest (informed by DGT measurements) and sediment elutriates.

Figure 7. Aluminium block used to assess the sensitivity of embryos to a range of temperatures. Photo credit: Cawthron Institute.

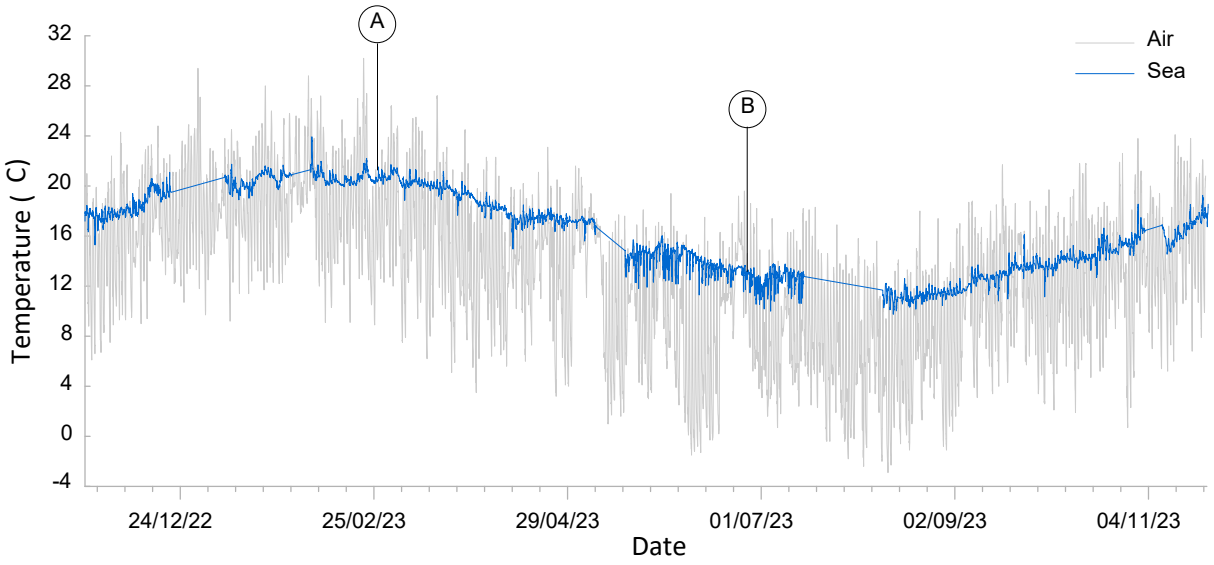


Figure 8. Daily temperature of air and sea (at 1 m depth) (Nov. 2022 to Nov. 2023) in the Tasman Bay / Te Tai-o-Aorere with time of testing (A and B). Data provided by Cawthron Institute (seawater) and NIWA (air).

Combination of temperature and single metal exposure

Metal test solutions were prepared in acid-rinsed 100 mL Schott bottles. The single metals tested on embryos were selected based on DGT-measured concentrations of a suite of metals from the study sites (see Section 3.1.1.).

A stock solution of each of the tested metals ($CoCl_2 \bullet 6H_2O$, $Pb(NO_3)$, $Zn(SO4) \bullet 7H_2O$) was prepared in type I water (ASTM 2011) at 2 g/L and 3 g/L, respectively. Six logarithmically equidistant concentrations (Table 1) for Co^{2+}, Pb^{2+} and Zn^{2+}, respectively, were prepared in reconstituted seawater. A volume of 10 mL of each were then transferred in five wells of six-well microplates, to obtain five replicates. The six concentrations of each metal were kept at 17 °C, 19 °C or 21 °C for 48 hours until the end of the test, when larval survival was assessed.

Combination of temperature and sediment elutriates from Marlborough Sounds

Sediment samples from sites with mussel farms (Skiddaw Bay, Garne Bay, Beatrix Bay) and an additional reference site without mussel farming (Waiona Bay) (Figure 3), were used to make elutriates as described in Section 2.3.1. The first experiment (range finding) was carried out to assess the range of effects from the dilutions of the 100% elutriate (Table 1) at 17 °C for 48 hours. From this range finding, six dilutions showing a high and intermediate survival were selected and used for the exposure with the three selected temperatures (17 °C, 19 °C and 21 °C for 48 hours).

Table 1. Nominal metal concentrations and sediment elutriate dilutions tested.

Test product	Concentration tested
Cobalt (Co^{2+}) (µg/L)	22.3, 37.1, 61.9, 103.3, 172.4, 287.5
Lead (Pb^{2+}) (µg/L)	81.8, 136.5, 227.6, 389.8, 653.3, 1056.6
Zinc (Zn^{2+}) (µg/L)	21.6, 34.6, 55.3, 88.5, 141.7, 226.7
Elutriate (%)	0.01, 0.02, 0.05, 0.1, 0.2, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50

2.3.4 Combined sediment elutriates and temperatures exposures of spat

Approximately 11,000 mussel spat (mean size of 2–5 mm; Figure 9A), produced in the hatchery about 10 weeks prior from wild parents caught in Golden Bay / Mohua, were exposed acutely to two different field-collected sediment samples at three different temperatures (17 °C, 19 °C, 21 °C) for 9 days. These mussels were previously cultured in our land-based facility in a tank supplied with 25 µm-filtered seawater at 15 °C (± 1) and fed *ad libitum* with a mixture of hatchery-grown microalgal feed and naturally occurring algal assemblages bloomed in earthen seawater ponds (French et al. 2021).

Figure 9. (A) Greenshell™ mussel spat used for the study; (B) experimental set up with three temperature holding tanks with experimental beakers. Photo credits: Julien Vignier, Cawthron Institute.

Experimental mussels were randomly divided into groups of 100 live individuals and transferred into clean glass beakers filled with 500 mL of FSW, which were kept at 17 °C for acclimation. During the 5 days acclimation, temperature was increased at a rate of 1.5 °C/day to reach the appropriate target temperature of 19 °C or 21 °C (Appendix Figure A.1) and maintained at the respective temperature by means of a water bath (Figure 9B) for 9 days. Two field-collected sediments – Skiddaw Bay (Site 1) and Beatrix Bay (Site 3) – were tested using the

elutriate procedure previously described in Section 2.2.3. The following elutriate dilutions were tested, in triplicates, at each temperature for each sediment: **0% (Control FSW) – 3.13%, 6.25%, 12.5%, 25%, 50%**.

Every other day, the content of each beaker was poured into a mesh (to retain the mussels), and seawater was fully exchanged to maintain acceptable water quality parameters. Mussels were fed daily by adding 10 mL of a bi-specific ration of microalgae (*Tisochrysis lutea*:*Chaetoceros muelleri*) per beaker. Mortality was assessed every other day by counting under magnification the number of spat with empty shells, and / or the absence of internal organisation or movement, and / or a failure to close their valves (South et al. 2021).

Finally, temperature, dissolved oxygen (DO; YSI probe), and chlorophyll-*a* (Turner Designs hand-held probe) water quality measurements were collected daily, whereas pH (hand-held Testo 206 pH meter) and total ammonia nitrogen (API® Ammonia test kit) were measured periodically throughout the trial.

2.3.5 Organic compounds exposure of sperm

To test the toxicity of field-collected extracts (Figure 10), we used a 'spermiotoxicity' assay, which characterises the health and viability of the sperm (via the use of flow cytometry) and the subsequent fertilisation success of exposed sperm with non-exposed eggs (Rolton et al. 2022).

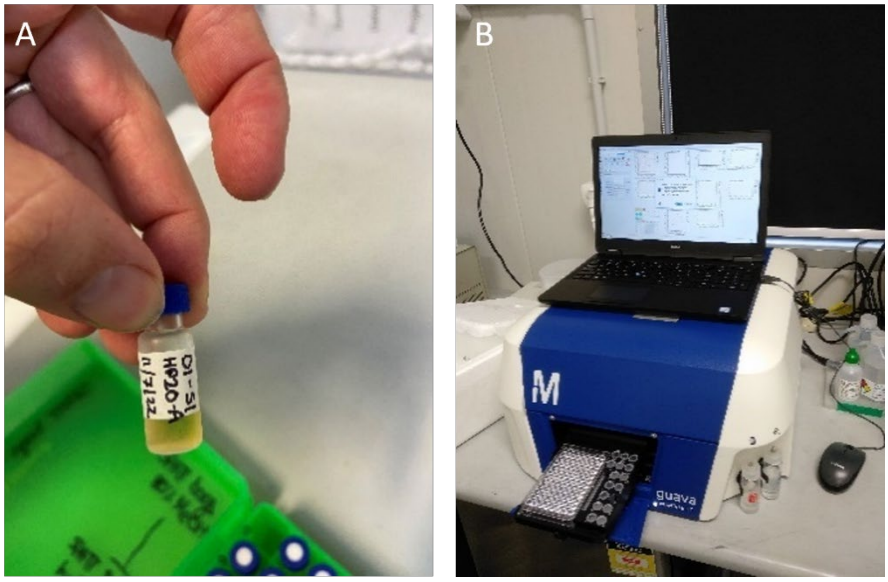


Figure 10. (A) Field-collected extract consisting of a cocktail of organic contaminants to be tested on Greenshell™ mussel sperm . (B) Guava® flow cytometer used for high-throughput cellular assessments of exposed sperm. Photo credits: Julien Vignier, Cawthron Institute.

Sperm collected from two to three males were pooled to make one replicate / pool of sperm, and three replicate pools of sperm were made (i.e. three pools of multiple males). Sperm from each pool (n = 3) was then either pre-diluted with FSW to reach a density of 1×10^6 cells/mL for the flow cytometry assessment, or to 1.25 x 10^8 sperm cells/mL for the fertilisation assay. Using Kimble® borosilicate glass tubes (9 mL), each pool of sperm was then exposed to the 48 different solutions extracted from Anion-SR and SDB-RPS PSDs, as well as a seawater control, a DMSO vehicle control (0.5% final concentration) and several field blanks(see Tables A3 and A4 for more details). Exposure was achieved by adding 45 µL of pure extracts or control treatment to each sperm solution to reach a final concentration of 0.5% DMSO. All exposures were carried out at ambient temperature (16–17 °C) for 60 minutes.

Sperm viability assay

Following the 60-minute exposure, the cellular characteristics of the spermatozoa (detailed below) were analysed by flow cytometry using a Guava® easyCyte™ 5HT flow cytometer equipped with a 488 nm blue laser and three detectors (FL1, green [525/30]; FL2, yellow [583/26]; FL3, red [695/50]). Samples were acquired during 30 seconds at a fixed flow rate of 0.24 µL s^{-1}. Assay incubations took place at 17 °C, in the dark, and results were analysed using Guavasoft™ 3.3 software.

Spermatozoa were identified and analysed according to their relative size and internal complexity, which correspond to the forward scatter and side scatter of light, respectively, and are presented in arbitrary units. The viability of spermatozoa was assessed using a LIVE/DEAD™ sperm viability kit (Molecular Probes, Invitrogen, L7011). Following 45 minutes of experimental exposure, 200 µL of sperm was double stained with SYBR® 14 at 0.5 µM final concentration (FC) and propidium iodide (PI) at 10 µg/mL FC for 15 minutes, before analysis (modified from Rolton et al. 2015). The membrane permeant dye SYBR® 14 stains cells with DNA, emitting at 516 nm (FL1), and PI enters cells with damaged membranes, emitting at 617 nm (FL3), thus allowing distinction between sperm that are alive and those that are dying and dead. Results are expressed as a percentage (%) of dead sperm (Rolton et al. 2022).

Fertilisation assay

Sperm at 1.25 x 10^8 cells/mL concentration were exposed as described above. Concomitantly, approximately 5,000 eggs pooled from multiple females were loaded in clean glass beakers filled with 10 mL of FSW (which had previously been treated with 12 µM EDTA) and had a pH of 8.35. Bovine serum albumin was added to each egg solution at 0.1% (v:v) to assist with fertilisation (Adams et al. 2009). Following the 60-minute exposure, sperm (40 µL) was added to each beaker at a pre-determined sperm:egg ratio of 1,000:1 to proceed to fertilisation. Fifteen minutes post-fertilisation (mpf), fertilised eggs were topped up with 90 mL of FSW (EDTA-treated and pH adjusted). After 60 mpf, a 2.5 mL aliquot was collected from each beaker and placed in a 24-well tissue culture plate and kept at 16 °C. After 180 mpf, all aliquots were fixed by adding 75 µL of 10% buffered formalin. Fertilisation success was determined from microscopic examination of the first 100 eggs from each aliquot presenting first cellular divisions (Figure 10A) and is expressed as a percentage.

2.4 Statistical analyses

Metal composition of surface seawaters (DGT) and mussel tissue were visualised for each deployment and site with a principal component analysis of the $\log_{10}$ DGT metal concentration data and $\log_{10}$ mean tissue metal concentration, respectively, using the package '*ggord'* for the ordination plots and '*prcomp'* function from the '*stats'* package with R (R Core Team 2023). Concentrations of zinc and lead measured in the water via DGTs and in the mussel tissue were compared through time and across sites, and the interactions of these two factors were assessed using two-way ANOVAs, while multiple pairwise comparisons were conducted nested for each time point / deployment (Holm-Sidak post-hoc test).

For the embryo-larval development assays (embryo exposure), calculation of the LTx (the temperature killing x% of the test population) or the LCx (the concentration killing x% of the test population) with associated 95% confidence intervals (95% CI) and model-based no (significant) effect concentration (N(S)EC) were carried out with R using bootstrap resampling with the 'drc' (Ritz et al. 2016) and the Bayesian 'Bayesnec' (Fisher et al. 2023) packages, respectively. Hypothesis testing (level of statistical significance of $P < 0.05$) to determine the no observed and lowest observed effect temperature / concentrations and interactions was based on the method described in Hall and Golding (1998) and conducted with Statistica 13 (TIBCO Inc., USA).

For the juvenile assay (spat exposure), survival functions were computed with R according to Kaplan and Meier (1958) using the '*Survival' package* (version 3.2-13) (Therneau and Grambsch 2000; Therneau 2023). Survival time was measured in days post-exposure to sediment elutriates. The data were read as the number of dead mussels within each beaker at each time point. Survival time curves were compared using the Cox regression model (Cox 1972) after adjusting for temperature, sediment type and concentration, and the random effect of the beakers was also considered. The proportionality of hazards (PH) was checked based on Schoenfeld residuals (Schoenfeld 1982). For the spermiotoxicity assays (sperm exposure), field-collected extracts were considered toxic when mean mortality rates of exposed sperm were significantly different from the most toxic field blank using one-way ANOVA (Holm-Sidak post-hoc test).

3 Results

3.1 Environmental chemistry

3.1.1 Metals concentrations in surface seawater

Principal component analyses of the DGT-based metal concentrations and associated measure of centrality showed that, across the three study sites, zinc (Zn) and lead (Pb) were the most predominant elements in water samples collected between November 2021 and June 2023 (Figure 11). Details are reported in Appendix Table A1.

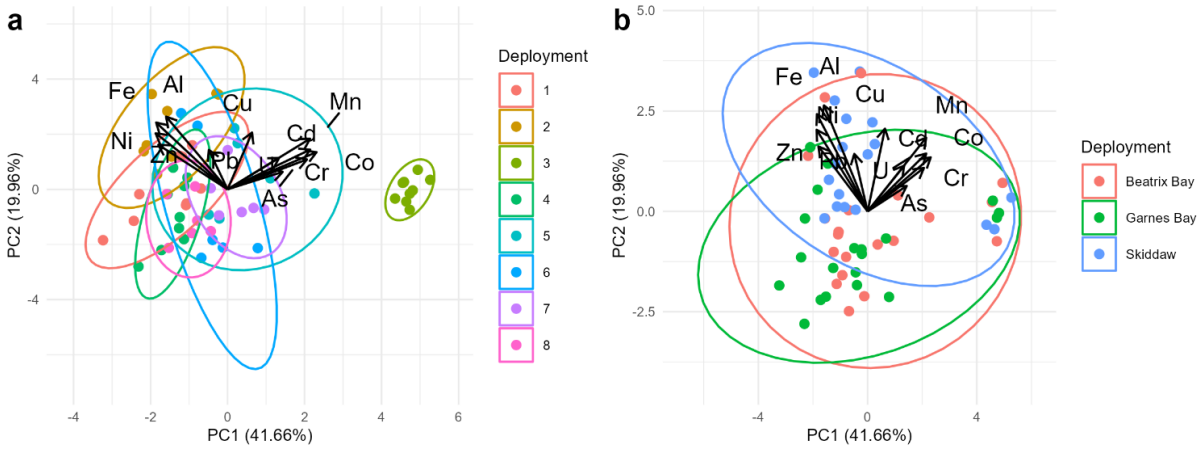


Figure 11. Principal component analysis of the log_{10} DGT bioavailable metal concentration data by (a) deployment and (b) site.

