Report for MASTS:

Marine Stressors Forum research grant 2019

"Investigating the effects of climate change on baseline biomarker performance utilising a multiple stressor approach" (£957.65)

&

Coastal Forum Flexible Small Grant 2020

"Effects of climate change on biomarker responses in coastal species (*Mytilus sp.*)" (£497.39)

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Project introduction

The effects of climate change are measurable in the marine environment in several ways and this research focuses on the following three factors: the temperature of the upper 75 m of the global ocean has increased by 0.11°C per decade since 1971; analysis of the ocean chemistry from time series stations and merged shipboard studies show consistent rates of decrease of 0.013-0.03 pH units per decade over 25 years in the ocean surface (both of these trends are predicted to continue (Wong *et al.*, 2014; Bindoff *et al.*, 2019); in addition, the near surface salinity of the ocean is predicted to reflect the increased hydrology cycle of the earth (changes in precipitation and increase in evaporation) with coastal and estuarine environments more likely to experience extreme salinity fluctuations as a result of extreme weather events and increased river runoff (Noyes *et al.*, 2009; Bindoff *et al.*, 2019). In turn, a change in these three factors might, separately, or in interaction with each other, affect marine organisms in a number of ways, described below:

Temperature: Increased temperatures likely lead to increases in chemical reaction rates and also affect biological processes such as metabolism, growth and reproduction. In addition, the solubility of chemicals is also temperature, oxygen, and pH-dependent; the solubility of oxygen in water decreases as temperature increases. Lower levels of dissolved oxygen potentially fall outside the suitable range of the present aquatic life leading to stress. Sessile species unable to migrate to areas with more favourable conditions will be especially susceptible to this. For the ectothermic marine invertebrates which make up a major part of macroscopic life in the ocean, increased temperatures could potentially have an impact on cellular permeability and membrane fluidity (Cossins and Prosser, 1978). This in turn could affect the bioavailability of contaminants and their toxicokinetics - how they behave inside organisms (Noyes *et al.*, 2009; Connell, Fernandes and Hartl, 2017).

Salinity fluctuations: Increased salinity stress might cause significant stress responses in osmoregulators (potentially already at or near their limit), due to an increase in energy spent on osmoregulation or exposure to osmotic stress (Kultz, 2015). Salinity might affect the behavioural and physiological responses of marine organisms as well as the bioavailability of contaminants (Leung *et al.*, 2002; Woodin *et al.*, 2020). Behavioural responses to salinity stress such as bivalves closing their valves might limit exposure to osmotic stress but lead to discontinued feeding and gas exchange (cease in energy intake and potentially introduce hypoxia).

Ocean acidification (OA): Whilst calcifying organisms have been argued to be most affected by OA (slowing of calcification rates, coral bleaching and potential dissolution) (Kuffner *et al.*, 2008; Hennige *et al.*, 2015), it also has several other direct or indirect adverse effects such as depressing immune responses and metabolic rates, hypercapnia, and other physiological responses, loss of habitat, decreased survival rates and reproduction, food web crashes and loss of biodiversity (Raven *et al.*, 2005; Kelly and Hofmann, 2013; Lopes *et al.*, 2018).

In ecotoxicology, a biomarker can be considered an objectively and quantitively measurable response (be it biochemical, physiological, behavioural, or histological etc.) to exposure. An ideal role of a biomarker is to be used as an early warning indicator before adverse consequences manifest themselves – especially in relation to chronic exposure to low-level pollution where organisms might look and behave normally as the pollution takes its toll over time.

The three climate related factors mentioned previously might cause biomarker responses to drift from the current baseline due to increased stress meaning they could potentially become less sensitive. In the future results will need to be contextualized appropriately to be able to distinguish responses causes by non-chemical confounding stressors from responses to pollutants or other anthropogenic activities. It might also mean that some endpoints will no longer be suitable for the biomarker task.

