Science of the Total Environment 892 (2023) 164485

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Immediate and delayed effects of a heatwave and *Prorocentrum lima* ((Ehrenberg) Stein 1878) bloom on the toxin accumulation, physiology, and survival of the oyster *Magallana gigas* (Thunberg, 1793)

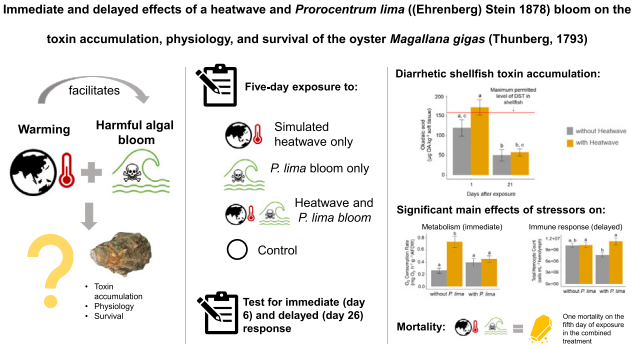
Ellen Grace M. Funesto [a,b,*], Adam M. Lewis [c], Andrew D. Turner [c], Tom C. Cameron [a], Michael Steinke [a]

[a] School of Life Sciences, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, United Kingdom
[b] Department of Biology and Environmental Science, College of Science, University of the Philippines Cebu, Gorordo Avenue, Lahug, Cebu City 6000, Philippines
[c] Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Barrack Road, The Nothe, Weymouth, Dorset DT4 8UB, United Kingdom

HIGHLIGHTS

- Exposure to warming led to higher DST accumulation in *Magallana gigas* (Pacific rock oysters).
- DST-producer *Prorocentrum lima* interferes with the normal metabolic response of *Magallana gigas* to warming.
- Combined heatwave and toxic algal bloom led to changes in the total hemocyte count of oysters.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Daniel Wunderlin

Keywords:
Diarrhetic shellfish poisoning
Harmful algal blooms
Heatwave
Prorocentrum lima
Magallana gigas
Climate change

ABSTRACT

Warming could facilitate the intensification of toxic algal blooms, two important stressors for marine organisms that are predicted to co-occur more frequently in the future. We investigated the immediate and delayed effects of a heatwave and a simulated bloom (3×10^6 cells L^{-1}) of the diarrhetic shellfish toxin (DST)-producing benthic dinoflagellate *Prorocentrum lima* on the survival, physiology (oxygen consumption rate, condition index, immune parameters), and toxin accumulation in the Pacific rock oyster *Magallana* (*Crassostrea*) *gigas*. Oysters exposed to both stressors contained higher mean DST concentrations (mean ± 1 SE: 173.3 ± 19.78 μg kg^{-1} soft tissue) than those exposed to *P. lima* bloom alone (120.4 ± 20.90 μg kg^{-1}) and exceeded the maximum permitted levels for human consumption. Exposure to individual stressors and their combination modified the physiology of *M. gigas*. Oysters exposed to heatwave alone had significantly higher oxygen consumption rates (0.7 ± 0.06 mg O_2 h^{-1} g^{-1}) than the control (0.3 ± 0.06 mg O_2 h^{-1} g^{-1}). However, this was not observed in oysters exposed to both heatwave and *P. lima* (0.5 ± 0.06 mg O_2 h^{-1} g^{-1}). This alteration of the metabolic response to warming in the presence of *P. lima* may affect the ability of rock oysters to adapt to environmental stressors (i.e., a heatwave) to ensure survival. Immunomodulation, through changes in total hemocyte count, was observed in oysters exposed to *P. lima* alone and in combination with warming. Individual stressors and their combination did not influence the condition index, but one mortality was recorded in oysters exposed to both stressors. The findings of this study highlight the vulnerability of rock oysters to the predicted increased frequency of heatwaves and toxic algal blooms, and the increased likelihood of shellfish containing higher than regulatory levels of DST in warming coasts.

* Corresponding author at: School of Life Sciences, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, United Kingdom.
E-mail address: emfunesto@up.edu.ph (E.G.M. Funesto).

http://dx.doi.org/10.1016/j.scitotenv.2023.164485
Received 21 February 2023; Received in revised form 4 May 2023; Accepted 24 May 2023
Available online 29 May 2023

1. Introduction

Planktonic microalgae are an important food source for primary consumers in the marine food web, including suspension-feeding bivalves. However, when microalgae proliferate, they can lead to 'algal blooms' that have both positive and negative impacts (Lloyd et al., 2013). Algal blooms can benefit aquaculture and wild fisheries, but they can also become harmful algal blooms (HAB) that cause economic damage to aquaculture, fisheries, and tourism, and negatively affect the environment, animal, and human health (Turner et al., 2021). There are 80 phytoplankton species that are known to produce harmful toxins that can bioaccumulate in finfish and shellfish, and cause disease and physical harm to a range of other animals, including humans (Turner et al., 2021). Often these toxin-producing phytoplankton have low biomass "blooms" where low cell densities of the toxic species can lead to the intoxication of shellfish, normally without detection via water discoloration or lack of specific dominance in the phytoplankton community (Berdalet et al., 2015; Hallegraeff et al., 2003).

One of the potentially harmful genera is the cosmopolitan dinoflagellate *Prorocentrum*, which includes several toxin-producing species. In temperate areas, they commonly become densely abundant in eutrophic marine waters in summer (Heil et al., 2005; Türkoğlu, 2010; Türkoğlu and Erdoğan, 2010), but blooms are also observed in late winter and early spring (Hu et al., 2022; Li et al., 2021; Taş and Okuş, 2011). *Prorocentrum lima*, one of the genus' potentially harmful species, is an epiphytic-benthic dinoflagellate and is common in UK waters (Coates et al., 2014, 2015; Harrison et al., 2016, 2017; Leftley and Hannah, 2009; Parks et al., 2018, 2019). It has been found to be associated with macroalgae and macrophytes in the Fleet Lagoon, Dorset, where shellfish production sites occasionally have to close their operation due to levels of diarrhetic shellfish poisoning (DSP)-associated toxins in shellfish above regulatory limits (Foden et al., 2005). This dinoflagellate produces okadaic acid (OA) and dinophysistoxins (DTXs), toxins that cause DSP in humans when ingested at high enough levels (Nishimura et al., 2020).

Although not yet fully established and understood, research has demonstrated the ability of climate-change co-stressors, specifically high CO_2 concentrations in seawater and coastal/ocean warming, to intensify and increase the frequency of HAB (Anderson et al., 2012; Brandenburg et al., 2019; Gobler et al., 2017; Hallegraeff, 2010; Moore et al., 2008). It is suggested that HAB occurrences are likely to increase at high latitudes as these areas become warmer and more suited for algal growth and decrease at lower latitudes as they become too warm (Brandenburg et al., 2019). Regardless of how they are affected by climate change, HAB have drawn attention in coastal communities worldwide because of their impact on marine ecosystems and human health (Wang and Wu, 2009), as well as aquaculture.

Oysters are suspension-feeding, sessile benthic inhabitants of the coast and, inevitably, challenged by the increased co-occurrence of HAB and climate-change stressors. One of the many climate-change stressors that threaten oysters is atmospheric heatwaves, which cause seawater temperatures to increase for several days via air-sea heat flux, one of the primary causes of marine heatwaves (Cronin et al., 2019). In recent years, mass mortalities of oysters and other bivalves have been reported during summer after an atmospheric heatwave (Colletta and Westfall, 2021; Hagenbuch, 2021; Timms, 2019; Troost, 2018). A number of studies suggest that such heatwaves are occurring more frequently and with greater intensity because of climate change (Herring et al., 2019; Meehl and Tebaldi, 2004; Schiermeier, 2018).

Despite the evidence of frequent co-occurrence of climate-change co-stressors with HAB in coastal zones, very little is known of how their combination affects aquatic organisms (Griffith and Gobler, 2017; Wells et al., 2015). It is in consideration of this knowledge gap that this study was initiated. This study investigated the combined effects of increased temperature (through a simulated heatwave) and the presence of the diarrhetic shellfish toxin (DST)-producing benthic dinoflagellate *P. lima* on the widely cultured and commercially important Pacific rock oyster *Magallana gigas*. This study aimed to test the immediate and delayed effects of these stressors. Specifically, this work assessed the physiological response of *M. gigas* including toxin accumulation, metabolic activity (through the measurement of oxygen consumption rate), body condition, immune function (total hemocyte count, hemocyte neutral red uptake, hemolymph protein concentration), and survival. The findings of this study offer some insight into the effect of heatwaves and DST algal blooms and provide baseline information for coastal managers and stakeholders for their evaluation of the dependability and viability of bivalve aquaculture in coastal seas affected by anthropogenic stressors.