When looking at concentrations of bioavailable zinc in surface waters, we found a strong influence of time / deployment ($F_{7\text{-}35}$ = 81.19; p < 0.001) as well as a significant influence of site ($F_{2\text{-}35}$ = 4.128; p = 0.025). However, no significant interaction of deployment and site was found ($F_{14\text{-}35}$ = 1.049; p = 0.432*)*. More specifically, the highest levels of zinc (> 60 µg Zn^{2+}/L) were detected during deployment 2 (February 2022), regardless of collection site (Figure 112A). Overall, no clear site-specific prevalence of zinc could be found, except for the latest deployments (7 and 8) where Beatrix Bay appears to have the lowest concentrations of zinc (Figure 112A).

When looking at concentrations of bioavailable Pb, we again found strong temporal variations, with deployment time significantly influencing lead concentrations ($F_{7\text{-}35}$ = 36.591, p < 0.001), whereas site collection had no significant influence ($F_{2\text{-}35}$ = 1.952; p = 0.157). Interactions of Site X deployments had a moderate effect on lead levels ($F_{14\text{-}35}$ = 1.850 p = 0.070) (Figure 12B). At some specific times of the year (e.g. deployment 2, February 2022), very high levels of lead (up to 1,550 µg Pb^{2+}/L at Beatrix Bay) were detected (Figure 12B). During deployment 5 (February 2023), high levels of lead were again measured in Beatrix Bay (241 µg Pb^{2+}/L) and Garne Bay (152 µg Pb^{2+}/L) (Figure 12B).

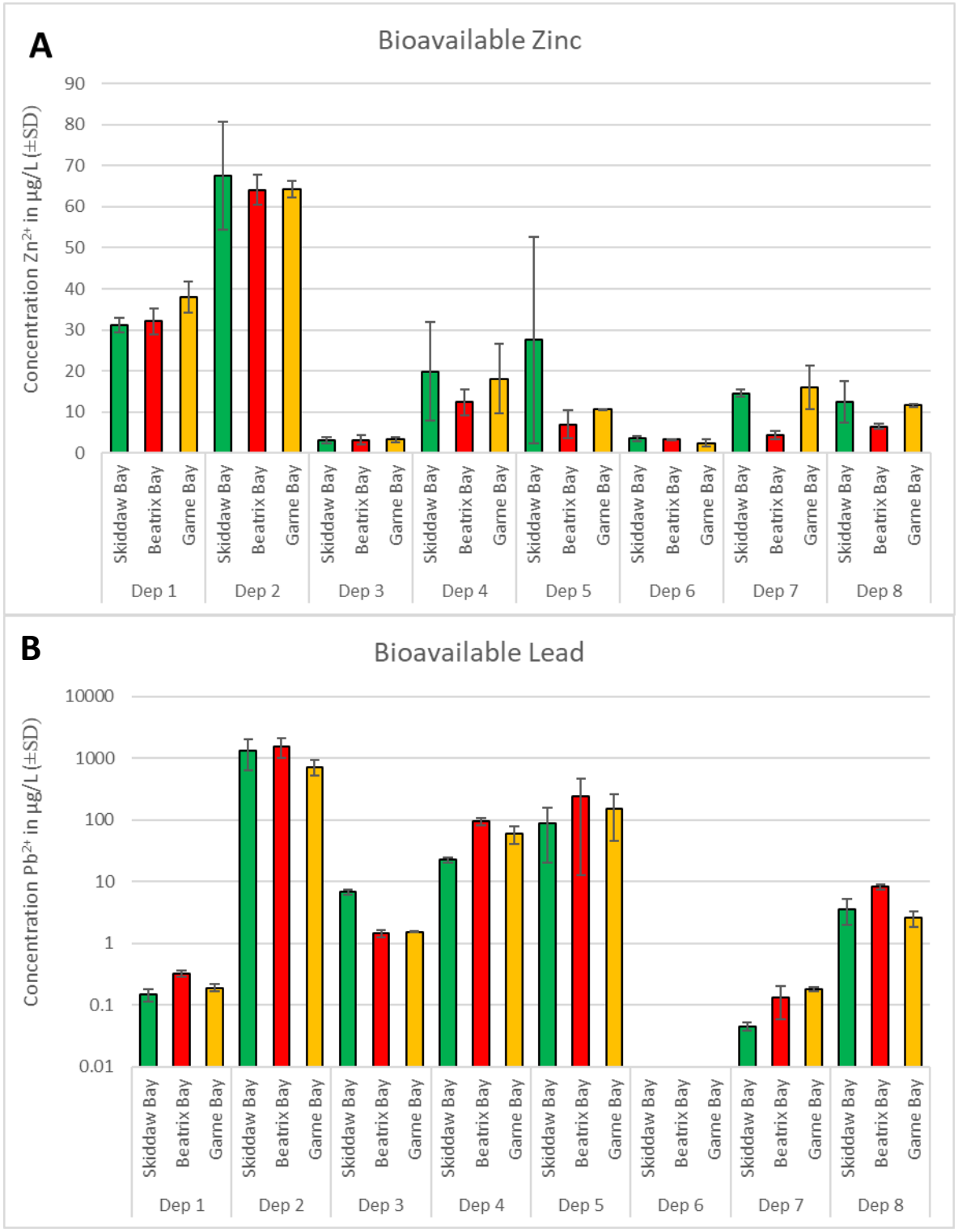


Figure 12. Mean concentrations (± SD) of bioavailable (A) zinc (Zn) and (B) lead (Pb) measured by DGT (n = 3) across three study sites – Skiddaw Bay (green); Beatrix Bay (red); Garne Bay (yellow) – throughout two catching seasons of Greenshell™ mussels (November 2021–May 2023 corresponding to eight deployments). Each field deployment (Dep) of DGT lasted 21 days. Note that the y-axis of graph B is on a log scale.

To link field-collected metal labile concentrations (i.e. bioavailable concentrations) measured by DGT with nominal concentrations used for embryo toxicity assays, we spiked artificial seawater with increasing concentrations of lead and zinc and placed a DGT probe at each concentration for 48 hours. Relationships between nominal and measured labile concentrations of Zn^{2+} and Pb^{2+} were determined and presented in Figure 13A and 13B, respectively.

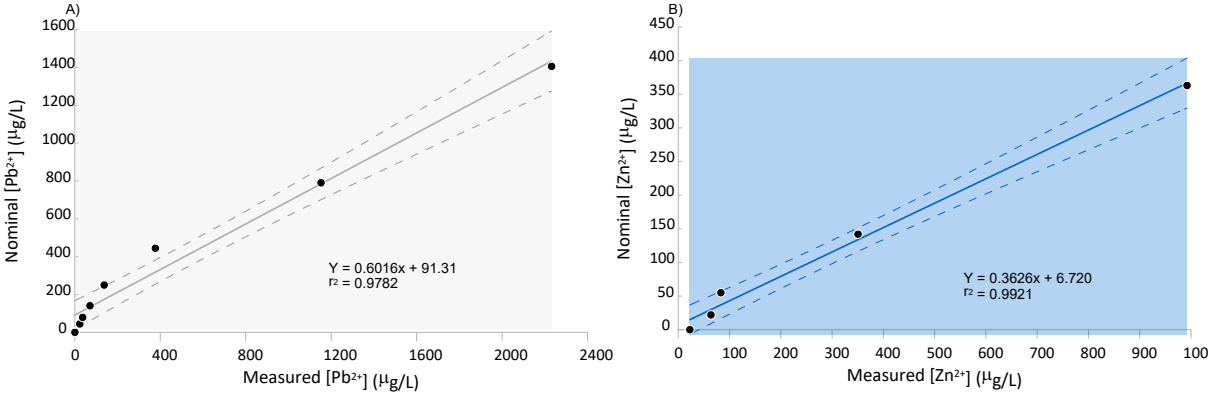


Figure 13. Nominal and DGT-measured concentrations in spiked seawater with (A) Pb^{2+} and (B) Zn^{2+}.

3.1.2 Metal concentrations in mussel tissue

Principal component analyses of the tissue metal concentrations measured between November 2021 and May 2023 showed that zinc (Zn), iron (Fe) and arsenic (As) were the most prevalent metals, across the three study sites (Figure 14; Appendices Table A2).

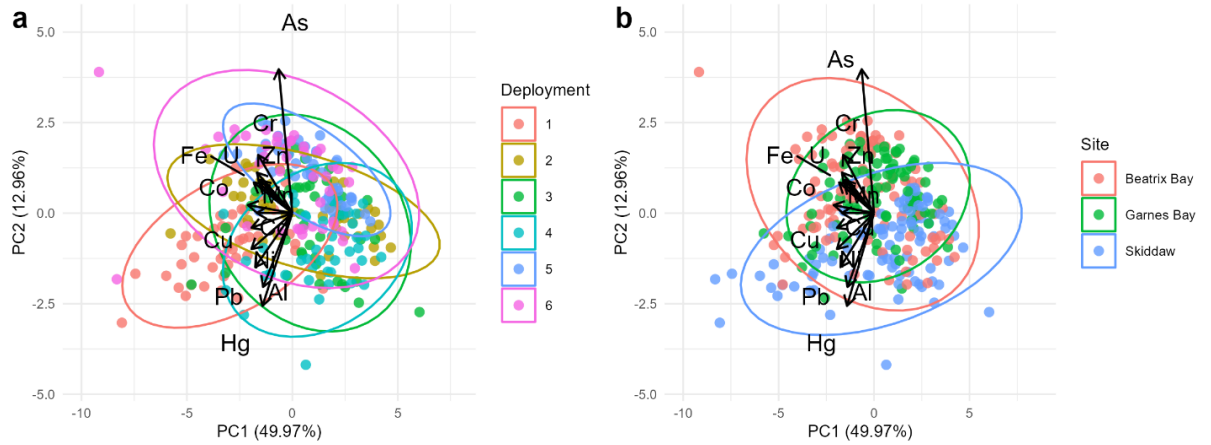
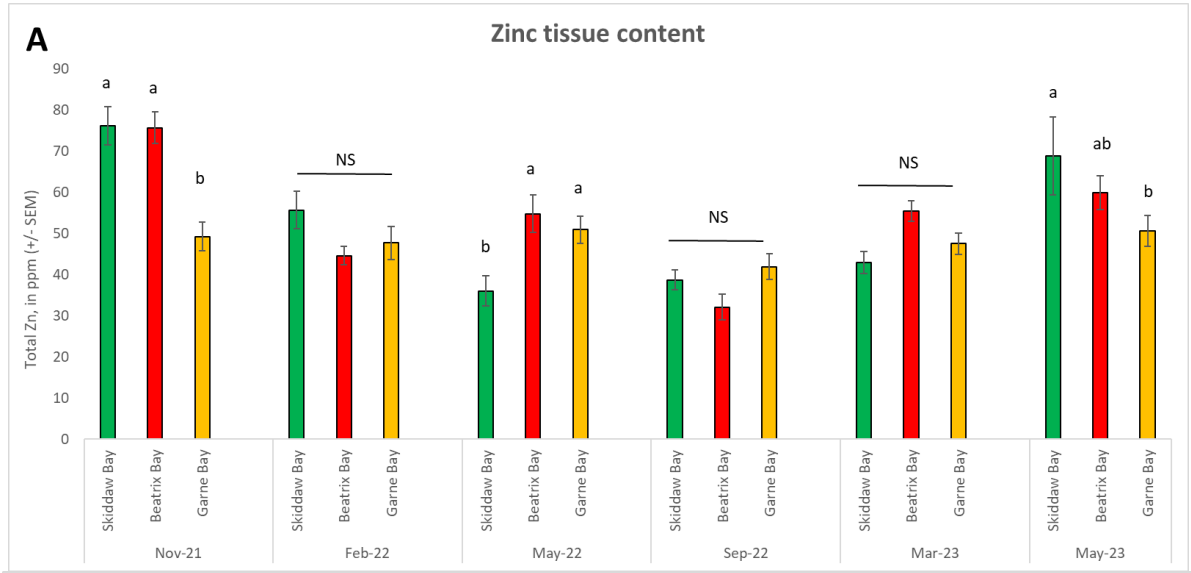


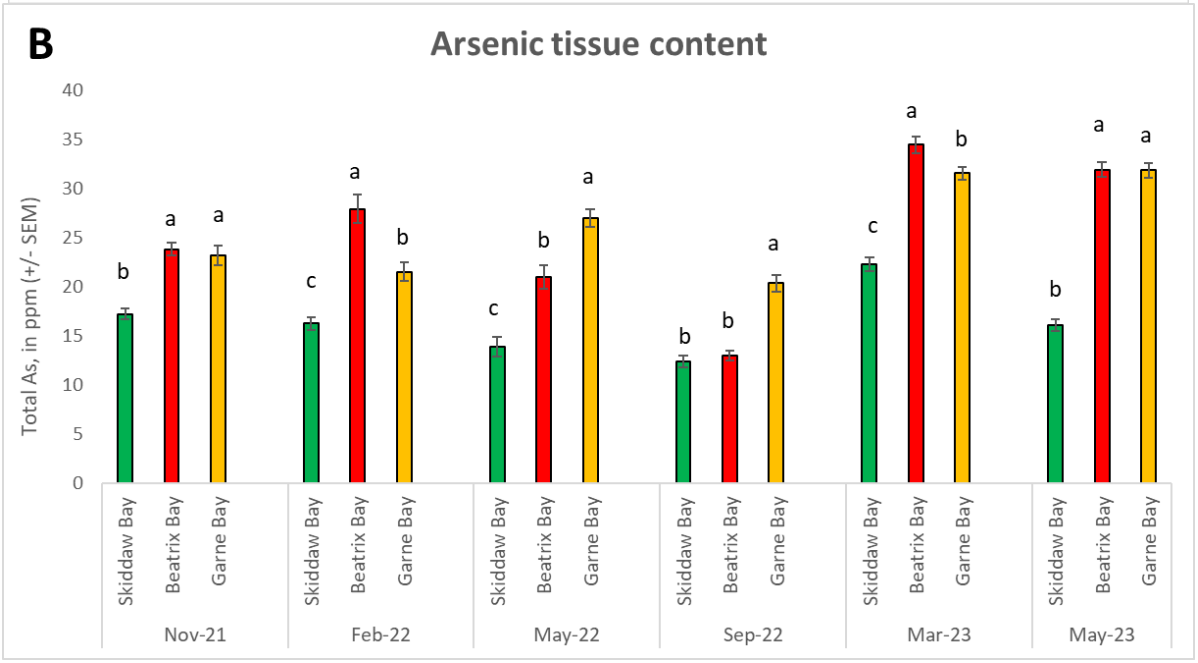
Figure 14. Principal component analysis of the log_{10} mean tissue metal concentration by (a) deployment and (b) site.

Specifically, zinc concentrations in mussel tissue ranged between 35 µg/g and 80 µg/g dry weight tissue during the study (Figure 15A). Tissue concentrations of zinc were significantly associated with time of sampling ($F_{5\text{-}252}$ = 19.105; p < 0.001), site ($F_{2\text{-}252}$ = 3.575; p = 0.029), and a strong interaction of these two factors ($F_{10\text{-}252}$ = 5.442; p < 0.001) was found (Figure 15A). No clear site-specificity was observed, with contrasting trends of zinc content in mussels grown in Skiddaw Bay (green bars), Beatrix Bay (red bars) and Garne Bay (yellow bars) (Figure 15A).