In this project, mussels (*Mytilus sp.*) are being exposed to current and predicted conditions in relation to climate change (increasing surface temperatures, OA and local fluctuations in salinity) using single- and multiple stressor scenarios to probe further how biomarkers may be impacted by climate change and to help provide key insights into how marine organisms will be affected by climate change. Biomarkers of interest include measures of genotoxicity due to the wide variety of chemicals that affect the DNA (Prá *et al.*, 2005) and oxidative stress, as this is arguably the most common mechanism in ecotoxicology (Samet and Wages, 2018).

Funding summary

A total of £1455.04 was received from the MASTS Marine Stressors Forum and Masts Coastal Forum and an additional £500 has been received from the school of Energy, Geoscience, Infrastructure and Societe, institute of Life and Earth Sciences.

This money was used to acquire a GHL Profilux Aquarium Controller and various accessories (pumps, piping, tubing etc.). This has allowed us to design and build and automated aquatic system (Figure 1) that is essential in the ocean acidification part of our research separately and in combination with the other stressors in question which would not be possible to do in a fully manual system. Due to the COVID-19 related lockdown of 2020 and ongoing restrictions this

project has been delayed substantially, the work that has been performed so far and preliminary results are presented below.

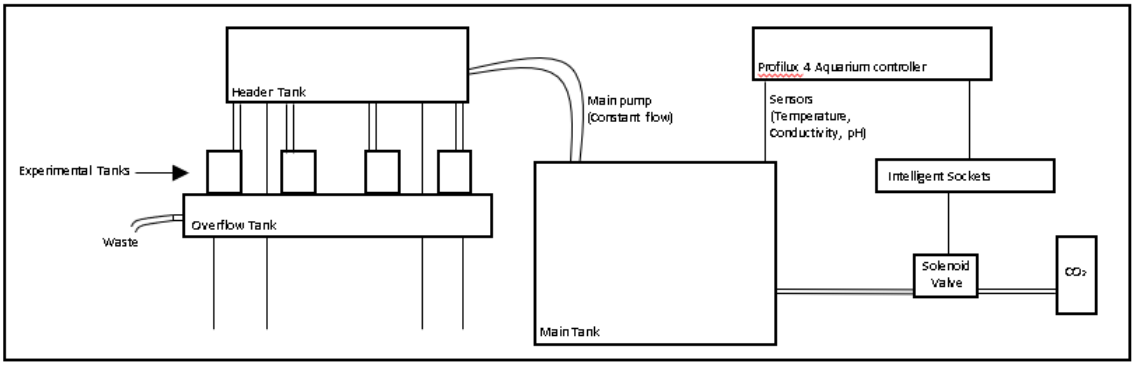

Figure 1: Experimental setup, GHL Profilux Aquarium Controller.

Materials and Methods

Mussel collection:

Mussels (*Mytilus sp.*) were collected for the study from Musselburgh at low tide, in early October 2020. Following collection, the mussels were kept in 50 l plastic tanks, half filled with aerated seawater from North Berwick filtered through a 30 µm Hydrotech hdf 501 filter. Both Musselburgh and North Berwick are situated in the Firth of Forth (Figure 2). After having recovered from industrial discharges, the waters in the Forth are now mostly classed as good (SEPA, 2006; Baxter *et al.*, 2011). One hundred percent water changes were performed twice weekly and the mussels were fed a diet of *Isochrysis galbana* and *Chaetoceros calcitrans* (herein after referred to as "marine algae") following water changes. Mussels were kept at these conditions for at least a week before being subjected to experimental conditions. No attempts were made to identify the mussels to species level or to check for hybridization.