2. Methods

2.1. Animal collection and husbandry

The experimental procedures in this study were reviewed and approved by the University of Essex Ethics Review and Management System under Application number ETH2021-1358. Adult Pacific rock oysters (*M. gigas*) (mean length: 92.0 ± SD: 17.24 mm) were obtained from Mersea Island, Essex, UK on 2 October 2021 and acclimated in the laboratory for 14 days in locally collected seawater, filtered through a polypropylene felt filter bag (pore size 25 μm; Cole-Palmer, UK), set to a controlled temperature of 14 °C, matching the field water temperature during collection. Starting from the second day of the acclimation period, the water temperature in the acclimation tank was raised by 1 °C every day until it reached 20 °C, the average water temperature of Pyefleet Creek, Mersea, in August 2020 (Funesto, 2023), and the control temperature in this experiment. Salinity was measured using the Practical Salinity Scale and adjusted to a salinity of 35 daily using reverse osmosis water. Concentrations of nitrate, nitrite, and ammonia were monitored every other day using a colorimetric test kit (Red Sea Marine Care Test Kit, Tel Aviv, Israel). During the acclimation period, the oysters were fed a commercial feed (Shellfish Diet 1800®; Reed Mariculture, California, USA).

2.2. Microalgae

The *P. lima* used in this study was isolated from Vigo, Spain; and purchased from the Centre for Culture of Algae and Protozoa (CCAP, Oban, Scotland, UK) in 2018. The dinoflagellate cultures were maintained at the University of Essex in Erlenmeyer flasks with 1 L K medium (Keller et al., 1987), at 15 °C and under a 12:12 light-dark cycle at mean light intensity (± standard error) of 221 ± 12 μmol m^{-2} s^{-1}, measured using a light sensor (Li-Cor Li-250A, California, USA) (Edullantes et al., 2023). In these standard culture conditions, we have quantified the total toxin concentration of the algae as 2.26 pg $cell^{-1}$ (OA: 1.84 pg $cell^{-1}$; DTX1: 0.42 pg $cell^{-1}$). The diatom *Thalassiosira pseudonana*, maintained in the same culture conditions as *P. lima,* was used as a non-toxic diet in the control treatment. Cell counts for both microalgal cultures were determined using a Neubauer hemocytometer and a compound microscope (Olympus BH-s BHT; Shinjuku, Japan) at 100× magnification. Cells were harvested by centrifugation (800 × *g* for 10 min) and adjusted to the required concentrations with filtered seawater (FSW).

2.3. Experimental design

The experiment consisted of five-day exposure of oysters to each of the four treatment combinations of two conditions: with or without the simulated heatwave; and with or without *P. lima* (Fig. 1). The work was conducted in a static system, with constant aeration, in 6 L plastic tanks (Exo Terra Faunarium; Montreal, Canada) with 5 L seawater. Each condition included three replicate tanks, and each tank contained six oysters (3 replicates each to quantify short-term and long-term effects, $N = 72$). The temperature in the tanks was maintained using independent thermostats (Ink bird Temperature Controller, Guangdong, China).

To simulate the local heatwave conditions observed in Pyefleet Creek, Essex in 2020 (Funesto, 2023), the water temperatures of the tanks assigned to the heatwave treatment were raised from 20 to 22 °C on day

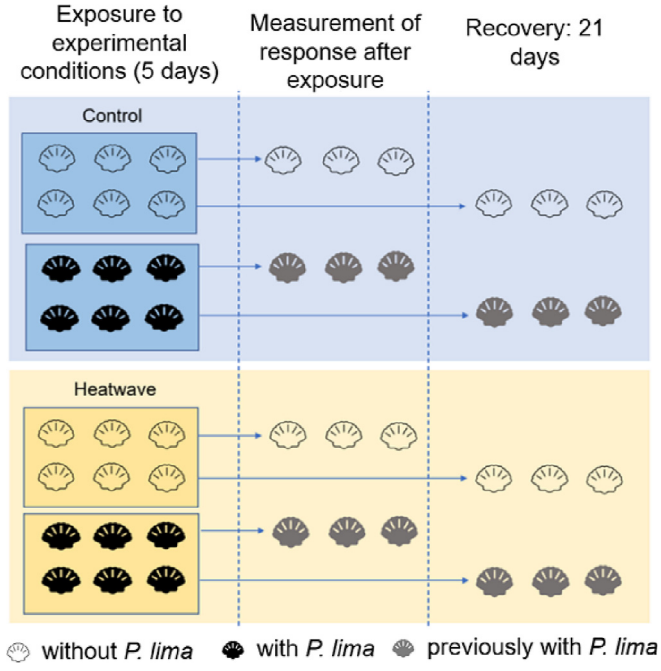


Fig. 1. Schematic diagram of the experimental design. The experiment had four treatment combinations of two conditions: with or without the simulated heatwave; and with or without *P. lima*. Each 6 L tank contained six oysters (*M. gigas*) exposed to experimental conditions for five days. All four conditions had three replicate tanks. Three oysters from each tank were evaluated for responses immediately after exposure and the rest were allowed to recover for 21 days to investigate possible delayed responses.

1, then increased to 24 °C on day 2, and then on day 3 raised to 25 °C, the peak of the heatwave. The tanks assigned to heatwave conditions were maintained at this temperature until the end of the experiment on day 5. The control treatment temperature was maintained at 20 °C.

Oysters in the toxic algal-bloom treatment were exposed to a daily suspension of *P. lima* at 3×10^6 cells L^{-1}, simulating cell densities occurring during a natural bloom (Türkoğlu, 2016). The control group was exposed to at an estimated equivalent cell concentration *T. pseudonana* of 27×10^6 cells L^{-1}, which was calculated based on the ratio of the equivalent spherical diameter (ESD) of *P. lima* (ESD: 35 μm) and *T. pseudonana* (ESD: 3.9 μm) cells. In addition, a fixed amount of *T. pseudonana* was added to all tanks (1×10^6 cells L^{-1}) to stimulate feeding. Before the microalgae were provided as a single dose once every 24 h, 100 % water changes (water adjusted to appropriate temperatures) were performed in all tanks. Other environmental parameters such as light cycles and salinity were maintained the same as during the acclimation period.

At the end of the exposure, on day 6, half of the oysters from each tank (i.e., three oysters) were haphazardously chosen for the measurement of their immediate response to experimental conditions, and the other half were transferred to fresh seawater at 20 °C and allowed to recover for 21 days and fed with the commercial feed every day. After the recovery period, their delayed response to the experimental conditions was evaluated.

2.4. Metabolic response - oxygen consumption rate

Oxygen consumption measurements were done with recirculating sealed chambers maintained in a water bath at the incubation temperature using FSW. The chambers were placed inside a water bath, where temperatures were maintained using a thermostat-linked heater (Schego 300 W titanium heater, D-D The Aquarium Solution Ltd., Germany). During the measurement, a single empty chamber was included, acting as a control to determine any microbial oxygen consumption associated with the chamber over time (background oxygen consumption).

Before observation, the oysters were placed in individual chambers overnight to acclimate to handling and were not fed to reduce digestion and feeding-associated metabolic responses. Using a PreSens O_2 sensor dot affixed inside the glass chamber and a Polymer Optical Fiber and Fibox system (PreSens Precision Sensing GmbH, Regensburg, Germany), the percentage oxygen saturation inside the chamber was measured every 2 s for 1 min, every hour. O_2 saturation was measured for a maximum of 3 h, and when a decline of 50 % saturation was observed in a chamber, the recording of oxygen consumption rates in that oyster was stopped. This gave rise to a time series of oxygen concentrations inside the chamber over a three-hour period.

The oxygen consumption rate (MO_2) of individual oysters was calculated using the following Eq. (1) (Bayne and Widdows, 1978; Sawusdee, 2015):

$$MO_2 = \frac{(Ct_0 - Ct_1)\left(\frac{V_c - V_a}{1000}\right)\left(\frac{60}{t_1 - t_0}\right)}{AFDW} \quad (1)$$

where MO_2 is the oxygen consumption rate in mg O_2 h^{-1} g^{-1}, Ct_0 is the oxygen concentration (mg L^{-1}) at the starting time (t_0), Ct_1 is the oxygen concentration at the end time point (t_1), V_c is the chamber volume and associated tubing volume (680 mL), and V_a is the mean volume of the oyster measured by three repeated measures of water displacement in mL. The background oxygen consumption rate from control chambers without oysters was subtracted from individual oyster oxygen consumption rates to calculate an oyster-only MO_2. The MO_2 was then divided by the ash-free dry weight of the soft tissue (AFDW) to calculate the oxygen consumption rate per g AFDW. The determination of AFDW is described in Section 2.5.