On the other hand, arsenic concentrations in mussel tissue ranged from 15 µg/g to 35 µg/g dry weight tissue over the course of the study (Figure 15B). Significant effects of time of sampling ($F_{5\text{-}251}$ = 69.39; p < 0.001), site ($F_{2\text{-}251}$ = 134.74; p < 0.001) and a strong interaction of these two factors ($F_{10\text{-}251}$ = 11.396; p < 0.001) were found to influence tissue uptake of arsenic (Figure 15B). In addition, mussels grown in Skiddaw Bay (green bars) had

consistently lower levels of arsenic than mussels grown in the other two sites, while mussels from Beatrix Bay (red bars) had the highest content of arsenic in their tissue (Figure 15B).





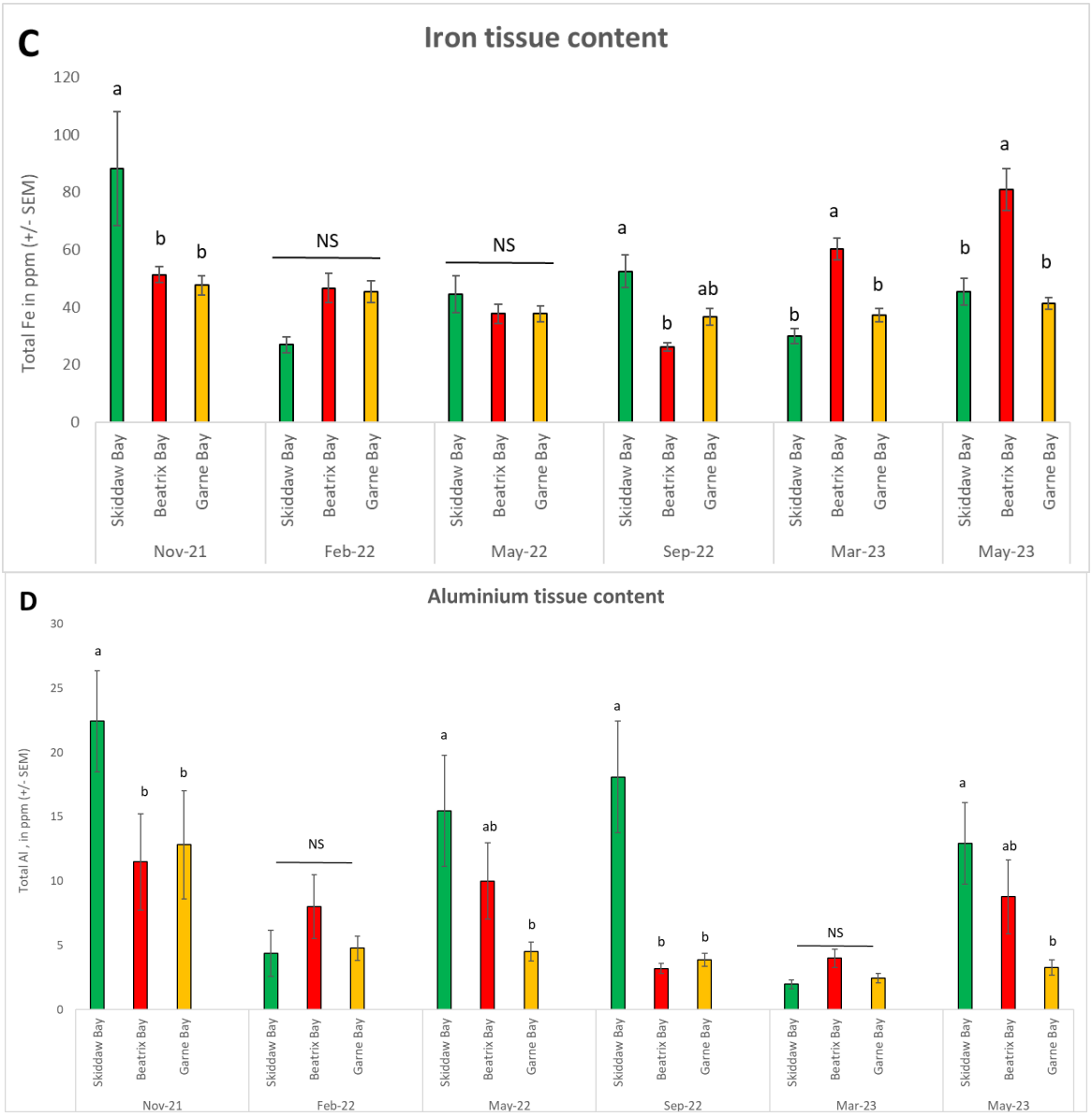


Figure 15. Concentrations of (A) zinc, (B) arsenic, (C) iron, and (D) aluminium in tissue from mussels collected at the study sites – Skiddaw (green); Beatrix Bay (red); Garne Bay (yellow) – between November 2021 and May 2023. Mean concentrations (± SEM, n = 15) are expressed as measured total metal in µg per g dry weight of mussel tissue (or ppm). Different superscript letters denote a significant difference at p = 0.05 (ANOVA Holm-Sidak, nested per time point). NS: not significant.

Iron concentrations measured in mussel tissue ranged from 30 µg/g to 90 µg/g dry weight tissue (Figure 15C). Tissue concentrations of iron were significantly associated with time of sampling ($F_{5\text{-}252}$ = 8.220; $p < 0.001$), site ($F_{2\text{-}252}$ = 3.907; p = 0.021) and a strong interaction of these two factors ($F_{10\text{-}252}$ = 7.478; $p < 0.001$) was found (Figure 15C). Overall, no clear site-specificity was observed even though mussels collected from Beatrix Bay in March and May 2023 showed the highest levels of iron compared to the other two study sites ($p < 0.05$; Figure 15C).

Finally, aluminium concentrations in tissue were highly variable, ranging from 2 µg/g to 28 µg/g of tissue, and strongly dependent on time of sampling ($F_{5\text{-}250}$ = 8.057; $p < 0.001$) and environment ($F_{2\text{-}250}$ = 11.97; $p < 0.001$; Figure 15D). A significant interaction of time and site was also found ($F_{10\text{-}250}$ = 2.375; p = 0.011). Overall, mussels grown at the Skiddaw Bay site had higher content in total recoverable aluminium than mussels collected from the other sites ($p < 0.05$; Figure 15D). Interestingly, aluminium levels in mussels from Skiddaw Bay dropped significantly during the summer periods (February 2022 and March 2023; Figure 15D)

3.1.3 Metal concentrations in sediment and elutriate samples

Results are provided in Table 2 along with the recommended default guideline values (DGV) for toxicants in sediment (Australian Government Initiative 2020). Aluminium and iron were the two metals with the highest concentrations found in sediment in all sites, with Skiddaw Bay having the highest concentration and Waiona Bay the lowest. Garne Bay and Beatrix Bay had nickel concentrations just above the DGV, while Skiddaw's nickel concentration was at the DGV-high. All other metal / metalloid concentrations were below their related DGV.

Table 2. Total recoverable concentrations of a series of metals and metalloids in the sediment samples collected at the study sites, along with the associated recommended default guideline values (DGV) in sediment.

		Garne Bay	Waiona Bay	Beatrix Bay	Skiddaw Bay	DGV	DGV-high
Dry matter	g/100 g	46	59	49	53		
Aluminium	mg/kg dw	19,800	13,200	22,000	27,000		
Cobalt	mg/kg dw	8	6.1	8.6	11		
Iron	mg/kg dw	29,000	22,000	31,000	37,000		
Manganese	mg/kg dw	340	280	360	420		
Molybdenum	mg/kg dw	0.42	0.32	0.32	0.8		
Silver	mg/kg dw	0.04	0.03	0.04	0.04	1	4
Total organic carbon	mg/kg dw	1.36	0.81	1.22	1.4		
Arsenic	mg/kg dw	4.2	3.2	3.9	6	20	70
Cadmium	mg/kg dw	0.075	0.035	0.063	0.062	2	25
Chromium	mg/kg dw	40	23	35	66	80	370
Copper	mg/kg dw	12	7	13.8	18	65	270
Lead	mg/kg dw	15.8	10.3	15.3	14.1	50	220
Mercury	mg/kg dw	0.04	0.04	0.04	0.06	0.15	1
Nickel	mg/kg dw	27	18.5	26	52	21	52
Zinc	mg/kg dw	65	51	66	72	200	410

After elutriation with the reconstituted seawater, water was sent for chemical analysis for the metals with the highest concentrations found in sediments. Results are reported in Table 3A for the embryo-larval development assay and Table 3B for the spat survival (average and standard deviation, $n = 5$). Concentrations found in suspension in seawater were 16,000 to 50,000 times lower than in sediments. Aluminium and iron remained the most prevalent metals. Sediment elutriates from Skiddaw Bay had the highest concentration of aluminium, followed by Waiona Bay, while Garne Bay and Beatrix Bay had the same and lower concentrations. Iron concentrations in sediment elutriates from Skiddaw Bay and Waiona Bay were comparable and higher than for Garne Bay and Beatrix Bay. The total organic content (non-purgeable organic carbon) of the seawater used to carry out elutriates is 1.13 ± 0.06 mg/L.

Table 3. Total recoverable concentrations of metals in sediment elutriates (undiluted) from the four study sites for A) the embryo-larval development assay and B) the spat survival assay.

A) Metal		Garne Bay	Waiona Bay	Beatrix Bay	Skiddaw Bay
Aluminium	mg/L	0.69	0.85	0.65	1.17
Cadmium	mg/L	< LOD	< LOD	< LOD	< LOD
Chromium	mg/L	0.0025	0.0022	0.0022	0.0026
Cobalt	mg/L	0.001	0.00068	< LOD	< LOD
Copper	mg/L	0.0023	< LOD	< LOD	< LOD
Iron	mg/L	0.67	0.78	0.56	0.72
Lead	mg/L	0.0012	0.0013	< LOD	< LOD
Manganese	mg/L	0.0088	0.0156	0.0086	0.135
Nickel	mg/L	< LOD	< LOD	< LOD	< LOD
Zinc	mg/L	0.0083	< LOD	< LOD	0.0044

B) Metal		Beatrix Bay	Skiddaw Bay
Aluminium	mg/L	1.006 ± 0.167	1.503 ± 0.499
Chromium	mg/L	0.003 ± 0.0005	0.003 ± 0.001
Iron	mg/L	0.664 ± 0.334	0.635 ± 0.287
Lead	mg/L	< LOD	< LOD
TOC	mg/L	5.53 ± 0.80	7.43 ± 1.33

Limit of detection (LOD) (mg/L): Aluminium < 0.00021; Cobalt < 0.00063; Copper / iron < 0.0011; Nickel < 0.007; Zinc < 0.0042

3.2 Effects of temperature on embryo-larval development

The survival of larvae after a 48-hour exposure to a range of 10 temperatures during two periods of the year are reported in Figure 16. The ecotoxicological parameters derived from the fitted model (three-parameter Weibull model) and hypothesis testing are reported in Table 4. The sensitivity to temperature is higher in early winter than in late summer, with all ecotoxicological parameters lower in early winter. The median lethal temperature ($LT_{50\text{-}48h}$) was 22.1 °C (22–22.2 °C) and 19.2 °C (18.4–19.4 °C) in February and June, respectively, and the no significant effective temperatures were 21 °C (20.9–21.1 °C) and 18.6 °C (17.5–19.5 °C) °C in February and June, respectively. All the testing with metals or sediment elutriate in combination with temperature were carried in autumn. To account for the increase in sensitivity, the three selected temperatures were reflecting a no significant effect (17 °C), the last no significant effect (19 °C) and a medium effect (21 °C).

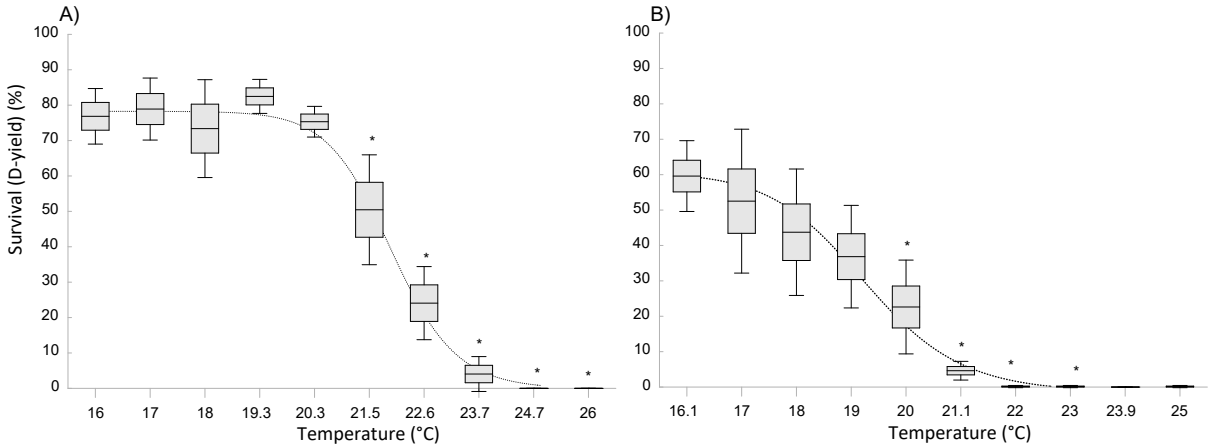


Figure 16. Mean survival (D-yield) (%) of larvae after a 48-hour exposure to a range of temperatures in (A) late summer (February 2023) and (B) early winter (June 2023) with fitted model (dashed line). The box plot represents the survival average (central horizontal line), the standard error (box) and standard deviation (whiskers). Asterisks represent a statistical difference (P < 0.05) from the lowest temperature tested.

Table 4. Embryo-larval development assay ecotoxicological parameters from the exposure to a range of temperatures (in °C)

Time of year	LT_{10} (95%CI)	LT_{25} (95%CI)	LT_{50} (95%CI)	N(S)ET	NOET	LOET
February 2023	20.3 (20.1–20.4)	21.2 (21.1–21.3)	22.1 (22–22.2)	21 (20.9–21.1)	20.3	21.5
June 2023	16.4 (15.9–16.9)	17.8 (17.5–18.2)	19.2 (18.4–19.4)	18.6 (17.5–19.5)	19	20

LTx: Lethal temperature causing x% mortality of the test population; N(S)ET: No significant effective temperature; NOET: No observed effective temperature; LOET: Lowest observed effective temperature; CI: Confidence intervals.

3.3 Combined effects of single metal toxicity and temperature on embryos

3.3.1 Chemical analysis

The average of the temperatures, with standard deviation, for the exposure to a range of metal concentrations were 16.1 ± 0.8 °C, 18.9 ± 0.3 °C and 21.2 ± 0.7 °C. The measured concentrations of cobalt (Co^{2+}), lead (Pb^{2+}) and zinc (Zn^{2+}) were within 20% of the nominal concentrations, results can be expressed as nominal concentrations. Nominal concentrations vs measured concentrations (total recoverable metal) are reported in Figure 17.