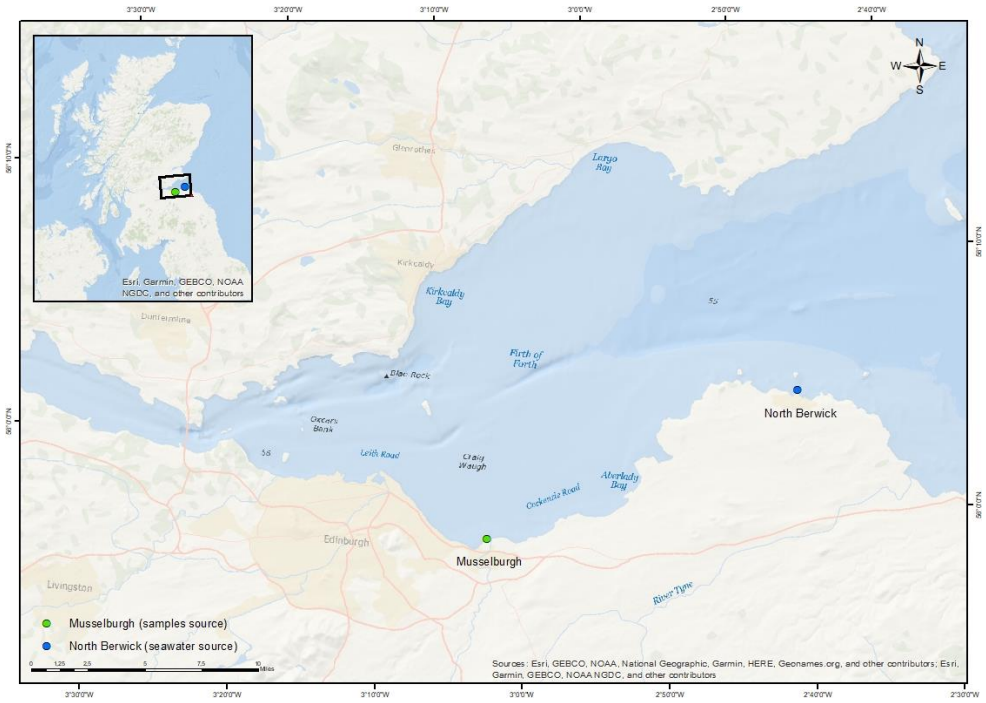

Figure 2: Map of the Firth of Forth (Scotland), Mussels sampled from Musselburgh (green), Seawater sourced from North Berwick (blue), (by Nine Le Reun).

Exposure conditions

For each treatment (pH 8.1 or 7.5), six animals were kept in each of four containers, containing 2 l seawater at a constant temperature (14..5±0.1 °C) in a flow through system with the pH in the main tank controlled using a GHL Profilux 4 aquarium controller and CO_2 gas (Figure 1) for 28 days. The animals in each container were fed a daily diet (Monday – Friday) of > 2.50*10^6 cells of marine algae, (determined using a haemocytometer). Water was changed at a flow rate of ≈ 15 mL^{-1} minute in each experimental tank, pseudo faeces and other detritus was removed daily (Monday – Friday) by siphoning water out of the experimental tanks. The water temperature in each experimental container, the header tank and main tank was recorded daily using a Fischer Scientific stainless-steel thermometer, in addition the temperature in each tank was measured every 15 minutes over a 24 hour period using a HOBO UA-001-08 Temp Logger; Salinity was recorded daily using a D-D H2Ocean Refractometer; temperature, dissolved oxygen, conductivity and salinity was recorded daily in each tank using a YSI Model 85; in the main tank, temperature, conductivity and pH was recorded hourly through the GHL Profilux 4, additionally the connected pH probe was used for spot checks in the other tanks. Mortalities were recorded daily; dead individuals were removed immediately, and 100% water changes were performed by siphoning out water and refilling it with water at the appropriate temperature and pH.

After 28 days of exposure two mussels from each beaker were sampled for analysis, haemolymph and the gill tissue of one valve was extracted for use in the comet assay, the gill tissue in the second valve was extracted, flash frozen in liquid nitrogen and stored at -80°C for biomarkers of oxidative stress.