2.5. Condition index and ash-free dry weight determination

Every morning (0900), the tanks were inspected for dead oysters, and those that died were immediately placed in a labelled bag in a freezer (−20 °C). Animals that were alive at the end of the study were first opened (shucked) and soft tissues and shells of oysters were separated. The wet weight of the soft tissue (WW_{Tissue}) was determined using an analytical balance. After weighing, the soft tissue was homogenized with mortar and pestle, and two grams of the soft tissue for each oyster were taken for toxin accumulation determination described in Section 2.6. The remaining soft tissue and shells were oven-dried at 80 °C on pre-dried foil dishes for 48 h. Animals from the freezer were recovered and included in this process. The dry weight of the shell (DW_{Shell}) and the tissue (DW-2) were determined using an analytical balance. DW_{Shell} and DW-2 measurements were used to obtain the condition index of each oyster, described below. Accounting for the 2 g reduction in soft tissue used for toxin quantification, the ratio of the wet weight after 2 g reduction (WW-2), the quantified dry weight of soft tissue (DW-2), and the true wet weight of the soft tissue (WW_{Tissue}) were used to obtain the true dry weight of soft tissue (DW_{Tissue}), following Eq. (2):

$$DW_{Tissue} = \frac{DW - 2}{WW - 2}\, x\, WW1 \quad (2)$$

The CI was calculated using Eq. (3):

$$CI = \frac{DW_{Tissue}}{DW_{Shell}} \times 100 \quad (3)$$

To obtain the AFDW, the dried soft tissues were placed in a muffle furnace for 6 h at 500 °C to remove organic content and reweighed for the ash weight. The ash weight was subtracted from the DW_{Tissue} to obtain the AFDW of the soft tissues.

2.6. Measurement of immune response

Immune response measurements were based on the work of Matozzo et al. (2012) with minor modifications. By prying the oysters open, approximately 700 μL of hemolymph per individual was collected from the

adductor muscle of the bivalves using a 19-gauge needle with a one mL disposable syringe and stored on ice. Total hemocyte count (THC) was determined using 5 μL of the hemolymph, and 500 μL was used for the neutral red (NR) uptake assay. As for the total protein concentration in the cell-free hemolymph (CFH) 190 μL of the pooled hemolymph was used.

To dilute samples and prevent clogging, 5 μL of the hemolymph was added to 195 μL of 0.38 % sodium citrate in filtered seawater (pore size: 0.45 μm) with a pH of 7.5. Twenty microliters of the hemolymph and sodium citrate mixture were placed on a hemocytometer. The number of hemocytes was quantified using a compound microscope (Olympus BH-s BHT; Shinjuku, Japan) at 100× magnification.

The hemolymph used for NR uptake was centrifuged at 780 ×*g* for 10 min. Hemocytes were then resuspended in an equal volume of 8 mg L^{-1} NR dye (Merck) solution in FSW and incubated at room temperature for 30 min. After this, the solution of hemocytes and NR dye was centrifuged at 780 ×*g* for 10 min, resuspended in distilled water, and lyzed using Precelly's ® Evolution homogenizer (Bertin Technologies Montigny-le-Bretonneux, France) at 6.5 m s^{-1} for 2 min, and centrifuged at 780 ×*g* for 10 min. The supernatant, corresponding to hemocyte lysate (HL), was then collected for the NR uptake assay. Absorbance at 550 nm was recorded on a microplate reader. The results were then expressed as optical density per mL hemolymph (OD mL^{-1} hemolymph).

The hemolymph used to determine protein concentration in CFH was first centrifuged at 780 ×*g* for 10 min. The supernatant corresponding to CFH was then collected for protein quantification. Through a BCA protein assay kit (Pierce™, Pennsylvania, USA), protein concentrations in CFH were measured using bovine serum albumin as standard. Five μL of CFH diluted with 20 μL deionized water were incubated at 37 °C for 30 min with 200 μL of the BCA kit working reagent. The absorbance was measured at 562 nm, and the results were expressed as mg protein mL^{-1} hemolymph.

2.7. Diarrhetic shellfish toxin determination

A three-step extraction based on the EU Reference Laboratory for Monitoring Marine Biotoxins protocol (EURL, 2015) was used. For this, 2.0 ± 0.01 g aliquots of each homogenized tissue were extracted in 6.0 mL methanol. After centrifugation at 2900 ×*g* (10 min at room temperature), the supernatants were removed and collected. The remaining solid pellets were subjected to a second and third extraction step involving the addition of a further 6.0 mL aliquot of methanol, before re-homogenization with a multi-tube vortex mixer. The supernatants from the three extraction steps were combined and diluted to 20.0 mL with methanol before they were filtered using 0.2 μm nylon syringe filters (Phenomenex, Manchester, UK). For Internal Quality Control purposes samples were again extracted in batches alongside positive and negative control reference tissues. Methanolic extracts from all samples and controls were hydrolyzed to allow determination of acyl esters of OA-group toxins as per EURL (2015). The total concentration of toxins, including both free form (not bound to fatty acids) and fatty acid esters, was measured using hydrolyzed samples.

Both unhydrolyzed and hydrolyzed extracts were analyzed from each sample using an Acquity I-class UHPLC system which was coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters Ltd., Manchester, UK). Chromatography of toxins was conducted using an Acquity BEH C18 column (1.7 μm × 2.1 mm × 50 mm) with a Waters BEH C18 guard cartridge. UHPLC and MS/MS conditions utilized are described by Dhanji-Rapkova et al. (2018, 2019). This method allowed the quantitation of the lipophilic toxins (LT) okadaic acid (OA), dinophysistoxins 1 and 2 together with associated acyl esters (DTX3), pectenotoxins (PTX1,2,11), yessotoxin analogues (YTX, h. YTX, 45 OH YTX, 45 OH h. YTX) and azaspiracids (AZA1-3) using a 6-point calibration curve prepared from dilutions of certified calibrants (NRC, Canada). Results were reported as μg toxin kg^{-1} soft tissue.

2.8. Calculation of survival-odds ratio and confidence intervals

The calculations of the survival-odds ratio (OR) and OR confidence intervals were based on a method described by Szumilas (2010). OR values equal to one meant that treatment did not affect odds of mortality, OR > 1 meant that the treatment was associated with higher odds of mortality, while OR < 1 meant that exposure to treatment was associated with lower odds of mortality. Since no mortality was observed in some groups, a Haldane-Anscombe correction was employed. Haldane-Anscombe correction adds 0.5 to all values in the OR equation if any of the values is equal to zero (Szumilas, 2010).

2.9. Data analysis

A linear mixed-effects analysis of the relationship between the treatment groups, specifically *P. lima* bloom alone, heatwave alone, combined *P. lima* and heatwave and the response variables of oxygen consumption rate, condition index, immune parameters, and toxin accumulation was performed in R (R Core Team, 2021) and using the R package "lme4" (Bates et al., 2015). The treatment group was entered as fixed effects, and the term "tank" as a random or grouped effect to account for multiple non-independent oysters in each tank. Pairwise comparisons of the responses between treatments were performed with the "emmeans" package (Lenth, 2021). Factorial ANOVA was performed to determine whether heatwave and *P. lima* have interactive effects when combined.

3. Results

3.1. DST accumulation and elimination

Soft tissues of *M. gigas* were evaluated for DST content one day and twenty-one days after exposure to *P. lima,* under control temperature and a heatwave. Most of the DST quantified was OA, but low levels of DTX1 were also detected. One day after exposure, oysters exposed to heatwave and *P. lima* reached a total OA concentration (mean ± 1 SE) of 173.3 ± 19.78 μg OA kg^{-1} soft tissue (Esterified: 65.3 ± 17.43, Free-form: 25.9 ± 6.31 μg OA kg^{-1} soft tissue), higher than the maximum permitted levels of DST in shellfish for safe consumption in Europe and other countries (160 μg OA eq. kg^{-1} soft tissue; Regulation EC No.853/2004, 29) (Fig. 2A). Oysters exposed to *P. lima* alone had lower total OA, with a mean of 120.3 ± 20.90 μg OA kg^{-1} soft tissue (Esterified: 44.1 ± 13.35, Free-form: 16.1 ± 6.42 μg OA kg^{-1} soft tissue), but this difference was not significant ($t_{32} = 2.22$, $p = 0.14$). After 21 days, the total OA in the group exposed to *P. lima* alone (Total: 50.6 ± 14.26; Esterified: 20.9 ± 5.31, Free-form: 6.39 ± 3.99 μg OA kg^{-1} soft tissue) and those exposed to both stressors (Total: 57.7 ± 9.23; Esterified: 28.5 ± 6.89, Free-form: 4.0 ± 1.18 μg OA kg^{-1} soft tissue) did not differ significantly ($t_{32} = -0.28$, $p = 0.99$).