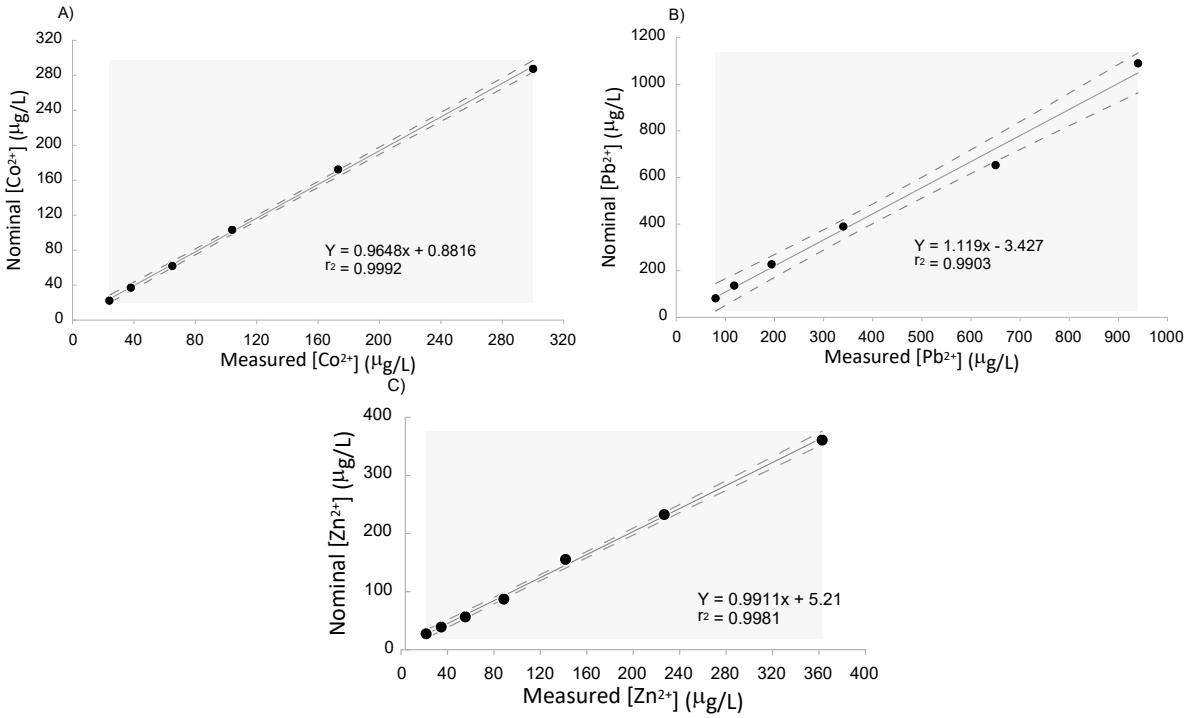


Figure 17. Relationship between the nominal and measured concentrations of (A) cobalt (Co^{2+}), (B) lead (Pb^{2+}) and (C) zinc (Zn^{2+}) in the test solutions, with fitted linear regressions (solid lines) and confidence intervals (dashed lines).

3.3.2 Toxicity testing

During testing, only embryos exposed to zinc (Zn^{2+}) at 17 °C developed (Figure 18). The calculated $LC10_{48h}$ and $LC50_{48h}$ were 16 (14.8–17.1) µg/L and 32 (31.2–33.6) µg/L, respectively. Despite conducting several trials, we have not been able to replicate the zinc exposure at multiple temperatures and obtain viable larvae outside the control. Survival of mussel larvae exposed to the other two metals (Co^{2+} and Pb^{2+}) in combination with temperatures are reported in Figure 19. Embryos were more sensitive to cobalt (Co^{2+}) than lead (Pb^{2+}), with lower LCx for most conditions (Table 5). The values for the LCx depending on the temperature are reported in Table 5 and represented in Figure 20. For both metals, mortality is highest at the highest tested temperature (21 °C). For lead, the survival rate increases as the temperature decreases. The effects of temperature and concentration and their interactions were significant on the embryo-larval development ($P < 0.05$), and the type of interaction was synergistic ($P = 0.18$) (effect is more than the addition of the two factors taken separately). However, for cobalt, the survival rate is higher ($P < 0.05$) at the intermediate tested temperature (19 °C) than at the lowest tested temperature (Figure 20). For cobalt, the effects of only temperature and concentration were significant ($P < 0.05$). No interaction between the tested temperatures and Co^{2+} was detected (statistical results are presented in Appendix Table A.5).

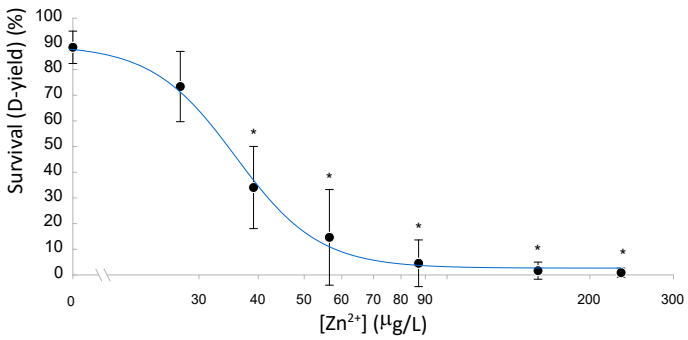


Figure 18. Mean survival (larval D-yield), with standard deviations, and fitted model (blue line) for embryos exposed to zinc (Zn^{2+}) at 17 °C. Asterisks represent a statistical difference from controls ($P < 0.05$)

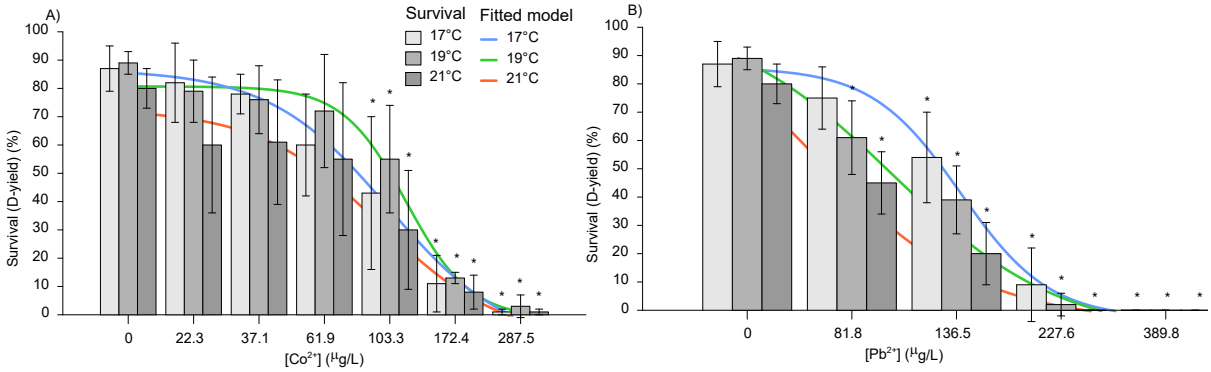


Figure 19. Mean survival (larval D-yield), with standard deviations and fitted model (coloured lines) for a range of concentrations of (A) cobalt (Co^{2+}) and (B) lead (Pb^{2+}), at three temperatures. Coloured lines represent the fitted dose-response models for each temperature. Asterisks represent a statistical difference from respective controls ($P < 0.05$).

Table 5. Embryo-larval development assay ecotoxicological parameters from a 48-hour exposure to cobalt and lead at three temperatures. Lethal concentrations (LCx) with associated 95% confidence intervals (CI) are expressed as measured total recoverable cobalt and lead (in µg/L).

	Cobalt (Co^{2+})			**Lead (Pb^{2+})**		
(µg/L)	**17 °C**	**19 °C**	**21 °C**	**17 °C**	**19 °C**	**21 °C**
LC_{10} (95%CI)	35 (32–39)	47 (42–52)	30 (24–35)	80 (73–86)	54 (50–59)	43 (38–49)
LC_{25} (95%CI)	61 (57–65)	78 (72–83)	55 (48–62)	118 (106–118)	83 (79–88)	68 (62–74)
LC_{50} (95% CI)	98 (94–102)	120 (115–125)	94 (87–101)	150 (146–155)	121 (117–126)	100 (96–105)

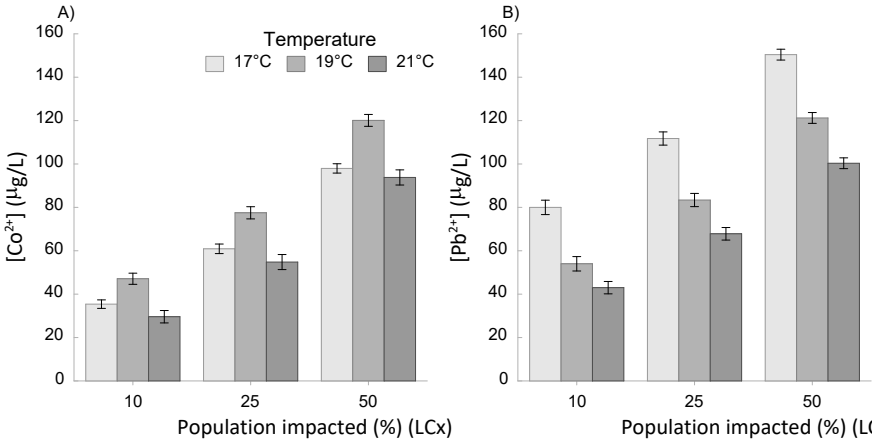


Figure 20. Lethal concentration impacting 10, 25 and 50% of the tested population values with associated standard error for the (A) cobalt (Co^{2+}) and (B) lead (Pb^{2+}) at the three temperatures tested.

3.4 Combined effects of field-collected sediment elutriates and temperatures on embryos

3.4.1 Range finding of sediment elutriates toxicity

A range finding of dilutions of the elutriate was carried out to determine the six concentrations that would be used for the combination with temperature. Larvae survival for the sediment elutriates of the four sites are

reported in Figure 21, and ecotoxicological parameters determined from the fitted model are presented in Table 6, with calculated aluminium concentration equivalent (Al eq).

At 17 °C, sediment elutriates from Garne Bay and Waiona Bay had the strongest impact on larvae survival (lowest LC50s). The sediment elutriate with the least impact was from Beatrix Bay. The impact on larvae survival from Skiddaw Bay sediment elutriate was intermediate.

For the test with the three temperatures, dilutions for the sediment elutriates showing high and intermediate survival were selected, corresponding to 0.1% to 3.13% for Garne Bay, 0.2% to 6.25% for Waiona Bay and 0.39% to 12.5% for Beatrix Bay and Skiddaw Bay.

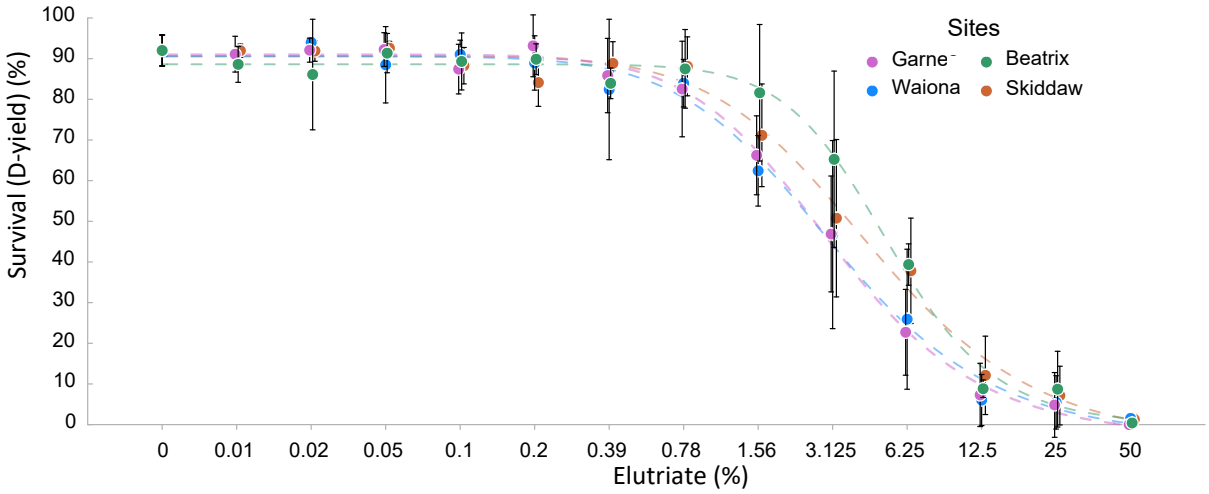


Figure 21. Mean survival (D-yield) (%) (dot), with associated standard deviations, of larvae after a 48-hour exposure to a range of dilutions of sediment elutriates from Garne Bay, Waiona Bay, Beatrix Bay and Skiddaw Bay, with related fitted model (dashed lines). Exposures were conducted at 17 °C.

Table 6. Embryo-larval development assay ecotoxicological parameters with associated 95% confidence intervals (CI) from a 48-hour exposure to dilutions of sediment elutriates from Garne Bay, Waiona Bay, Beatrix Bay and Skiddaw Bay at 17 °C.

Site	LC_{10} (95% CI)	LC_{25} (95% CI)	LC_{50} (95% CI)	LC_{50} (µg Al eq./L)	N(S)EC (95%CI)
Garne Bay	0.64 (0.58–0.71)	1.36 (1.26–1.46)	2.88 (2.73–3.04)	19.9	2.49 (2.04–2.82)
Waiona Bay	0.56 (0.49–0.62)	1.28 (1.17–1.39)	2.94 (2.77–3.12)	25	1.37 (0.72–1.31)
Beatrix Bay	1.45 (1.32–1.58)	2.69 (2.52–2.86)	4.99 (4.77–5.21)	32.5	2.49 (2.03–2.82)
Skiddaw Bay	0.84 (0.75–0.92)	1.82 (1.70–1.95)	3.98 (3.79–4.17)	46.6	1.25 (1.02–1.48)

3.4.2 Combined effects of sediment elutriate toxicity and temperatures on embryos

Chemical analysis

To assess the accuracy of dilutions, concentrations of aluminium in the test water was used as a proxy (other metals such as iron or manganese were not suitable, as they showed either elevated concentrations in blanks during the chemical analysis, or had similar concentration levels across the dilutions, indicating external contamination). Measured concentrations against expected concentrations of aluminium, determined from the concentration of the 100% sediment elutriate (Table 3), are presented in Figure 22. Skiddaw Bay elutriate dilutions were not analysed after mishandling, but considering the three other sites, it is likely they would behave similarly.

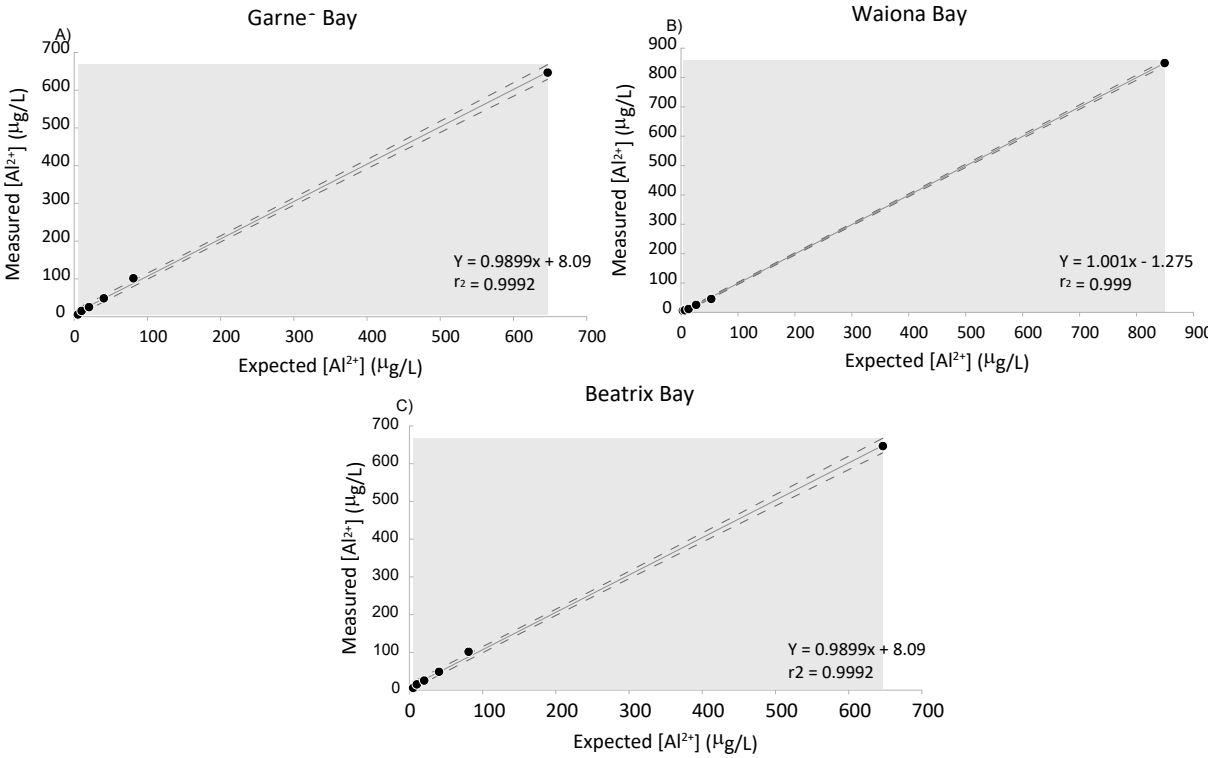


Figure 22. Expected and measured concentrations of aluminium (Al^{2+}) in sediment elutriates from (A) Garne Bay, (B) Waiona Bay and (C) Beatrix Bay.