Comet Assay

The comet assay was adapted from (Woods *et al.*, 1999; Coughlan *et al.*, 2002) and performed as described in (Hartl, Grigson and Sinet, 2010). Hamelymph and gill tissue cell suspensions were incorporated into a 1% (w/v) PBS agarose "sandwich" with 3 layers: 100 µL normal gelling agarose, 30 µL cell suspension + 70 µL low melting point agarse (LMP), 100 µL LMP. The prepared slides were lysed in a high-salt buffer (1 M NaCl, 100 mM EDTA, 10 mM TRIS, 1% Sarcosinate, 1% (v/v) Triton X-100 and 10% (v/v) DMSO at pH 10.0) for at least 90 minutes and up to 48 hours in dark lysis tanks at 4°C. Following lysis, the slides were placed in a horizontal electrophoresis tank and covered in an alkaline solution (0.3 N NaOH, 1 mM EDTA) for 30 minutes at 4°C in the dark (to allow for alkaline unwinding) following alkaline unwinding, 25 V was applied and alkaline solution was added to afford a current of 300 mA for 25 minutes. Following this, each slide was neutralised 3 times using a Tris buffer (0.4 M Tris-HCl at pH 7.5), the slides were stained with Gelred and stored in a humidified atmosphere for up to 72 hours before scoring. The DNA damage of 50 random cells per slide was recorded as %Tail intensity using a ZEISS Axio Sxcope.A1 using the Perceptive instruments, Comet Assay IV software.

All statistical testing was performed using R [version 3.4.1, R Core team (2017)].

Interpretating preliminary results

The number of DNA single strand breaks (expressed as %Tail DNA) was found to increase significantly following 28 days exposure to pH 7.6, when compared to pH 8.1 in both haemolymph and gill tissue of blue mussels (*Mytilus sp.*) (t-test, $p < 0.05$, $n = 4$) (Figure 3).

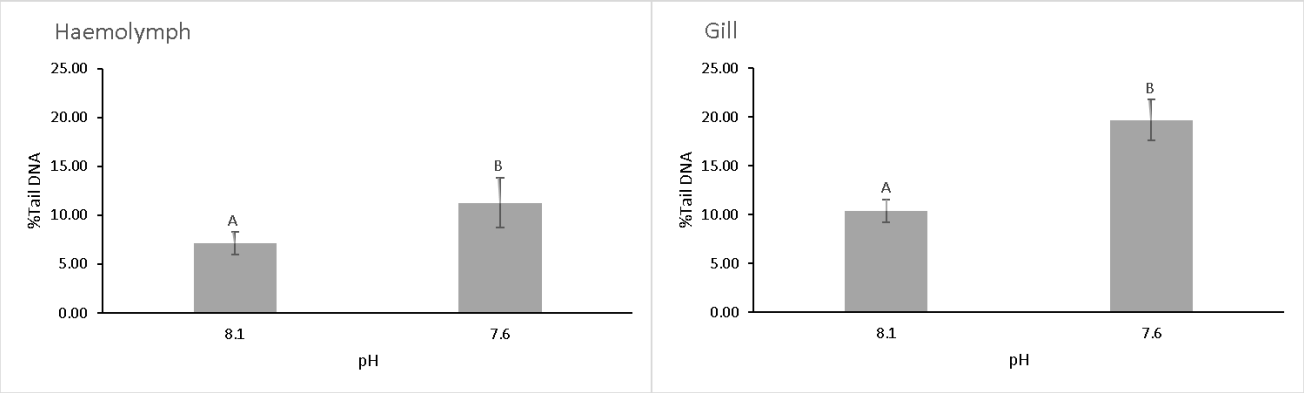

Figure 3: DNA damage, Ocean Acidification: Average %Tail DNA ± SD, n = 4. DNA damage (as %Tail DNA) was significantly higher in mussel subjected to lower pH, compared to the control in haemolymph (t-test, $p < 0.05$) and gill tissue (t-test, $p < 0.001$). Groups that do not share a letter are significantly different ($p \leq 0.05$).

Discussion and conclusions

These initial results indicate that ocean acidification was found to cause an increase in the number of DNA single strand breaks in both the haemolymph and gill tissue of blue mussels, a similar trend has previously been observed in another marine mollusc (*Crassostrea gigas*) (Cao *et al.*, 2018). Going into 2021, the plan is to run multiple stressor scenarios (ocean acidification combined with other stressors) and perform a range of oxidative stress assays (e.g. catalase, GSH and TBARS). Previous experiments have indicated that as multiple stressors are combined the observed responses become more complex (increasing temperatures and fluctuating salinity, presented at the MASTS annual

science meeting 2020), it is expected that this will also be the case when mussels are exposed to ocean acidification in combination with other stressors (increasing temperatures and salinity fluctuations). The results of these experiments will be highly relevant due to the multiple stressor nature of the marine environment.

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