One day after exposure, the concentration of total DTX1 in the group exposed to *P. lima* alone (Total: 21.6 ± 3.94; Esterified: 8.2 ± 2.57, Free-form: 2.6 ± 1.26 μg DTX1 kg^{-1} soft tissue) was lower than those exposed to both stressors (Total: 27.9 ± 4.61; Esterified: 11.5 ± 3.42, Free-form: 3.2 ± 0.80 μg DTX1 kg^{-1} soft tissue, respectively), but this difference was not significant ($t_{31} = 1.27$, $p = 0.59$) (Fig. 2B). Twenty-one days after exposure, the total DTX1 in oysters exposed to *P. lima* alone (Total 10.3 ± 2.73; Esterified: 4.5 ± 1.34, Free-form: 0.6 ± 0.63 μg DTX1 kg^{-1} soft tissue) and both stressors had very similar values (Total: 9.7 ± 1.65; Esterified: 4.9 ± 1.45, Free-form: 0.1 ± 0.07 μg DTX1 kg^{-1} soft tissue, respectively) and did not differ significantly ($t_{32} = 0.11$, $p = 0.99$).

3.2. Physiological responses

One day after exposure, the oysters not exposed to heatwave nor *P. lima* (henceforth the control group) had the lowest MO_2 (0.3 ± 0.06 mg O_2 h^{-1} g^{-1} AFDW) among the four treatment groups (Fig. 3A). The treatment type had a significant main effect on MO_2 one day after stressor-exposure ($F_{3,20} = 8.52$, $p < 0.001$) but not 21 days after exposure ($F_{3,20} = 0.27$, $p = 0.85$) (Fig. 4A). Only the group exposed to heatwave alone had a significantly higher MO_2 than the control, with a mean of 0.7 ± 0.24 mg O_2

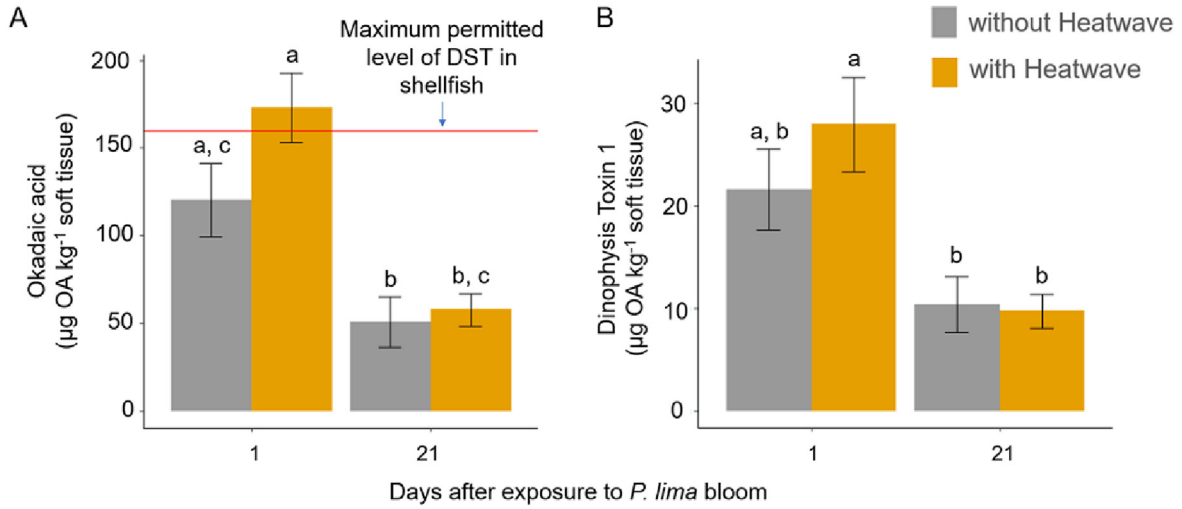


Fig. 2. Total Okadaic acid (A) and Dinophysistoxin 1 (B) content of the soft tissue of *Magallana gigas* exposed under control temperature (20 °C) and a Heatwave (peak: 25 °C), one and twenty-one days after exposure to *Prorocentrum lima* (3×10^6 cells L^{-1}). Data presented are means with ±1 SE ($n = 9$). Statistical groupings ($p < 0.05$) are indicated by letters above the bars. The red line in panel A represents the maximum permitted level of DST in shellfish meat set by the UK Government.

h^{-1} g^{-1} AFDW ($t_8 = -4.90$, $p = 0.005$). Specifically, *P. lima* alone and heatwave alone increased MO_2 one day after exposure by 0.1 and 0.5 mg O_2 h^{-1} g^{-1} AFDW, respectively, suggesting a 0.6 mg O_2 h^{-1} g AFDW^{-1} increase when applied in combination if they did not interact. Instead, putting the two stressors together had an antagonistic effect, with the mean in the combined stressors treatment showing only a 0.2 mg O_2 h^{-1} g AFDW^{-1} increase. The factorial ANOVA revealed an interactive effect of stressors ($F_{1,19} = 8.76$, $p = 0.008$), in which combined treatment had a higher MO_2 than the control but less than the expected additive effect.

Results revealed that the treatment groups did not have a significant main effect on the CI (Immediate: $F_{3,33} = 0.40$, $p = 0.75$; Delayed: $F_{3,31} = 2.39$, $p = 0.09$), hemocyte NR uptake (Immediate: $F_{3,30} = 1.13$, $p = 0.35$; Delayed: $F_{3,31} = 0.24$, $p = 0.86$), and the total protein concentration in CFH (Immediate: $F_{3,32} = 0.18$, $p = 0.90$; Delayed: $F_{3,31} = 1.22$, $p = 0.32$) of *M. gigas* (Fig. 3B, D, & E and Fig. 4B, D, & E).

No effect of treatment groups was found for THC a day after exposure ($F_{3,32} = 0.68$, $p = 0.57$) (Fig. 3C), but a significant main effect was detected after 21 days ($F_{3,31} = 4.80$, $p = 0.007$) (Fig. 4 C). The THC of the group exposed to *P. lima* alone was significantly lower than those exposed to heatwave alone and both stressors, 21 days after exposure ($t_{31} = -2.69$, $p = 0.05$; and $t_{31} = -3.59$, $p = 0.006$, respectively) (Fig. 4 C). If there was no interaction, the expected additive effect of the combined treatment 21 days after exposure is a THC decrease of 2.23×10^6 cells mL^{-1} relative to the control. Instead, the combined treatment had higher THC compared to the control, which suggests an interactive effect between the stressors. This interaction is confirmed by the factorial ANOVA performed ($F_{1,31} = 6.24$, $p = 0.018$).

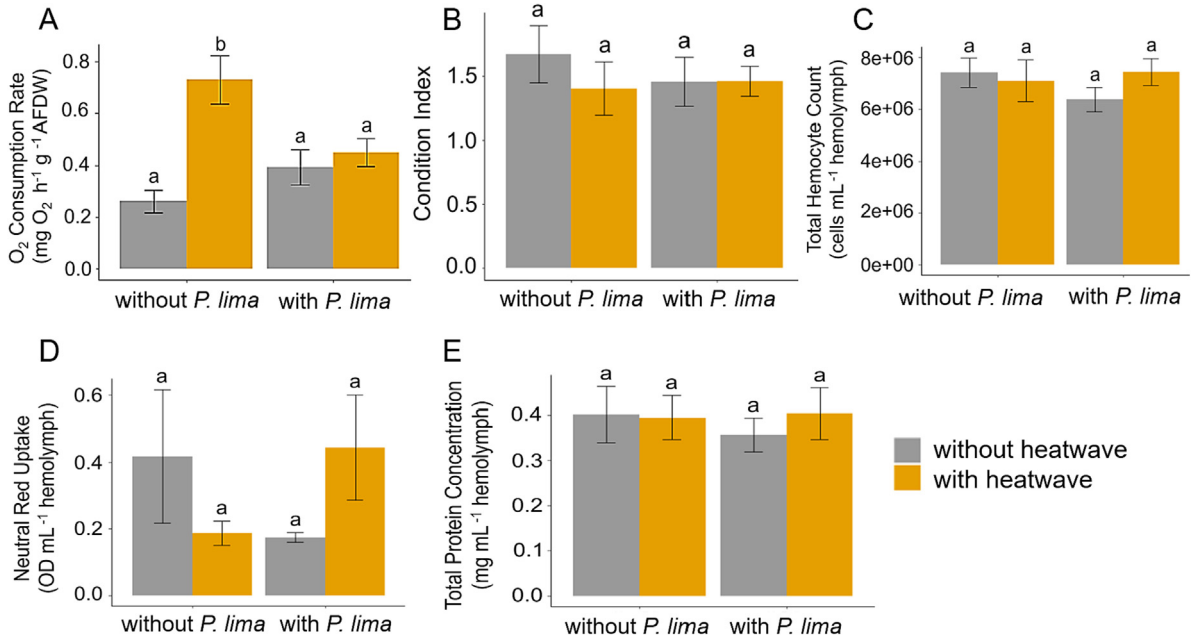


Fig. 3. Immediate effects of a heatwave and *Prorocentrum lima* on the oxygen consumption rate (A), Condition Index (B), Total Hemocyte Count (C), Hemolymph Neutral Red Uptake (D), and Hemolymph total protein concentration (E) of Pacific rock oysters (*Magallana gigas*). Data presented are means with ±1 SE ($n = 9$). Statistical groupings ($p < 0.05$) are indicated by letters above the bars.