Toxicity testing

The toxicity of sediment elutriates at 17 °C and 19 °C was similar for all sites (Figure 23). Temperature and concentration of elutriate had a separate significant effect ($P < 0.05$) on the embryo-larval development of the GSM, but no interaction between the two factors could be observed ($P > 0.05$). The same result was observed for the sediment elutriates from the four study sites (statistical analysis are provided in Table A.6). However, the lowest observed effective concentrations (LOEC) at 21 °C were lower than those for the two other temperatures, with toxicity thresholds for Garne Bay and Waiona Bay at 0.78% and 1.56%, respectively, and 3.13% dilution for Beatrix Bay and Skiddaw Bay (Figure 23).

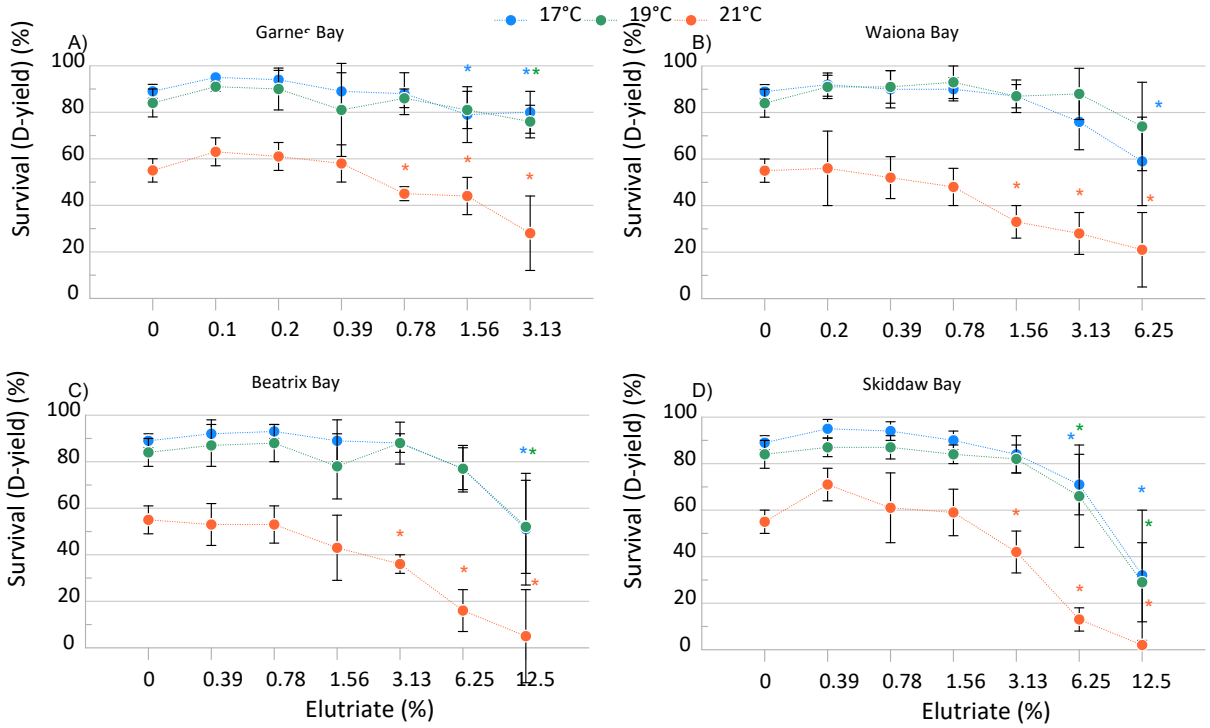


Figure 23. Average survival (with standard deviation) of embryos exposed to a range of dilution of sediment elutriates from (A) Garne Bay, (B) Waiona Bay and (C) Beatrix Bay at three different temperatures. Asterisks represent a statistical difference from the related control (P < 0.05).

3.5 Combined effects of elutriates from field-collected sediment and temperatures on spat

3.5.1 Water quality parameters

During the 9 days exposure, temperatures in the 17 °C, 19 °C, and 21 °C treatments were maintained at 16.8 °C (± 0.7), 19.3 °C (± 0.6) and 21.2 °C (± 0.8), respectively (Appendices Figure A.1). Spat were fed adequately, with fluorometer readings decreasing significantly for all treatments (down to 5 µg chl-*a*/L). Dissolved oxygen remained at acceptable levels above 40% (Li et al. 2022), whereas pH ranged from 7.55 to 8.15 across all treatments. In addition, NH_3/NH_4^+ levels remained ≤ 1 mg/L during acclimation and in the control vessels.

3.5.2 Spat survival

Overall, we found a significant influence of temperature on the survival of mussel spat regardless of the presence or absence of sediment elutriates (17 > 19 > 21; Log-Rank: $X^2 = 268$; $p < 0.0001$). For instance, spat maintained in clean seawater (no sediment) for 9 days at 17 °C, 19 °C, and 21 °C survived at 95.8% (94.2–97.4), 92.5% (89.7–95.4) and 84.8% (81.3–88.4), respectively (Figure 24).

In addition, sediment type (i.e. Skiddaw Bay or Beatrix Bay) had a significant effect on survival of exposed spat (Skiddaw Bay > Beatrix Bay; Log-rank: $X^2 = 88.9$; $p < 0.0001$) compared to spat exposed to control FSW. Toxicity of sediment was also significantly different between Skiddaw Bay and Beatrix Bay (Log-rank: $X^2 = 88.9$; $p = 0.0000014$). Finally, all three factors (i.e. temperature, sediment, concentrations) interacted significantly on spat survival (Log-rank: $X^2 = 1{,}631$; $p < 0.0001$).

For most sediments and temperatures tested, spat survival was dose-dependent, meaning that mortality of spat increased with increasing concentrations of sediment elutriates ($X^2 = 778$; $p < 0.001$). However, in the case of the Beatrix Bay sediment at 19 °C, we found a more severe effect, with only 14% (10.1–19.5) of spat exposed to 50% elutriate surviving compared to 52.4% (45.7–59.9) surviving at 21 °C. Spat exposed to elutriates made from Skiddaw Bay sediment tend to experience increasing mortality at increasing temperatures (Figure 24).

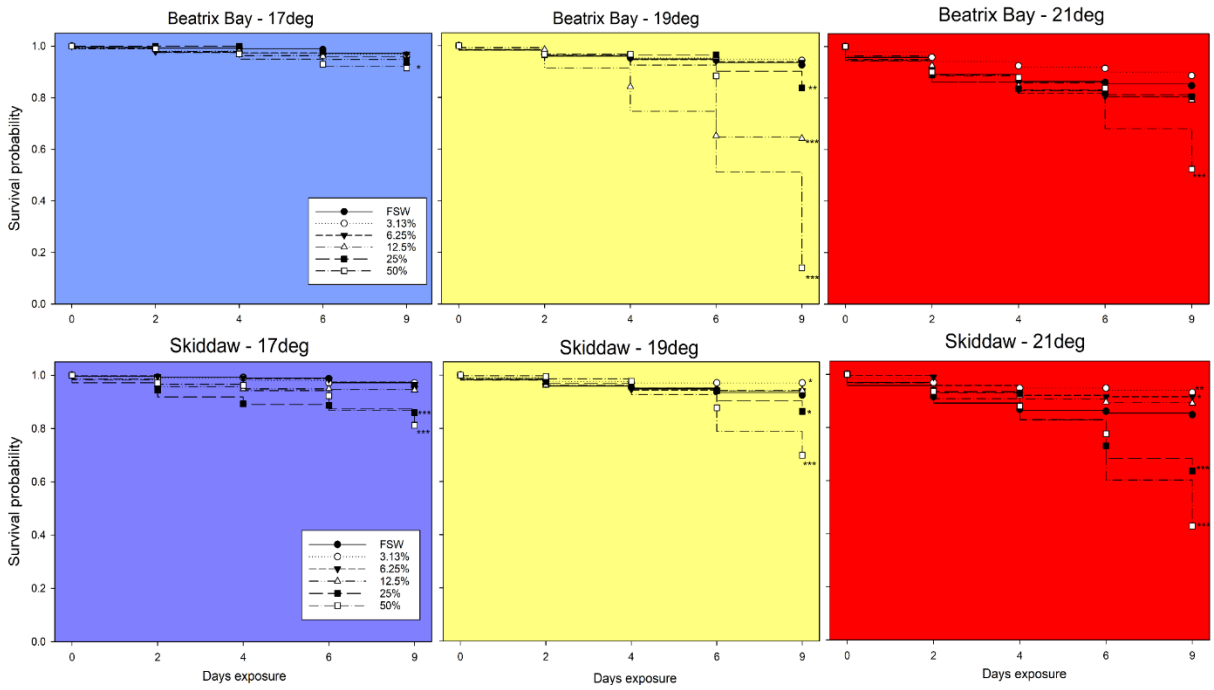


*Figure 24. Kaplan-Meier survival curves for Greenshell™ mussel spat exposed to increasing concentrations of Beatrix Bay (top) and Skiddaw Bay (bottom) sediment elutriates at 17 °C (blue), 19 °C (yellow), and 21 °C (red). Concentrations tested were 0% (filtered seawater [FSW]), 3.13%, 6.25%, 12.5%, 25% and 50% of an 100% elutriate stock (250 g of raw sediment per L of seawater). Asterisks denote a statistical differences: *= $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$, using Cox regression model to compare survival time curves with the FSW control.*

3.6 Toxicity of organic contaminants from field-collected extracts on sperm

3.6.1 Anion-SR passive samplers

Four of the extracts of organic compounds recovered from Anion-SR PSDs, which were deployed at the four different sites on four separate occasions, caused significant mortality of GSM sperm. Extracts collected from Skiddaw Bay and Garne Bay during deployment 1 induced high sperm mortality (> 90%; Figure 23) after a 1-hour exposure. From deployment 2, extract collected in Garne Bay induced around 60% sperm mortality, as did the sample collected from Skiddaw Bay during deployment 3.

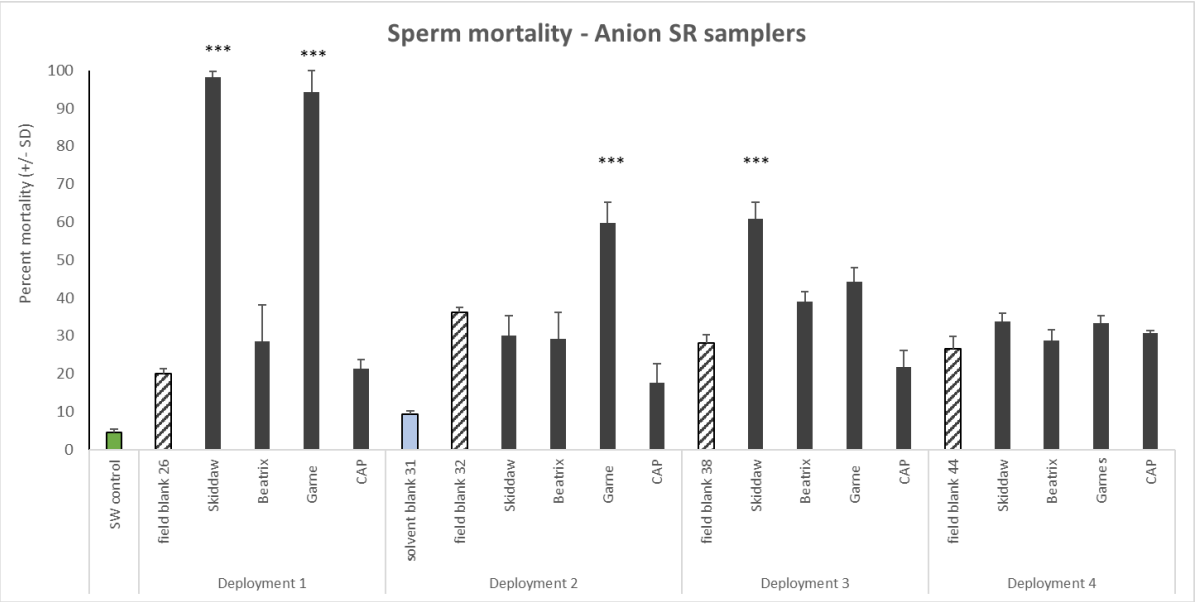


Figure 25. Mean mussel sperm mortality (± SD, n = 3), expressed in percent, following a 60-minute exposure to a suite of field-collected extracts. Passive sampler devices fitted with Anion-SR discs were deployed at four separate locations for 3 weeks. Control treatment consisting of seawater only (SW, green) and 0.5% dimethyl sulfoxide (DMSO) (solvent blank, blue), and respective field blanks (hashed) for each deployment were also tested. Asterisks denote a significant difference at p < 0.001. CAP = Cawthron Aquaculture Park.

Although not significant, a trend of reduced fertilisation success was also observed in these same samples (Figure 24)

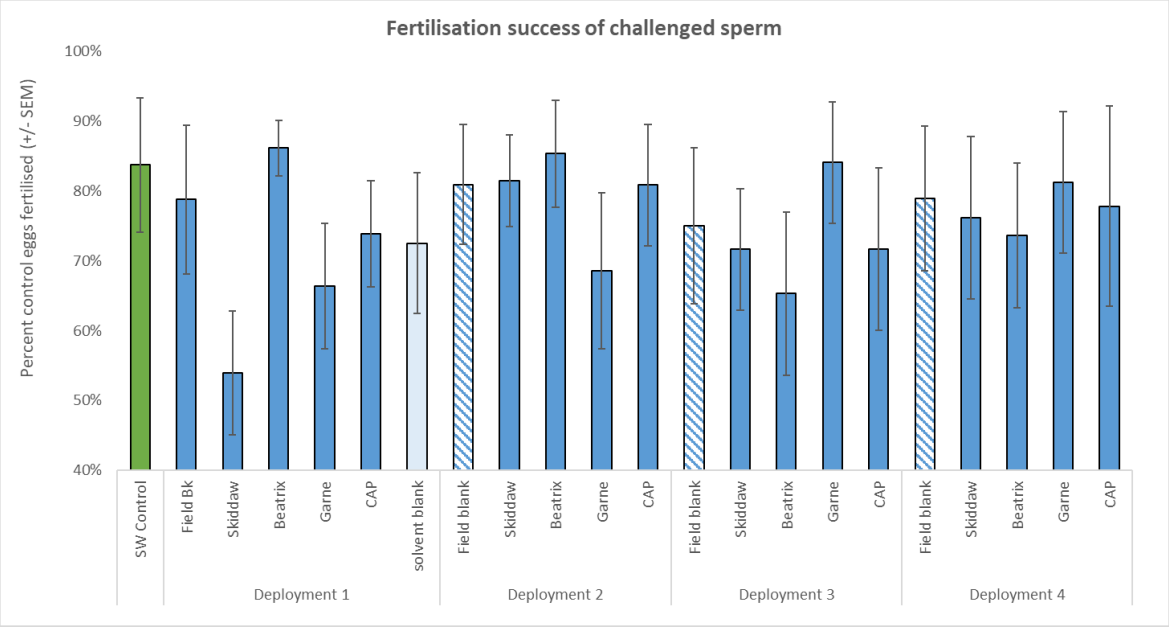


Figure 26. Mean fertilisation success (± SEM, n = 3), expressed in percent, of sperm previously exposed for 60 minutes to a suite of field-collected extracts fertilised with unexposed eggs. Control treatment consisting of seawater only (SW, green) and 0.5% dimethyl sulfoxide (DMSO) (solvent blank or vehicle control [VC], blue), and respective field blanks (hashed) for each deployment were also tested.