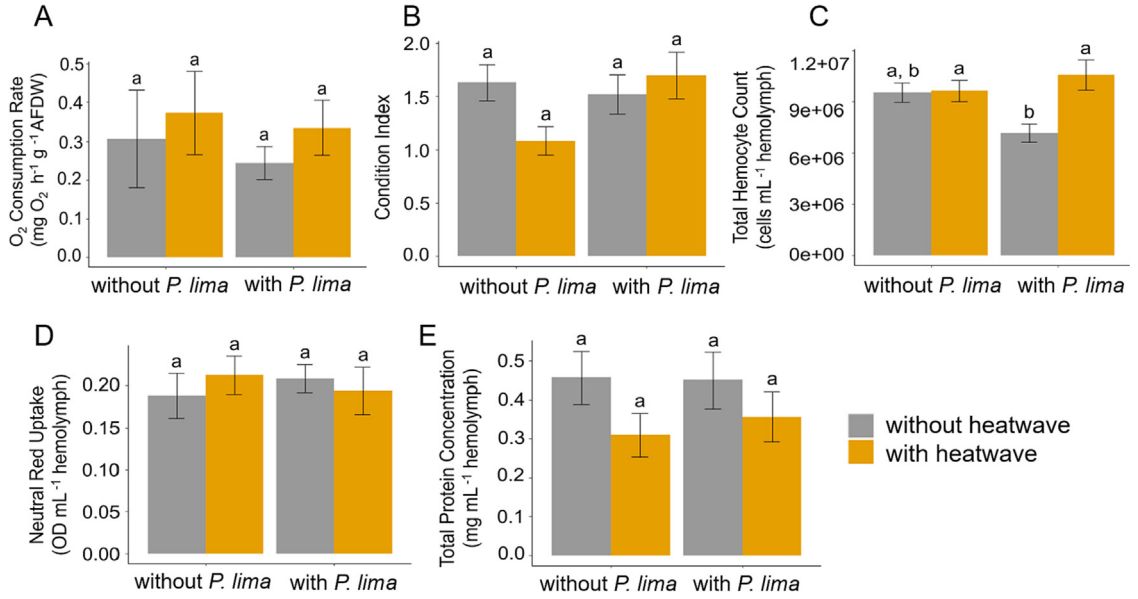


Fig. 4. Delayed effects of a heatwave and *Prorocentrum lima* on the oxygen consumption rate (A), Condition Index (B), Total Hemocyte Count (C), Hemolymph Neutral Red Uptake (D), and Hemolymph total protein concentration (E) of Pacific rock oysters (*Magallana gigas*). Data presented are means with ±1 SE (n = 9). Statistical groupings ($p < 0.05$) are indicated by letters above the bars.

3.3. Mortality

On the fifth day of stressor exposure, one mortality was observed out of the 18 oysters assigned to heatwave and *P. lima* bloom. All other oysters in the experiment survived the stressor exposure and recovery period. Calculating the survival-odds ratio in oysters exposed to both stressors gave a value of 3.2 [95 % Confidence Interval: 0.12–83.17]. Although the calculated OR was positive, the 95 % Confidence interval includes 1.0, indicating our experimental exposure to both stressors may not lead to a higher chance of death in oysters over the total period of study.

4. Discussion

4.1. M. gigas exposed to P. lima and heatwave show high levels of toxins above the safe consumption limit

The results of this study showed that exposure of Pacific rock oysters to *P. lima*, with or without a concurrent heatwave, resulted in the accumulation of diarrhetic shellfish toxins (DSTs) in their soft tissues. Most of the DSTs quantified were okadaic acid (OA), with low levels of dinophysistoxin 1 (DTX1).

Although this study did not find a statistically significant difference in the DST content of *P. lima*-exposed oysters with or without the presence of a heatwave, it is noteworthy that the mean DST accumulation was higher in oysters exposed to high temperatures. Of particular concern, one day after exposure, the concentration of total OA in oysters exposed to both *P. lima* and heatwave was higher than the maximum permitted levels of DST in shellfish for safe consumption in Europe and other countries. This was not the case for the oysters not exposed to heatwave. This finding highlights the potential risk to public health, as even brief exposure to toxic blooms, especially under stressful environmental conditions, could result in DST accumulation above regulatory limits in Pacific rock oysters.

Remarkably, the warming conditions did not appear to affect the ability of rock oysters to eliminate DST, as evidenced by the negligible difference in total DST levels between oysters exposed to only *P. lima* and those exposed to both *P. lima* and a heatwave, 21 days after exposure. This suggests that, despite the challenging environmental conditions, the rock oysters maintained their capacity to process harmful toxins. Depuration or purification, which makes use of the bivalve's natural ability to expel bacteria or toxins, is a technique used in many parts of the world to remove microbial contaminants from bivalves by placing them in tanks of clean seawater, for several hours to a few days. However, this technique is not presently considered an effective means of lowering biotoxin contamination to safe levels due to the variation of depuration rate between the toxin and the species of bivalves (Lee et al., 2008). The Food and Agriculture Organization (FAO) suggests that toxin elimination in the natural environment may be quicker than in tanks due to the presence of natural food (Lee et al., 2008).

As depuration cannot reliably remove biotoxins, and with the possibility of toxin accumulation exceeding safe levels at high temperatures, more frequent and constant monitoring of shellfish meat and toxic algal bloom in coastal waters should be employed. For some countries in Europe, monitoring of toxic algae concentration in the water is done weekly in the summer and less frequently in other seasons. If algal cells are detected to be higher than warning levels, shellfish meat is tested for toxins. However, there are cases when toxins can accumulate in bivalve meat in less than a week (Fernandes-Salvador et al., 2021; Whyte et al., 2014). One of the possible methods for conducting constant monitoring toxic algal blooms in the water is the use of biosensors.

To further investigate DST accumulation and elimination in oysters exposed to warming, future work should involve exposing the animals to different concentrations of *P. lima*, e.g., 100 cells L^{-1} which is the trigger level for *P. lima* cell counts in European waters, to establish whether oysters can still accumulate high toxin levels at lower *P. lima* concentration.

4.2. P. lima silence oysters' metabolic response to the heatwave

This study demonstrated that exposure to both heatwave and *P. lima* bloom had a negative effect on the metabolic response of *M. gigas* to heat stress. The substantial upregulation of metabolic activity (demonstrated by an increase in oxygen consumption of 133 %) of oysters exposed to a heatwave alone is a typical response of aquatic ectotherms to higher temperatures (Rubalcaba et al., 2020). However, this expected upregulation of metabolic activity under heat stress was not observed in the oysters exposed to both heatwave and *P. lima*. Instead, the combined stressors resulted in only a 67 % increase in oxygen consumption, which is much lower than the 133 % increase observed in oysters exposed to heatwave alone.

This finding suggests that exposure to DST-producing dinoflagellates changes the metabolic response of the oysters to heat stress and may have decreased the oysters' metabolic capacity. The toxic algae appear to silence the physiological response of the oysters to heat stress, thus may influence

the ability of the oysters to survive in a stressful environment that requires a fast metabolic response (e.g., an abrupt temperature increase during emersion while in a heatwave). The lack of increased metabolic rate in oysters exposed to *P. lima* suggests that toxin produced by this dinoflagellate may be inhibiting the normal metabolic response of oysters to heat stress. Similar silencing of physiological response was also observed in the clam *Meretrix meretrix* exposed to the toxic dinoflagellate *Alexandrium* sp. (paralytic shellfish toxin (PST)-producer), where the clams do not respond to increased temperature or combined treatments of increased temperature, acidification, and decreased salinity (Turner et al., 2019).

It is worth noting that the metabolic response to the combined stressors is not simply the sum of the individual responses to each stressor. Instead, the factorial ANOVA revealed an interactive effect of stressors, in which the combined treatment had a higher oxygen consumption rate than the control but less than the expected additive effect. This suggests that the combination of heatwave and *P. lima* has a unique effect on the metabolic response of the oysters that cannot be predicted based on the individual effects of each stressor.

The observed lack of increase in metabolic rate in oysters exposed to both stressors can be explained by the interaction between the two stressors. *P. lima* produces potent toxins, including okadaic acid and dinophysistoxins, that can disrupt cellular signaling pathways and impair physiological processes such as feeding, respiration, and reproduction (He et al., 2019; Lv et al., 2021). It is possible that exposure to these toxins, even in the absence of a heatwave, may have already decreased the metabolic capacity of the oysters, making them less able to respond to an additional stressor such as a heatwave. This is supported by previous studies that have shown that exposure to harmful algal blooms can lead to reduced metabolic rates (Basti et al., 2019; Bricelj and Kuenstner, 1989; Colin and Dam, 2003; Haque et al., 2023). Therefore, the combined exposure to *P. lima* and heatwave may have resulted in a synergistic effect, with the toxic effects of the algae exacerbating the negative impact of heat stress on the oysters' metabolism.

Finally, it is important to note that no detectable difference in oxygen consumption was found between treatments in the delayed response measurement, suggesting that exposure of the oyster to heat stress, toxic algal bloom, and their combination do not have a long-term effect on metabolic activity. Whether this result is maintained with longer heatwaves or different toxins remains to be tested.

4.3. Condition Index is not an effective tool in assessing heatwave and/or P. lima effect on bivalve health

In this study, the treatments did not affect the CI immediately after exposure and after recovery. There are no published studies investigating the combined effects of a heatwave and toxic algal bloom on the CI of bivalves. Yet, some studies involve heat stress alone and toxic algae alone or combined with other stressors. These studies involved both short and long-duration of exposure to stressors. Among these studies, some report a decrease in CI after heat stress (Hiebenthal et al., 2012; Lemasson et al., 2018; Mackenzie et al., 2014; Shpigel et al., 1992; Taylor et al., 2017), and one study reports an increase (Payton et al., 2016). Most studies investigating the effects of toxic algae observed no impact on bivalve CI, similar to the findings presented here (Borcier et al., 2017; Haberkorn et al., 2011; Hégaret et al., 2009; Lassudrie et al., 2014, 2015). One study reports lower CI for *Ruditapes philipinarum* (Manila clams) that were exposed to an *Alexandrium minutum* strain that contains bioactive extracellular compounds with cytotoxic properties (*BEC*) only without PST (Castrec et al., 2018). Our results demonstrate that CI may not be an effective tool for monitoring the physiological effects of heat stress and DST-producing algae in *M. gigas*.

4.4. Total hemocyte count is modulated by P. lima and combined heatwave and P. lima exposure

Hemocyte functionality is important in investigating potential impact of environmental stressors. They indicate changes in physiological performance, which increase the vulnerability to diseases and decrease survival capacity (Munari et al., 2019). Hemocyte NR uptake assays indicate damage to membranes, including lysosomes. Lysosomes serve an important role in receiving and degrading macromolecules, be they secretory, endocytic, autophagic, or phagocytic membrane trafficking pathways (Zhao et al., 2011). They are responsible for detoxification and defense against pathogens in marine organisms (Zhao et al., 2011). This study found no difference in NR uptake, indicating that the stressors employed did not affect the lysosomal membrane stability of the hemocytes.

As the majority of proteins found in the hemolymph play a role in immune defense against microbes, fungi, protozoans, and viruses, hemolymph protein concentration (HPC) is considered a useful biomarker when evaluating changes in the immune and physiological functions of bivalves (Gianazza et al., 2021). In the present study, the stressors were found to have no effect on HPC for all treatment groups. However, an effect on THC was observed in the delayed response. Oysters exposed to *P. lima* bloom alone had lower THC in the delayed response compared to those exposed to heatwave alone and both stressors. An interaction of stressors was detected in the delayed effects, showing an antagonistic response. This finding suggests a modulation mechanism of circulating hemocyte numbers in the recovery period after exposure to the combined stressors. In bivalves, an increase in THC is commonly thought to result from the production or movement of cells from tissues to hemolymph (Matozzo et al., 2012). At the same time, a decrease is likely because of cell lysis or the movement of cells from hemolymph to tissues (Matozzo et al., 2012).

Mortality was observed in those oysters exposed to both stressors, but odds ratio analysis reveals that this was not statistically different to other treatment or control groups. As far as we are aware, the effects of DST algal bloom and high temperature on bivalve survival has not been investigated. Published studies which investigate PST algal blooms do not report mortalities. Additional studies with higher sample size should be conducted to confirm the effects of exposure to both stressors on survival.

5. Conclusion

This study describes the combined effects of a heatwave and DSP toxic algal bloom on the physiology and toxin accumulation of bivalves. The results suggest that exposure to a *P. lima* bloom alters the normal response of Pacific rock oysters to increased temperature during a simulated heatwave. Additionally, the study demonstrates the immunoregulation of *M. gigas* when exposed to a *P. lima* bloom, as well as combined heatwave and *P. lima*. Notably, *M. gigas* exposed to higher temperatures during the simulated heatwave accumulated more DSP-associated toxins OA and DTX1 than those not exposed to heatwave, leading to toxin levels higher than the regulatory limit in oysters exposed to both stressors. This trend could result in more shellfish production site closures and possibly higher DSP occurrence under climate-change scenarios even when HAB do not become more frequent or intense. Consistent and constant monitoring of toxic algal cells in the water and shellfish product prior to sale should be imposed, especially during warmer seasons, when they may accumulate more toxin within a shorter period. Moreover, techniques for efficient and continuous monitoring of toxic algal blooms and toxin concentrations in coastal seas should be developed.

This study highlights the vulnerability of bivalves to warming and HAB and the looming challenges bivalve aquaculture may face in the future, which could impact human health, especially in areas that rely on shellfish as a major protein source and livelihood. Further work is needed to address whether the combined effects of heat stress and *P. lima* lead to higher chances of mortality in *M. gigas*.

CRediT authorship contribution statement

Ellen Grace M. Funesto: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Adam M. Lewis:** Methodology, Formal analysis, Writing – review & editing. **Andrew D. Turner:** Methodology, Formal analysis, Writing – review & editing. **Tom**

C. Cameron: Conceptualization, Methodology, Writing – review & editing, Supervision. **Michael Steinke:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Data availability

Data will be made available on request.

Declaration of competing interest

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

Acknowledgement

This work was supported by a doctoral fellowship from the University of the Philippines awarded to Ellen Grace M. Funesto.

References

Anderson, D.M., Cembella, A.D., Hallegraeff, G.M., 2012. Progress in understanding harmful algal blooms (HABs): Paradigm shifts and new technologies for research, monitoring and management. Annu. Rev. Mar. Sci. 4, 143–176. https://doi.org/10.1146/annurev-marine-120308-081121.

Basti, L., Nagai, K., Segawa, S., Tanaka, Y., Suzuki, T., Nagai, S., 2019. Harmful algal blooms and shellfish aquaculture in changing environment—United States-Japan Natural Resources Panel on Aquaculture (NOAA-FRA) [Conference paper]. The 46th UJNR Scientific Symposium "Marine Aquaculture in a Changing Environment Hiroshima, Japan.

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67, 1–48. https://doi.org/10.18637/jss.v067.i01.

Bayne, B.L., Widdows, J., 1978. The physiological ecology of two populations *of Mytilus edulis* L. Oecologia 37, 137–162. https://doi.org/10.1007/BF00344987.

Berdalet, E., Fleming, L.E., Gowen, R., Davidson, K., Hess, P., Backer, L.C., Moore, S.K., Hoagland, P., Enevoldsen, H., 2015. Marine harmful algal blooms, human health and wellbeing: challenges and opportunities in the 21st century. J. Mar. Biol. Assoc. 96, 61–91. https://doi.org/10.1017/S0025315415001733.

Borcier, E., Morvezen, R., Boudry, P., Miner, P., Charrier, G., Laroche, J., Hegaret, H., 2017. Effects of bioactive extracellular compounds and paralytic shellfish toxins produced by *Alexandrium minutum* on growth and behaviour of juvenile great scallops *Pecten maximus*. Aquat. Toxicol. 184, 142–154. https://doi.org/10.1016/j.aquatox.2017.01.009.

Brandenburg, K.M., Velthuis, M., Van de Waal, D.B., 2019. Meta-analysis reveals enhanced growth of marine harmful algae from temperate regions with warming and elevated CO_2 levels. Glob. Chang. Biol. 25, 2607–2618. https://doi.org/10.1111/gcb.14678.

Bricelj, V.M., Kuenstner, S.H., 1989. Effects of the "brown tide" on the feeding physiology and growth of bay scallops and mussels. In: Cosper, E.M., Bricelj, V.M., Carpenter, E.J. (Eds.), Novel Phytoplankton Blooms, Coastal and Estuarine Studies. Springer, Berlin, Heidelberg, pp. 491–509 https://doi.org/10.1007/978-3-642-75280-3_28.