3.6.2 SDB-RPS passive samplers

Similarly, four extracts from the first three deployments of the SDB-RPS PSDs induced significant mortality. Extracts collected from Skiddaw Bay and Garne Bay during deployment 1 (Appendices Table A.4) caused around 50% and 30% mortality, respectively, in GSM sperm following a 1-hour exposure. In deployment 2, extract from Site 1 – Skiddaw Bay – resulted in 30% mortality of sperm, whereas extract collected from Beatrix Bay during deployment 3 caused the highest morality of sperm, at 60%.

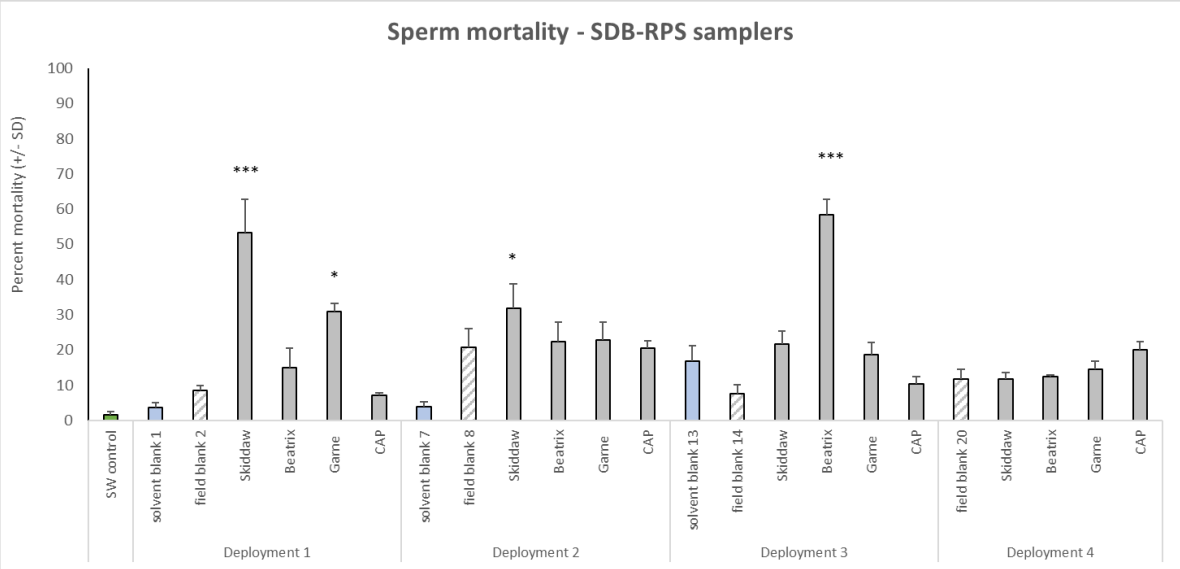


*Figure 25. Mean mussel sperm mortality (± SD, n = 3), expressed in percent, following a 60-minute exposure to a suite of field-collected extracts. Passive sampler devices fitted with SDB-RPS discs were deployed at four separate locations for 3 weeks. Control treatment consisting of seawater only (SW, green) and 0.5% dimethyl sulfoxide (DMSO) (solvent blank, blue), and respective field blanks (hashed) for each deployment were also tested. Asterisks denote a significant difference: * = p ≤ 0.05; *** = p < 0.001.*

4 Discussion

4.1 Chemical characterisation of the environment

In the Marlborough Sounds, there has been a decline from great abundance of marine life to relative scarcity, resulting in reduced allowable catches of fish and shellfish (Urlich and Handley 2020). The top anthropogenic stressors in the Sounds have been extirpations of marine megafauna, overharvesting of exploited species, and disruption to ecological functioning through ongoing clear-felling of terrestrial and marine biogenic communities since European settlement in the 1800s. This is in addition to the effects of ocean acidification and warming sea surface temperatures, and sedimentation and dredging, which have been identified in the national assessment of the direct threats to NZ's aquatic ecosystems and biodiversity (Urlich and Handley 2020). It has been established that land-derived stressors and natural environmental variables across multiple spatio-temporal scales can shape patterns of compositional turnover in estuarine macroinvertebrate communities across NZ (Clark et al. 2021). Our integrated study targeting multiple compartments aimed to investigate the contribution of time-integrated levels of a suite of stressors (i.e. from the water column, mussel tissue and sediment) to the decline of settling mussel larvae.

The study area (Pelorus Sound / Te Hoiere) was primarily selected due to the ongoing decline in wild spat recruitment, an assessment based on historical data and reports from the industry. In particular, Beatrix Bay and Garne Bay have experienced a severe lack of spat settlement in the last decade (Figure 2), whereas Skiddaw Bay (Site 1) is one of the last remaining catching sites in the Marlborough Sounds (Ned Wells, pers. comm.). The main contaminants of interest were trace metals that were quantified in surface waters using DGT. We also measured metals accumulated in adult mussels (used as bio-indicators) that had resided at each site for many months prior to sampling and in sediments below each study site.

4.1.1 Trace metals concentrations in mussel tissue

Tissue uptake of metals by mussels is governed by the bioavailability of these chemicals and the capacity of mussels to filter large volumes of seawater and thus accumulate these compounds (Rainbow 2002) . The three main elements detected in mussel tissue – arsenic, zinc and iron – varied significantly between sites but also over time. For arsenic, tissue levels were high (ranging from 15 µg/g to 35 µg/g), with some spatial differences and the highest concentrations detected in mussels collected in Beatrix Bay. The essential metal zinc was also observed at high levels (30–80 µg/g) in mussels, with some temporal and spatial variations. These levels are in agreement with mean concentrations of arsenic and zinc measured in *Perna canaliculus* during a contaminant monitoring programme (1999–2005) in the Auckland area (Kelly 2007).

Arsenic and zinc are naturally present in marine environments, and generally originate from mining activities and soil abrasion. The toxic properties of arsenic are dependent on the chemical form (speciation) in which it is found (e.g. toxic inorganic arsenicals vs nontoxic arsenobetaine) (Whaley-Martin et al. 2012). Tissues of marine invertebrates and fish contain high concentrations of arsenic, usually in the range of about 1 µg/g to 100 µg/g dry weight, and most in the form of organoarsenic compounds, particularly arsenobetaine (Neff 1997). Arsenobetaine is not toxic or carcinogenic to mammals, including humans; therefore, marine arsenic represents a low risk to human consumers of fishery products (Neff 1997). It has been demonstrated that the green mussel *Perna viridis* can maintain zinc at a constant tissue concentration of around 100 µg/g of dry tissue (Chan 1988). As such, we can expect *Perna canaliculus* to possess similar regulation mechanisms.

4.1.2 Trace metals concentrations in surface waters

Zinc and lead were the two elements detected in surface waters during two successive catching / recruitment seasons of GSM. These essential (zinc) and non-essential (lead) metals are naturally occurring trace elements, found throughout the environment. In addition to anthropogenic sources, such as mining and industrial activities, urban run-offs, or agro-fertilisers, these metals can be derived from natural sources and processes such as erosion of rocks and soils. The land surrounding the mussel growing area selected in our study is very sparsely populated with land cover, including mono-specific exotic forestry, native vegetation and pastoral agricultural land; therefore, we can assume that the origin of these metals is a natural geological source and the result of rainfall-derived run-offs from land.

Concentrations of zinc and lead were variable over the course of the study, but some clear spikes with elevated levels were found, notably during deployment 2 (February 2022). Meteorological data show that for this specific deployment in February 2022, a cumulative 392 mm of precipitation was recorded in the Pelorus Sound / Te Hoiere, with a total of 115 mm of rain in a single day (Table 7). By comparison, the total rainfall during the 4-day exceptional flood of August 2022 was 468 mm. With that in mind, we can expect that considerable sediment loading from run-offs would have occurred during deployment 2, depositing terrestrial metal-rich sediments in mussel growing areas and our study sites.

Our water quality measurements were conducted at a depth ranging between 4 m and 8 m. This depth was based on historical spat catching data (https://cawthron.shinyapps.io/BMOP/). Mussels are broadcast spawners, meaning they release their eggs and sperm in the water column where fertilisation occurs, followed by a 3- to 5-week long pelagic phase when mussel larvae swim in the water column and are exposed to any potential chemical and climate-related stressors. Field-collected measurements of zinc and lead via DGT are time integrated and reflect the bioavailable fraction of these metals, the most toxic form for aquatic organisms. Using toxicity thresholds determined in our lab-based toxicity assays, it was possible to benchmark / link concentrations causing adverse effects to early life stages of mussels with environmentally relevant levels of metals.

Table 7. Weather conditions at the time of each deployment. Mean temperature (±SD), in °C, measured during the 21-day deployment at a depth of ~6 m. Max: maximum temperature recorded during the 21-day deployment. Cumulative precipitation, in mm, over each of the 21-day deployments recorded at the Crail Bay weather station in Pelorus Sound / Te Hoiere (latitude -41.1031; longitude 173.9641) accessed via the NIWA's CliFlo database.

Deployment	Time	Site	Temperature (°C)	Max	Cumulative precipitation (mm)
Deployment 1	Dec. 2021	Skiddaw Bay	17.4 (0.4)	18.5	
		Beatrix Bay	16.9 (0.5)	18.1	237
		Garne Bay	16.3 (0.5)	17.2	
Deployment 2	Feb. 2022	Skiddaw Bay	19.4 (0.4)	20.3	
		Beatrix Bay	18.6 (0.4)	19.9	392
		Garne Bay	18.5 (0.5)	19.6	
Deployment 3	Mar. 2022	Skiddaw Bay	18.6 (0.6)	19.8	
		Beatrix Bay	18.6 (0.3)	19.2	44
		Garne Bay	18.3 (0.4)	19.7	
Deployment 4	May 2022	Skiddaw Bay	17.1 (0.4)	17.9	
		Beatrix Bay	17.1 (0.3)	17.7	48
		Garne Bay	17.1 (0.3)	17.5	
Deployment 5	Feb. 2023	Skiddaw Bay	19.7 (0.4)	21.1	
		Beatrix Bay	19.5 (0.5)	20.9	81
		Garne Bay	19.3 (0.3)	20.1	
Deployment 6	Mar. 2023	Skiddaw Bay	19.2 (0.4)	19.9	
		Beatrix Bay	19.0 (0.3)	19.7	183
		Garne Bay	18.7 (0.2)	19.3	
Deployment 7	Apr.- 2023	Skiddaw Bay	17.2 (0.2)	17.6	
		Beatrix Bay	17.3 (0.2)	17.7	175
		Garne Bay	17.4 (0.2)	17.7	
Deployment 8	May 2023	Skiddaw Bay	16.8 (0.3)	17.1	
		Beatrix Bay	16.9 (0.4)	17.4	243
		Garne Bay	16.9 (0.6)	17.5	

4.2 Impacts of environmentally relevant metals and temperature stress on early life stages of Greenshell™ mussels

This work using embryo-laval development assays of the GSM is the first of its kind in NZ. Previous studies have only used natural seawater (McDougall et al. 2022), but the drawback of using natural seawater is the change of quality during the year, mostly influenced by weather conditions. To mitigate this fluctuating water quality, hatchery rearing operations for GSM often treat natural seawater with EDTA to remove potential risks of poor water quality and to obtain good mussel yields all year round (McDougall et al. 2020b). However, using EDTA in ecotoxicological testing of metals underestimates the toxicity, mainly due to the chelating properties of EDTA, which can reduce metal bioavailability (Knepper 2003). The use of reconstituted seawater can circumvent this issue, allowing a controlled and standardised chemical environment, with an EDTA concentration (48 nM) below the guideline level allowance for metal testing (150 nM) (OECD 2011). The survival of larvae (D-yield) after 2 days in controls with normal conditions and reconstituted seawater was > 80%, which is higher than previous attempts without EDTA in seawater (Gale et al. 2016; McDougall et al. 2020a; McDougall et al. 2022).

4.2.1 Impact of temperature on embryo-larval development

Temperature influences growth (Hoegh-Guldberg and Pearse 2015) and is a predominant factor in the development of marine invertebrates (Nguyen et al. 2012), as larvae and juveniles are more sensitive to thermal stress than adults. The larval development of the NZ sea urchin / kina (*Evechinus chloroticus*) showed a similar response to GSM following a 24-hour exposure to a range of temperatures (15.7 °C to 24.2 °C) (Delorme and Sewell 2013). The lowest observed effect temperature (LOET) for the sea urchin was 20.5 °C compared to a LOET for the GSM in the current study of 21.5 °C and 20 °C in summer and winter, respectively. The LOET value obtained is dependent on the experimental design and the selected temperatures. The no significant effect temperature (N(S)ET) is derived from a non-linear regression fitted to observations and will be a closer fit to the description of the 'pejus temperature' (Pörtner and Farrell 2008), as well as provide associated uncertainties. In these experiments, the N(S)ET (with associated 95% CI) were 21 °C (20.9–21.1 °C) and 18.6 °C (17.5–19.5 °C) in summer and winter, respectively; these temperatures were lower than the LOETs. The non-overlap of the confidence interval also indicates a significant difference between the values (Cumming 2009).

The ecotoxicological values derived for the embryo-larval development assays with GSM show a differential sensitivity depending on the season. The importance of seasonal status when assessing ecotoxicity of contaminants in conjunction with environmental stressors has been highlighted by Nardi et al. (2018). The parents (or broodstocks) used for the present assays were acclimatised to environmental conditions at the time of the experiment, influencing the characteristics of the gametes. Thermotolerance responses of early life stages of the neogastropod *Ocenebra erinaceus* is influenced by the geographic origin, with a wider (12–18°C) or narrower (14–16°C) window of tolerance depending on whether the population originated from a warmer (Southwest of France) or colder (South of England) environment (Mardones et al. 2021). When parents of the sea urchin embryos (*Strongylocentrotus intermedius*) were exposed to elevated temperatures, embryos showed significantly different gene expression patterns at ambient vs high developmental temperatures, indicating complex molecular mechanism of intergenerational effects of impact of temperature changes (Shi et al. 2020). Likewise, recent work exposing GSM parents to 22 °C for 1 week identified transgenerational mechanisms where offspring performed better (i.e. better growth and survival) in a warmer environment when their fathers were also challenged to heat stress (Kozal et al. 2023 [forthcoming]). If rising seawater temperature is recognised to have an impact on future population distribution, the speed of occurrence will be most decisive. Work carried on the influence of marine heatwaves on GSM (Venter et al. 2023), which considers population-specific performance, provides a background for further multi-generational and phenotypic plasticity studies.

4.2.2 Impact of temperature in combination with single metals on embryo-larval development

The *Perna canaliculus* used in this study were less sensitive than other bivalve species, with $LC50_{48h}$ values for GSM embryos exposed to lead at our three temperatures higher (100–150 µg/L) than the $LC50_{48h}$ values of the mussel *Xenostrobus securis* and the scallop *Scaeochlamys livida* (species that are mostly found in Northland, NZ and Australia) (Markich 2021). However, the lead sensitivity of GSM is similar to that of the Mediterranean blue mussel *M. galloprovincialis* (Nadella et al. 2013).