Castrec, J., Soudant, P., Payton, L., Tran, D., Miner, P., Lambert, C., Le Goïc, N., Huvet, A., Quillien, V., Boullot, F., Amzil, Z., Hégaret, H., Fabioux, C., 2018. Bioactive extracellular compounds produced by the dinoflagellate *Alexandrium minutum* are highly detrimental for oysters. Aquat. Toxicol. 199, 188–198. https://doi.org/10.1016/j.aquatox.2018.03.034.

Coates, L., Stubbs, B., Turner, A.D., Williams, O., Milligan, S., Algoet, M., 2014. Annual report on the results of the Biotoxin and Phytoplankton Official Control Monitoring Programmes for England & Wales – 2014. Centre for Environment, Fisheries and Aquaculture Science, UK.

Coates, L., Wilkinson, T., Stubbs, B., Turner, A.D., Milligan, S., Algoet, M., 2015. Annual Report on the Results of the Biotoxin and Phytoplankton Official Control Monitoring Programmes for England and Wales – 2015. Centre for Environment, Fisheries and Aquaculture Science, UK.

Colin, S.P., Dam, H.G., 2003. Effects of the toxic dinoflagellate *Alexandrium fundyense* on the copepod *Acartia hudsonica*: a test of the mechanisms that reduce ingestion rates. Mar. Ecol. Prog. Ser. 248, 55–65. https://doi.org/10.3354/meps248055.

Colletta, A., Westfall, S., 2021. "Crushing heat wave in Pacific Northwest and Canada cooked shellfish alive by the millions". Seattle Times. https://www.seattletimes.com/seattle-news/environment/crushing-heat-wave-in-pacific-northwest-and-canada-cooked-shellfish-alive-by-the-millions/. (Accessed 14 June 2022).

Cronin, M.F., Gentemann, C.L., Edson, J., Ueki, I., Bourassa, M., Brown, S., Clayson, C.A., Fairall, C.W., Farrar, J.T., Gille, S.T., Gulev, S., Josey, S.A., Kato, S., Katsumata, M., Kent, E., Krug, M., Minnett, P.J., Parfitt, R., Pinker, R.T., Stackhouse, P.W., Swart, S., Tomita, H., Vandemark, D., Weller, A.R., Yoneyama, K., Yu, L., Zhang, D., 2019. Air-sea fluxes with a focus on heat and momentum. Front. Mar. Sci. 6.

Dhanji-Rapkova, M., O'Neill, A., Maskrey, B.H., Coates, L., Teixeira Alves, M., Kelly, R.J., Hatfield, R.G., Rowland-Pilgrim, S.J., Lewis, A.M., Algoet, M., Turner, A.D., 2018. Variability and profiles of lipophilic toxins in bivalves from Great Britain during five and a half years of monitoring: Okadaic acid, dinophysistoxins and pectenotoxins. Harmful Algae 77, 66–80. https://doi.org/10.1016/j.hal.2018.05.011.

Dhanji-Rapkova, M., O'Neill, A., Maskrey, B.H., Coates, L., Swan, S.C., Teixeira Alves, M., Kelly, R.J., Hatfield, R.G., Rowland-Pilgrim, S.J., Lewis, A.M., Turner, A.D., 2019. Variability and profiles of lipophilic toxins in bivalves from Great Britain during five and a half years of monitoring: azaspiracids and yessotoxins. Harmful Algae 87, 101629. https://doi.org/10.1016/j.hal.2019.101629.

Edullantes, E., Low-Decarie, E., Steinke, M., Cameron, T., 2023. Comparison of thermal traits between non-toxic and potentially toxic marine phytoplankton: Implications to their responses to ocean warming. J. Exp. Mar. Biol. 562, 104294. https://doi.org/10.1016/j.jembe.2023.151883.

EURL, 2015. EU reference laboratory for monitoring marine biotoxins. https://www.aesan.gob.es/en/CRLMB/web/public_documents/seccion/crlmb_standard_operating_procedures.htm. (Accessed 30 November 2022).

Fernandes-Salvador, J.A., Davidson, K., Sourisseau, M., Revilla, M., Schmidt, W., Clarke, D., Miller, P.I., Arce, P., Fernández, R., Maman, L., Silva, A., Whyte, C., Mateo, M., Neira, P., Mateus, M., Ruiz-Villarreal, M., Ferrer, L., Silke, J., 2021. Current status of forecasting toxic harmful algae for the north-east atlantic shellfish aquaculture industry. Front. Mar. Sci. 8, 666583. https://doi.org/10.3389/fmars.2021.666583.

Foden, J., Purdie, D.A., Morris, S., Nascimento, S., 2005. Epiphytic abundance and toxicity of *Prorocentrum lima* populations in the Fleet Lagoon, UK. Harmful Algae 4, 1063–1074. https://doi.org/10.1016/j.hal.2005.0s3.004.

Funesto, E.G., 2023. Impacts of Heatwaves and Toxic Algal Blooms on the Physiological Performance and Future Aquaculture of the Oysters *Ostrea edulis* and *Magallana* (*Crassostrea*) *gigas*. (Doctoral Dissertation).University of Essex.

Gianazza, E., Eberini, I., Palazzolo, L., Miller, I., 2021. Hemolymph proteins: an overview across marine arthropods and molluscs. J. Proteome 245, 104294. https://doi.org/10.1016/j.jprot.2021.104294.

Gobler, C.J., Doherty, O.M., Hattenrath-Lehmann, T.K., Griffith, A.W., Kang, Y., Litaker, R.W., 2017. Ocean warming since 1982 has expanded the niche of toxic algal blooms in the North Atlantic and North Pacific oceans. Proc. Natl. Acad. Sci. 114, 4975–4980. https://doi.org/10.1073/pnas.1619575114.

Griffith, A.W., Gobler, C.J., 2017. Transgenerational exposure of North Atlantic bivalves to ocean acidification renders offspring more vulnerable to low pH and additional stressors. Sci. Rep. 7, 11394. https://doi.org/10.1038/s41598-017-11442-3.

Haberkorn, H., Tran, D., Massabuau, J.-C., Ciret, P., Savar, V., Soudant, P., 2011. Relationship between valve activity, microalgae concentration in the water and toxin accumulation in the digestive gland of the Pacific oyster *Crassostrea gigas* exposed to *Alexandrium minutum*. Mar. Pollut. Bull. 62, 1191–1197. https://doi.org/10.1016/j.marpolbul.2011.03.034.

Hagenbuch, B., 2021. Pacific Northwest heat wave causes vibrio bacteria outbreak in oysters. Seafood Source. https://www.seafoodsource.com/news/food-safety-health/pacific-northwest-heatwave-causes-vibrio-bacteria-outbreak-in-oysters. (Accessed 14 June 2022).

Hallegraeff, G.M., 2010. Ocean climate change, phytoplankton community responses, and harmful algal blooms: a formidable predictive challenge. J. Phycol. 46, 220–235. https://doi.org/10.1111/j.1529-8817.2010.00815.x.

Hallegraeff, G., Andersson, D., Cembella, A., 2003. Manual on Harmful Marine Microalgae. 2nd ed. UNESCO.

Haque, Md.N., Nam, S.-E., Lee, M., Kim, H.-W., Gil, H.-W., Park, H.S., Rhee, J.-S., 2023. Chronic exposure to environmental concentrations of harmful algal bloom-forming dinoflagellates induces oxidative stress and reduces immune and hepatic functions in red seabream. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 266, 109573. https://doi.org/10.1016/j.cbpc.2023.109573.

Harrison, K., Wilkinson, T., Coates, L., Turner, A.D., Milligan, S., Algoet, M., 2016. Annual Report on the Results of the Biotoxin and Phytoplankton Official Control Monitoring Programmes for England & Wales – 2016. Centre for Environment, Fisheries and Aquaculture Science, UK.

Harrison, K., Walton, A., Coates, L., Turner, A., Algoet, M., 2017. Annual Report on the Results of the Biotoxin and Phytoplankton Official Control Monitoring Programmes for England & Wales – 2017. Centre for Environment, Fisheries and Aquaculture Science, UK.

He, Z.-B., Duan, G.-F., Liang, C.-Y., Li, H.-Y., Liu, J.-S., Yang, W.-D., 2019. Up-regulation of Nrf2-dependent antioxidant defenses in *Perna viridis* after exposed to *Prorocentrum lima*. Fish Shellfish Immunol. 90, 173–179. https://doi.org/10.1016/j.fsi.2019.05.003.

Hégaret, H., da Silva, Sunila, I., Shumway, S.E., Dixon, M.S., Alix, J., Wikfors, G.H., Soudant, P., 2009. Perkinsosis in the Manila clam *Ruditapes philippinarum* affects responses to the harmful-alga, *Prorocentrum minimum*. J. Exp. Mar. Biol. 371, 112–120. https://doi.org/10.1016/j.jembe.2009.01.016.