The temperatures selected for our multi-stressor toxicity assays (i.e. 17 °C, 19 °C and 21 °C) are representative of sea surface temperatures experienced in a typical catching season in the Marlborough Sounds. The highest temperature tested of 21 °C is occasionally reached during the height of the summer (see maximum temperature of deployment 5; Table 7). This temperature, on its own, is not a cause for concern, as we found that 21 °C (20.9–21.2) had no significant effect on embryo-larval development during the summer season (Table 4). However, in combination with a single chemical stressor, such as lead, normal embryo-larval development was markedly impaired with lethal concentrations (LC_{50}) decreasing from 150 µg Pb^{2+}/L at 17 °C down to 100 µg Pb^{2+}/L at 21 °C. In other words, rising temperature increased the toxicity of lead to mussel embryos. A similar trend was observed when *M. galloprovincialis* embryos were exposed to single metals (Ag and Cu) in combination with increasing temperatures (18 °C to 24 °C) (Boukadida et al. 2016). The authors attributed the increased sensitivity to metals to global warming but did not consider the seasonal variations of sensitivity.

Natural heavy metal concentrations in seawater collected in locations historically associated with high commercial catches of settling GSM larvae were suggested as a cause of low survival of larval mussel (McDougall et al. 2022). In the present study, we have used a passive sampler approach (DGT) to characterise *in situ* trace levels of different heavy metals at key locations with poor spat settlement. As mentioned

previously, lead was one of the most prevalent metals detected at our study sites with elevated levels of up to 1,550 µg Pb^{2+}/L measured in February 2022, and 241 µg Pb^{2+}/L in Beatrix Bay during deployment 5 a year later. Mean temperatures recorded during these respective deployments were between 19 °C and 20 °C. After conversion from nominal to measured labile Pb^{2+}, toxic levels impairing embryo-larval development ($LC50_{48h}$) were estimated to be 316 µg Pb^{2+}/L (309–326) at 19 °C, and 277 µg Pb^{2+}/L (269–286) at 21 °C; these concentrations were well within the range of those detected in the field by DGT samplers.

We can therefore infer that mussel early life stages are adversely impacted by real-world levels of lead encountered at many locations in the Marlborough Sounds. As demonstrated by our lab-based assays, these toxic effects will be compounded by summer seawater temperatures., Significantly, additional abiotic stressor, such as the prolonged low salinity that occurred in February 2022, will exacerbate these negative effects on mussels, as evidenced with lead in embryos (Hrs-Brenko et al. 1977; Nadella et al. 2013) and adult *M. galloprovincialis* (Freitas et al. 2019b).

In addition to lead, zinc was also detected at significant levels at our study sites, and we have evidence that zinc is toxic to marine mussels' embryo-larval development (Nadella et al. 2013; Markich 2021). Our previous work showed that increasing temperature had an interaction with zinc toxicity on embryos of *M. galloprovincialis,* presenting a similar response pattern to that of GSM for cobalt . The LC_{50}s at 17 °C, 20 °C and 23 °C were 170 µg/L, 184 µg/L and 154 µg/L, respectively (Champeau, unpublished data). Unfortunately, we were not able to successfully conduct our GSM embryo-larval development assays with zinc in interaction with the two other temperatures, which impeded the field-relevant comparison with DGT-measured zinc concentrations. Nonetheless, earlier work using GSM embryos exposed to zinc at 17 °C (Figure 18) gave $LC10_{48h}$ and $LC50_{48h}$ of 16 µg /L and 32 µg /L, respectively, which converted in labile concentrations of Zn^{2+} corresponding to 39 µg /L and 83.5 µg /L. These toxic levels were somewhat overlapping with field levels of Zn^{2+} measured by DGT in February 2022 (60–80 µg/L), indicating a possible role of land-derived zinc in the lack of competent mussel larvae in the study area.

Furthermore, compared to other species, the $LC50_{48h}$ values for GSM embryos exposed to cobalt (at 17 °C or 21 °C) are similar to the $LC50_{48h}$ results reported for Australasian mussel and scallop species (Markich 2021). Interestingly, no temperature-dependent response was found with GSM embryos exposed to cobalt, with embryos better tolerating 19 °C than 17 °C or 21 °C (Figure 20A). Similarly, the ciliate *Euplote crassus* exposed to a range of copper at different temperatures showed a decrease of sensitivity to copper at intermediate tested temperature (Gomiero and Viarengo 2014). Shellfish are naturally rich in zinc and cobalt, metals that can be used in metabolism. Cobalt is the core atom of the cobalamin molecule (vitamin B12), of which shellfish are particularly rich (Vogeler et al. 2022). During stressful events, organisms may use certain essential metals to produce molecules entering in the defence mechanism to maintain hemostasia.

4.3 Impacts of field-collected complex mixtures and temperature on multiple life stages of Greenshell™ mussels

Environmental relevance is paramount for accurate ecotoxicological assessment studies. As such, we opted for a time-integrated monitoring approach using multiple compartments of which sediment was an integral part. Sediments are often considered a sink for many persistent contaminants, including heavy metals and organic chemicals such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides and herbicides (Lee et al. 1978; Rubio et al. 2000). We used a proven methodology, sediment elutriation, to evaluate the toxicity of field-collected sediments on mussel embryos and spat, in combination with a range of temperatures. For the elutriation, raw sediment and seawater were mixed in a standardised manner (ratio of 250 g per litre) and the supernatant was collected as the exposure solution to mimic the natural resuspension process occurring during storms and extreme weather (Geffard et al. 2002). A cocktail of organic contaminants, including herbicides, were extracted from the rest of the passive samplers deployed in the field, and then tested in the lab on mussel sperm to evaluate cellular and reproductive toxicity.

4.3.1 Impact of temperature in combination with sediment elutriates on embryo-larval development

All elutriates tested had some toxic effects on embryo-larval development of GSM, with some dose-dependent survival responses. Based on LC_{50} values, sediment toxicity ranked as follows, from most toxic to least toxic: Garne Bay = Waiona Bay < Skiddaw Bay < Beatrix Bay. There was no significant interaction of the temperatures

on the survival of GSM embryos exposed to sediment elutriates, with overlapping survival dose responses at 17 °C and 19 °C. However, regardless of the presence of sediment, embryos subjected to 21 °C were significantly impacted, with survival in control seawater estimated at 55% ± 5. This trial was carried out at the end of May 2023 with gametes obtained from broodstock held in colder conditions, which was reflected in the reduced thermal tolerance of the offspring (N(S)ET and LOET of 18.6 °C and 20 °C, respectively, in June 2023). This finding again highlights the importance of seasonality when conducting ecotoxicity tests using temperature as a co-stressor, and the need to replicate experiments to account for seasonal status of model organisms.

The timing of the collection, in January 2023, is significant, as it captured the end of an exceptionally wet year for Te Tauihu o Te-Waka-a-Māui (the top of the South Island), with two major heavy rainfall events occurring in February and August 2022. As such, we can expect the sediment to have collected substantial contaminants associated with run-off from the catchments. Sediment analyses indicated that aluminium and iron were the most prevalent metals, particularly in the sample collected from Skiddaw Bay. Of note, tissue analyses also showed elevated levels of aluminium, particularly in mussels collected from Skiddaw Bay, and iron, particularly in mussels from Skiddaw Bay and Beatrix Bay. This apparent relationship between mussel tissue and sediment implies a potential transfer of metals present in the nearby sediments being resuspended in the water column and then taken up by filter-feeding mussels. In our study, highest measured concentrations of total aluminium for Garne Bay (6.25% of raw elutriate), Waiona Bay (6.25% raw elutriate), Beatrix Bay (12.5% of raw elutriate) and Skiddaw Bay (12.5% of raw elutriate) were 0.023 mg/L, 0.053 mg/L, 0.081 mg/L and 0.146 mg/L, respectively. Furthermore, our $LC50_{48h}$ estimates for each sediment elutriate tested ranged from 19.9 µg/L to 46.6 µg Al^{3+}/L and are below toxicity values (EC/LC50s) of total aluminium reported for many marine animals (Table 8).

In seawater, aluminium is present as mineral, as $Al(OH)_3$ predominating at alkaline pH, and can provoke respiratory disturbances and smothering (Gensemer and Playle 1999). In marine organisms, aluminium concentrations in tissues and organs are heterogeneous, particularly for molluscs. Aluminium accumulates on gills, the digestive gland and the gonad complex (Botté et al. 2022). Mantle and gill tissues were specifically dissected from our sentinel mussels for metal burden analysis (Figure 15D), confirming previous reports by Botté et al. (2022). When analysing the dissolved fraction, aluminium was below the limit of detection. The concentrations of dissolved aluminium in coastal seawater around the globe ranged from 0.6 µg/L to 131.96 µg/L, while the concentrations in the open ocean are between 0.09 µg/L and 2.03 µg/L (Botté et al. 2022), suggesting that aluminium is brought to the marine environment from land (Hydes and Liss 1977).

Iron was the second most prevalent metal measured in the undiluted sediment elutriates, with total concentrations of 0.67 mg/L, 0.78 mg/L, 0.56 mg/L and 0.72 mg/L for Garne Bay, Waiona Bay, Beatrix Bay and Skiddaw Bay, respectively. Similar to aluminium, in coastal and shelf waters, substantial external inputs of iron come from riverine sources and bottom sediments, leading to markedly higher dissolved and particulate iron concentrations (Wells et al. 1995); however, iron is an essential trace element for both plants and animals, required by most organisms for essential growth and development (Oborn 1960; Wells et al. 1995). Sediment-associated aluminium originating from land may be a contributing factor to the high toxicity of the elutriates tested. However, sediment elutriates are complex mixtures that include metals as well as other organic chemicals and minerals; therefore, these mixtures combined with abiotic stressors may have further interactions on marine biota, including the sensitive early life stages of the GSM, as demonstrated in our current study.

Table 8. Toxicity of total aluminium for marine species.

Species	Duration	pH	LC/EC_{50} (mg/L)	Reference
Polychaete worm *Capitella capitata*	96 h		0.405	Petrich and Reish (1979)
Polychaete worm *Ctenodrilus serratus*	96 h		0.097	Petrich and Reish (1979)
Polychaete worm, *Neanthes arenaceodentata*	96 h		> 0.405	Petrich and Reish (1979)
Copepod adult *Niktora spinipes*	96 h	8	10	Bengtsson (1978)
American oyster (embryos) *Crassostrea virginica*	42–48 h	7–8.5	> 1.518	Calabrese et al. (1973)
Pacific oyster *Crassostrea gigas*	48 h	7.85	0.195	Markich (2021)
Australian mussel *Xenostrobus secures*	48 h	7.85	0.228	Markich (2021)
Shrimp (embryos) *Palaemonetes pugio*	72 h	7.6–8.1	1.075	Rayburn and Aladdin (2003)

4.3.2 Impact of temperature in combination with sediment elutriates on mussel spat

As well as the decline in commercial catching of settling mussel larvae, the industry is facing another challenge that is hampering production: unexplained summer mortality of spat. Thus, we were interested in further evaluating the potential detrimental effects of flood-associated stressors (namely sediment), combined with rising temperatures typically encountered in the summer, on mussel spat survival. Given the scale of the trial (over 11,000 spat distributed across 126 beakers), we decided to only assess the toxicity of two field-collected sediments (Skiddaw Bay and Beatrix Bay) at our three main temperatures: 17 °C, 19 °C and 21 °C.

Aligning with our previous multi-stress trial on embryos, temperature alone had a strong effect on spat survival, with 21 °C leading to about 20% cumulative mortality of spat after 9 days. In the context of marine heatwaves, current research on GSM has identified an important 'tipping point' temperature for adults, where heavy mortalities are observed following several days at a sustained temperature of 26 °C (Ericson et al. 2023a). Previous research has shown that adult GSM held for 4 months at 21 °C experienced significant stress and their reproductive performance was impacted, despite most individuals surviving (Ericson et al. 2023b). Juvenile mussels (40 mm shell length) were found to be more vulnerable than adults when challenged for 21 days at a sustained 26 °C (~90% mortality), whereas small spat (4 mm) challenged acutely to heat stress (> 30 °C) for 3 hours had a thermal tolerance (LT_{50}) of approximately 33 °C (Ericson et al. 2023a). In the present study, we challenged 4 mm spat to our different temperatures for 9 days following a 5-day acclimation at the appropriate target temperature (Appendix Figure A.1). Our findings indicate that small spat appear more sensitive than adults to thermal stress. The spats used in our study were bred from a wild population from Golden Bay / Mohua in May 2023, and were maintained in our land-based facilities for 10 weeks at approximately 15 °C. The effect of season status, namely winter, on the thermotolerance of young spat is again highlighted in this trial, and we can consider whether spat reared at a different season and / or from a different source (e.g. Kaitaia origin) may have a higher tolerance to thermal stress.

The type of sediment tested also had a varied impact on the survival of spat, with Beatrix Bay's elutriate having overall the most deleterious effect on mussel spat after 9 days. Unfiltered elutriates from Skiddaw Bay and Beatrix Bay had a similar composition in aluminium and iron compared to those tested on embryos, although Skiddaw Bay's aluminium content was slightly higher. Both sediments had a dose-dependent effect on survival, with the highest concentration tested (50%) – corresponding to 0.5 mg/L (Beatrix Bay) and 0.75 mg/L (Skiddaw Bay) of total aluminium (Table 3 B) – causing the highest mortalities at all temperatures tested. As discussed previously, aluminium could be partly responsible for the observed toxic effects, especially if we compare these levels with toxic thresholds (EC_{50}) determined for other marine animals (Table 8). Increasing temperatures had a compounding effect on the toxicity of both sediments on spat after only 9 days, indicating that young spat subjected to environmentally relevant stressors (run-off-associated sediment combined with summer

temperature) are at a higher risk of death. Further work should study the potentially compounding effects of an additional abiotic stress, such as low salinity (< 25‰) or decreased pH (7.8), on the fitness of GSM spat.

4.3.3 Impact of environmentally relevant organic compounds on mussel sperm

Passive samplers are a powerful and promising tool for the measurements of organic micropollutants in surface waters, particularly in areas heavily influenced by land-derived stressors. The passive sampling approach is being accepted as a novel contaminant monitoring technique around the world, particularly in Europe, where many inter-laboratory initiatives are continuing to validate the approach (Poulier et al. 2014; Gonzalez et al. 2022). The samplers used in our study were specifically designed to target acidic herbicides (Anion-SR), and a broad range of polar organic chemicals including other types of pesticides, hydrocarbons or even terpene, a chemical associated with pines (SDB-RPS). These samplers were deployed for 21 days, alongside the other metal-specific samplers (DGT), during two reproductive seasons of GSM. The advantage of PSDs compared to DGTs is that they can be extracted in a suitable solvent, which can then be tested in the lab via bioassays. The disadvantage of PSDs is that, because they contain a complex mixture of many organic compounds, it is very challenging to analyse their composition unless a non-targeted analysis (NTA) approach using expensive analytical techniques is used. We opted for a two-tiered approach, which allowed us to test multiple samples and assess their toxicity on relevant life stages of mussels; this approach also used a duplicate sample that is archived for NTA analysis once toxicity is known.

Prior to testing the different extracts, we conducted several pilot trials to establish the safe levels of solvent (DMSO) that did not induce toxicity on sperm; 0.5% final solvent concentration was not toxic to sperm after 1 hour of exposure. We were then able to screen a whole suite of field-collected extracts and test their acute toxicity on mussel sperm using a high-throughput validated spermiotoxicity assay (Rolton et al. 2022). Out of over 40 extracts analysed, a few extracts caused high mortality in mussel sperm cells after only 1 hour. Subsequent fertilisation of mussel eggs with exposed sperm was generally lower than unexposed sperm, confirming some reproductive toxicity associated with certain extracts. The most toxic extracts specifically targeting acid herbicides (Anion-SR) were collected from Skiddaw Bay and Garne Bay in December 2021, February 2022 and March 2022. The extracts targeting a broader spectrum of organic contaminants (SDB-RPS) were also the most toxic in samples collected in December 2021, February 2022 and March 2022, particularly those collected from Skiddaw Bay and Beatrix Bay in March 2022. Some recent studies have evidenced that forestry herbicide mixtures (atrazine, hexazinone, indaziflam and bifenthrin) can pose an environmental risk to shellfish populations, with environmentally relevant concentrations impacting fitness and survival of soft-shell clams (Tissot et al. 2022). We are researching this toxicity screening approach by analysing key archived extracts using non-targeted analyses via high resolution mass spectrometry to identify the specific chemicals that are causing the spermiotoxicity on mussels.

5 Conclusion

Intensifications of human activities in the Marlborough Sounds combined with the recent increase in atmospheric carbon dioxide and its consequences (increase of the global temperature and sea level rises) may have pushed GSM to a tipping point. The speed of the environmental changes and the interactions with anthropogenic stressors may also be contributing to the decline, jeopardising the adaptation of local populations to the rapidly changing environment. One of the key knowledge gaps is the characterisation of the contributions of each stressor under a multiple-stressor situation. This study aimed to assess the risk of chemical contaminants on the sensitive early life stages of the GSM.

The new method using embryo-larval development assays with GSM facilitated the assessment of a wider range of chemicals under varying conditions; This approach was used because the impact on the embryo-larval development of GSM appears to be dependent on parental acclimatisation to their environment and the type of chemicals present. In this study, the cause of poor recruitments in the Marlborough Sounds could not be directly attributed to metals measured in sediment and higher temperatures. On the other hand, discharges of run-off-associated sediments, in conjunction with rising seawater temperature, impacted the fitness of the different GSM life stages tested, suggesting that climate-driven stressors may largely contribute to the negative effects on population recruitment in coastal waters.

Our results highlighted the importance of investigating the effects of complex mixtures, at environmentally relevant levels, combined with climate-change condition scenarios on native species. Studies focusing on the effects of individual compounds over short periods of time and under one temperature can miss important details including sub-lethal effects of chronic exposure or synergistic effects of multiple compounds / abiotic stressors. We believe that toxicity assessments of field-collected sediments or passive samplers and associated extracts are the most appropriate and robust approach to accurately predict the effects of climate change on

marine pollution. The toxicity thresholds established for ecologically relevant contaminants will assist regulators and industry as well as inform decision-making on environmental policies and land-use management.

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8 Glossary

Abbreviation	Terminology
°C	Degree Celsius
95%CI	95% confidence interval
Ag	Silver (ion)
Al	Aluminium (ion)
ASTM	American Society for Testing and Materials
Cd	Cadmium (ion)
Chl-*a*	Chlorophyll-*a*
Co	Cobalt (ion)
Cr	Chromium (ion)
Cu	Copper (ion)
DGT	Diffusive gradients in thin films
DGV	Default guideline value
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dw	Dry weight
EDTA	Ethylenediaminetetraacetic acid
FSW	Fresh seawater
g.	Gravitational acceleration unit
g/L	Gram per litre
GSM	Greenshell™ mussel
Hg	Mercury (ion)
HNO_3	Nitric acid
ICP-MS	Inductively coupled plasma-mass spectrometry
IFREMER	Institut Français de Recherche pour l'Exploitation de la Mer (French Research Institute for Exploitation of the Sea)
L	Litre
ECx/LCx	x% effect/lethal concentration – the concentration that is expected to cause a specified effect in x% (ECx) of a group of organisms, or to be lethal to x% of a group of organisms (LCx)
LOD (LD)	Limit of detection
LOEC	Lowest observed effect concentration – the lowest tested concentration of a test substance or material which is observed to have a statistically significant adverse effect on the test organisms for a defined time of exposure and under the test conditions, relative to the control (hypothesis testing based)
LTx	Lethal Temperature affecting x% of the population
m	Metre
MBIE	Ministry of Business, Innovation & Employment
mg/kg	Milligram per kilogram
mg/L	Milligram per litre
mJ/cm^2	Millijoule per centimetre square
mL	Millilitre

mm	Millimetre
Mn	Manganese (ion)
mpf	Minutes post-fertilisation
N(S)EC	No significant effect concentration – the concentration at which the modelled mean response is statistically indistinguishable from the mean control response
N(S)ET	No significant effect temperature – the temperature at which the modelled mean response is statistically indistinguishable from the mean control response
NH_3/NH_4^+	Ammonium ion
Ni	Nickel (ion)
NIWA	National Institute of Water and Atmospheric Research
NOEC	No observed effect concentration – the highest tested concentration of a test substance or material which is observed not to have a statistically significant adverse effect on the test organisms for a defined time of exposure and under the test conditions, relative to the control (hypothesis testing based)
NOET	No observed effective temperature
Pb	Lead
ppb	Part per billion
ppm	Part per million
PSD	Passive sampler device
PSU	Practical salinity unit
S	Sulfur
SDB-RPS	Styrenedivinylbenzene- reverse phase sulfonate (extraction discs are used for extraction of polar and nonpolarorganic compounds)
Se	Selenium
U	Uranium
mL	Microlitre (10^{-9} L)
mm	Micrometre (10^{-9} m)
V	Vanadium
v:v	Volume to volume

9 Appendices

Table A.1. Percentiles of mean (with standard deviation [SD]) measured concentrations of metals by diffusive gradients in thin films (DGT) ordered by their median (p50, in bold) as measure of centrality for non-normal data. Most prevalent elements in surface waters are highlighted in bold. Manganese was excluded from the analyses due to abnormally high levels in some samples.

Metal	n_missing	complete_rate	Mean	SD	p0	p25	p50	p75	p100
Zn	0.0	1.0	21.0	21.1	1.8	4.2	**11.9**	32.9	76.9
Pb	6.0	0.9	207.5	469.2	0.0	0.3	**6.8**	80.2	2108.4
Fe	0.0	1.0	5.7	6.2	0.2	1.5	3.6	8.5	29.9
Ni	0.0	1.0	2.5	1.3	0.1	1.9	2.6	3.3	6.5
Al	9.0	0.8	4.4	5.0	0.0	1.2	**2.4**	5.6	22.1
Cu	0.0	1.0	0.9	0.5	0.1	0.6	0.8	1.3	2.5
Co	12.0	0.8	1115.5	2352.2	0.0	0.2	0.3	0.6	7612.8
U	12.0	0.8	0.3	0.7	0.0	0.1	0.2	0.3	4.1
Cd	12.0	0.8	5.3	11.7	0.1	0.1	0.2	0.3	43.5
Cr	9.0	0.8	9.2	24.1	0.0	0.1	0.1	0.2	100.3
As	8.0	0.9	0.1	0.1	0.0	0.0	0.0	0.1	0.4

Table A.2. Percentiles of mean (with standard deviation [SD]) measured concentrations of metals in mussel tissue ordered by their median (p50) as measure of centrality for non-normal data. Most prevalent elements in mussel tissue are highlighted in bold.

Metal	n_missing	complete_rate	Mean	SD	p0	p25	p50	p75	p100
Zn	0	1	51.6	19.5	11.0	38.6	**49.1**	60.9	190.1
Fe	0	1	46.5	27.9	7.9	30.9	**42.6**	56.9	362.1
As	0	1	22.5	7.5	7.9	16.4	**22.1**	28.9	40.7
Mn	0	1	7.0	8.1	0.7	3.4	6.3	8.5	115.7
Al	0	1	9.9	22.7	0.5	2.5	**4.4**	8.7	316.5
Cu	0	1	3.7	1.6	0.3	2.6	3.4	4.5	10.5
Co	0	1	0.6	0.5	0.0	0.2	0.4	0.9	3.2
Ni	0	1	0.7	0.9	0.0	0.2	0.4	0.8	7.5
Cd	0	1	0.7	1.0	0.0	0.1	0.3	0.9	6.1
Cr	0	1	0.3	0.2	0.0	0.1	0.2	0.3	1.9
Pb	0	1	0.1	0.2	0.0	0.0	0.1	0.2	1.5
Hg	0	1	0.0	0.0	0.0	0.0	0.0	0.1	0.2
U	0	1	0.0	0.0	0.0	0.0	0.0	0.0	0.1

Table A.3. List of extracts tested on mussel sperm and corresponding Anion-SR passive sampler devices, deployment number and study site (Site 1: Skiddaw Bay; Site 2: Beatrix Bay; Site 3: Garne Bay; Site 4: Cawthron Aquaculture Park). NA corresponds to solvent blank (DMSO) or field blanks.

Extract ID	Sample type	Deployment no.	Site
26	Anion-SR Field blank	1	NA
27	Anion-SR PSD	1	1
28	Anion-SR PSD	1	2
29	Anion-SR PSD	1	3
30	Anion-SR PSD	1	4
31	Solvent blank	2	NA
32	Anion-SR Field blank	2	NA
33	Anion-SR PSD	2	1
34	Anion-SR PSD	2	2
35	Anion-SR PSD	2	3
36	Anion-SR PSD	2	4
38	Anion-SR Field blank	3	NA
39	Anion-SR PSD	3	1
40	Anion-SR PSD	3	2
41	Anion-SR PSD	3	3
42	Anion-SR PSD	3	4
44	Anion-SR Field blank	4	NA
45	Anion-SR PSD	4	1
46	Anion-SR PSD	4	2
47	Anion-SR PSD	4	3
48	Anion-SR PSD	4	4

Table A.4. List of extracts tested on mussel sperm and corresponding SDB-RPS passive sampler devices, deployment number and study site (Site 1: Skiddaw Bay; Site 2: Beatrix Bay; Site 3: Garne Bay; Site 4: Cawthron Aquaculture Park). NA corresponds to solvent blank (DMSO) or field blanks.

Extract ID	Sample type	Deployment no.	Site
1	Solvent blank	1	NA
2	SDB-RPS Field blank	1	NA
3	SDB-RPS PSD	1	1
4	SDB-RPS PSD	1	2
5	SDB-RPS PSD	1	3
6	SDB-RPS PSD	1	4
7	Solvent blank	2	NA
8	SDB-RPS Field blank	2	NA
9	SDB-RPS PSD	2	1
10	SDB-RPS PSD	2	2
11	SDB-RPS PSD	2	3
12	SDB-RPS PSD	2	4
13	Solvent blank	3	NA
14	SDB-RPS Field blank	3	NA
15	SDB-RPS PSD	3	1
16	SDB-RPS PSD	3	2
17	SDB-RPS PSD	3	3
18	SDB-RPS PSD	3	4
20	SDB-RPS Field blank	4	NA
21	SDB-RPS PSD	4	1
22	SDB-RPS PSD	4	2
23	SDB-RPS PSD	4	3
24	SDB-RPS PSD	4	4

Figure A.1. Temperature profiles of each treatment (17 °C, 19 °C, 21 °C) measured during the Greenshell™ mussel spat survival assay. Acclimation period lasted 5 days.

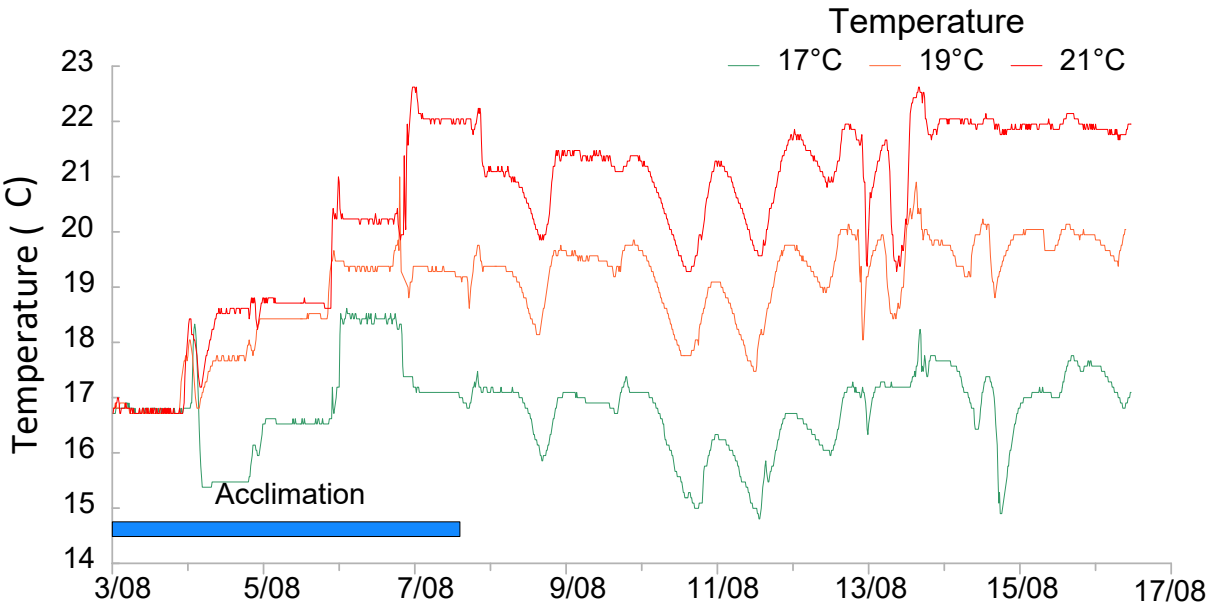

Table A.5. Statistical results for embryo-larval development assay with a combination of temperature and single metals cobalt and lead.

Effect	Univariate Tests of Significance for CoSurv_transf (Spreadsheet in Metals) Sigma-restricted parameterization Effective hypothesis decomposition SS	Degr. of Freedom	MS	F	p
Intercept	45.40838	1	45.40838	1456.653	0.000000
CoTemp	0.38359	2	0.19179	6.153	0.003711
CoConc_val	11.83180	6	1.97197	63.259	0.000000
CoTemp*CoConc_val	0.14134	12	0.01178	0.378	0.966500
Error	1.87039	60	0.03117		

Effect	Univariate Tests of Significance for PbSuv_transf (Spreadsheet in Metals) Sigma-restricted parameterization Effective hypothesis decomposition SS	Degr. of Freedom	MS	F	p
Intercept	13.73812	1	13.73812	1425.962	0.000000
Pb_conc_val	16.98861	6	2.83143	293.892	0.000000
Pb_Temp	0.30668	2	0.15334	15.916	0.000003
Pb_conc_val*Pb_Temp	0.34862	12	0.02905	3.015	0.002209
Error	0.59733	62	0.00963		

Table A.6. Statistical results for embryo-larval development assay with a combination of temperature and sediment elutriates.

Univariate Tests of Significance for Trans (Garnes in ELS TempXElutriates) Sigma-restricted parameterization Effective hypothesis decomposition					
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	81.55357	1	81.55357	6088.714	0.000000
Temperature	2.91005	2	1.45503	108.631	0.000000
Concentration	0.67963	5	0.13593	10.148	0.000001
Temperature*Concentration	0.08915	10	0.00892	0.666	0.750737
Error	0.72329	54	0.01339		

Univariate Tests of Significance for Var4 (Waiona in ELS TempXElutriates) Sigma-restricted parameterization Effective hypothesis decomposition					
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	74.89397	1	74.89397	4306.361	0.000000
Temperature	4.43898	2	2.21949	127.619	0.000000
Concentration	1.05085	5	0.21017	12.085	0.000000
Temperature*Concentration	0.14058	10	0.01406	0.808	0.621551
Error	0.93914	54	0.01739		

Univariate Tests of Significance for transformed (Beatrix in ELS TempXElutriates) Sigma-restricted parameterization Effective hypothesis decomposition					
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	66.33252	1	66.33252	3748.437	0.000000
Temperature	4.58899	2	2.29450	129.661	0.000000
Concentration	2.30420	5	0.46084	26.042	0.000000
Temperature*Concentration	0.15391	10	0.01539	0.870	0.566204
Error	0.95559	54	0.01770		

Univariate Tests of Significance for Trans (Skiddaw in ELS TempXElutriates) Sigma-restricted parameterization Effective hypothesis decomposition					
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	63.85954	1	63.85954	3475.862	0.000000
Temperature	2.79802	2	1.39901	76.148	0.000000
Concentration	5.08371	5	1.01674	55.341	0.000000
Temperature*Concentration	0.22371	10	0.02237	1.218	0.301026
Error	0.99210	54	0.01837		