Heil, C.A., Glibert, P.M., Fan, C., 2005. *Prorocentrum minimum* (Pavillard) Schiller: a review of a harmful algal bloom species of growing worldwide importance. Harmful Algae 4, 449–470. https://doi.org/10.1016/j.hal.2004.08.003.

Herring, S.C., Hoell, A., Hoerling, M., Christidis, N., Stott, P.A., 2019. Introduction to explaining extreme events of 2017 from a climate perspective. Bull. Am. Meteor. Soc. 100, S1–S4. https://doi.org/10.1175/BAMS-D-18-0307.1.

Hiebenthal, C., Philipp, E.E.R., Eisenhauer, A., Wahl, M., 2012. Interactive effects of temperature and salinity on shell formation and general condition in Baltic Sea *Mytilus edulis* and *Arctica islandica*. Aquat. Biol. 14, 289–298. https://doi.org/10.3354/ab00405.

Hu, Z., Liu, Y., Deng, Y., Tang, Y.Z., 2022. The notorious harmful algal blooms-forming dinoflagellate *Prorocentrum donghaiense* produces sexual resting cysts, which widely distribute along the coastal marine sediment of China. Front. Mar. Sci. 9, 826736. https://doi.org/10.3389/fmars.2022.826736.

Keller, M.D., Selvin, R.C., Claus, W., Guillard, R.R.L., 1987. Media for the culture of oceanic ultraphytoplankton. J. Phycol. 23 (4), 633–638. https://doi.org/10.1111/j.1529-8817.1987.tb04217.x.

Lassudrie, M., Soudant, P., Richard, G., Henry, N., Medhioub, W., da Silva, P.M., Donval, A., Bunel, M., Le Goïc, N., Lambert, C., de Montaudouin, X., Fabioux, C., Hégaret, H., 2014. Physiological responses of Manila clams *Venerupis* (=*Ruditapes*) *philippinarum* with varying parasite *Perkinsus olseni* burden to toxic algal *Alexandrium ostenfeldii* exposure. Aquat. Toxicol. 154, 27–38. https://doi.org/10.1016/j.aquatox.2014.05.002.

Lassudrie, M., Wikfors, G.H., Sunila, I., Alix, J.H., Dixon, M.S., Combot, D., Soudant, P., Fabioux, C., Hégaret, H., 2015. Physiological and pathological changes in the eastern oyster *Crassostrea virginica* infested with the trematode *Bucephalus* sp. and exposed to the toxic dinoflagellate *Alexandrium fundyense*. J. Invertebr. Pathol. 126, 51–63. https://doi.org/10.1016/j.jip.2015.01.011.

Lee, R., Lovatelli, A., Ababouch, L., 2008. Bivalve Depuration: Fundamental and Practical Aspects (Technical Paper). FAO Fisheries, Rome.

Leftley, J.W., Hannah, F., 2009. Evidence, A Literature Review of the Potential Health Effects of Marine Microalgae and Macroalgae (Government Report). UK Environment Agency.

Lemasson, A.J., Hall-Spencer, J.M., Fletcher, S., Provstgaard-Morys, S., Knights, A.M., 2018. Indications of future performance of native and non-native adult oysters under acidification and warming. Mar. Environ. Res. 142, 178–189. https://doi.org/10.1016/j.marenvres.2018.10.003.

Lenth, R.V., 2021. emmeans: estimated marginal means, aka least-squares means. R package version 1.7.0. https://CRAN.R-project.org/package=emmeans.

Li, M., Zhang, F., Glibert, P.M., 2021. Seasonal life strategy of *Prorocentrum minimum* in Chesapeake Bay, USA: validation of the role of physical transport using a coupled physical–biogeochemical–harmful algal bloom model. Limnol. Oceanogr. 66, 3873–3886. https://doi.org/10.1002/lno.11925.

Lloyd, J.K., Duchin, J.S., Borchert, J., Quintana, H.F., Robertson, A., 2013. Diarrhetic shellfish poisoning, Washington, USA, 2011. Emerg. Infect. Dis. 19, 1314–1316. https://doi.org/10.3201/eid1908.121824.

Lv, J., Yuan, K., Lu, M., He, Z., Li, H., Yang, W., 2021. Responses of JNK signaling pathway to the toxic dinoflagellate *Prorocentrum lima* in the mussel *Perna viridis*. Ecotoxicol. Environ. Saf. 227, 112905. https://doi.org/10.1016/j.ecoenv.2021.112905.

Mackenzie, C.L., Ormondroyd, G.A., Curling, S.F., Ball, R.J., Whiteley, N.M., Malham, S.K., 2014. Ocean warming, more than acidification, reduces shell strength in a commercial shellfish species during food limitation. Plos One 9, e86764. https://doi.org/10.1371/journal.pone.0086764.

Matozzo, V., Chinellato, A., Munari, M., Finos, L., Bressan, M., Marin, M.G., 2012. First evidence of immunomodulation in bivalves under seawater acidification and increased temperature. PLOS ONE 7 (3), e33820. https://doi.org/10.1371/journal.pone.0033820.

Meehl, G.A., Tebaldi, C., 2004. More intense, more frequent, and longer lasting heat waves in the 21st century. Science 305, 994–997. https://doi.org/10.1126/science.1098704.

Moore, S.K., Trainer, V.L., Mantua, N.J., Parker, M.S., Laws, E.A., Backer, L.C., Fleming, L.E., 2008. Impacts of climate variability and future climate change on harmful algal blooms and human health. Environ. Health 7, S4. https://doi.org/10.1186/1476-069X-7-S2-S4.

Munari, M., Matozzo, V., Chemello, G., Riedl, V., Pastore, P., Badocco, D., Marin, M.G., 2019. Seawater acidification and emerging contaminants: a dangerous marriage for haemocytes of marine bivalves. Environ. Res. 175, 11–21. https://doi.org/10.1016/j.envres.2019.04.032.

Nishimura, T., Uchida, H., Noguchi, R., Oikawa, H., Suzuki, T., Funaki, H., Ihara, C., Hagino, K., Arimitsu, S., Tanii, Y., Abe, S., Hashimoto, K., Mimura, K., Tanaka, K., Yanagida, I., Adachi, M., 2020. Abundance of the benthic dinoflagellate *Prorocentrum* and the diversity, distribution, and diarrhetic shellfish toxin production of *Prorocentrum lima* complex and *P. caipirignum* in Japan. Harmful Algae 96, 101687. https://doi.org/10.1016/j.hal.2019.101687.

Parks, R., Walton, A., Coates, L., Maskrey, B., 2018. Annual Report on the Results of the Biotoxin and Phytoplankton Official Control Monitoring Programmes for England & Wales – 2018. Centre for Environment, Fisheries and Aquaculture Science, UK.

Parks, R., Bear, E., Coates, L., Maskrey, B., 2019. Annual Report on the Results of the Biotoxin and Phytoplankton Official Control Monitoring Programmes for England & Wales – 2019. Centre for Environment, Fisheries and Aquaculture Science, UK.

Payton, S.L., Johnson, P.D., Jenny, M.J., 2016. Comparative physiological, biochemical and molecular thermal stress response profiles for two unionid freshwater mussel species. J. Exp. Biol. 219, 3562–3574. https://doi.org/10.1242/jeb.140129.

R Core Team, 2021. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rubalcaba, J.G., Verberk, W.C.E.P., Hendriks, A.J., Saris, B., Woods, H.A., 2020. Oxygen limitation may affect the temperature and size dependence of metabolism in aquatic ectotherms. Proc. Natl. Acad. Sci. 117, 31963–31968. https://doi.org/10.1073/pnas.2003292117.

Sawusdee, A., 2015. Restoration of the European Flat Oyster *Ostrea edulis* Using Elevated Broodstock Reefs. (Doctoral Dissertation).University of Southampton.

Schiermeier, Q., 2018. Droughts, heatwaves and floods: How to tell when climate change is to blame. Nature 560, 20–23.

Shpigel, M., Barber, B.J., Mann, R., 1992. Effects of elevated temperature on growth, gametogenesis, physiology, and biochemical composition in diploid and triploid Pacific oysters, *Crassostrea gigas* Thunberg. J. Exp. Mar. Biol. Ecol. 161, 15–25. https://doi.org/10.1016/0022-0981(92)90186-E.

Szumilas, M., 2010. Explaining odds ratios. J. Can. Acad. Child Adolesc. Psychiatry 19, 227–229.

Taş, S., Okuş, E., 2011. A review on the bloom dynamics of a harmful dinoflagellate *Prorocentrum minimum* in the Golden Horn Estuary. Turk. J. Fish. Aquat. Sci. 11, 673–681.

Taylor, A.M., Maher, W.A., Ubrihien, R.P., 2017. Mortality, condition index and cellular responses of *Anadara trapezia* to combined salinity and temperature stress. J. Exp. Mar. Biol. Ecol. 497, 172–179. https://doi.org/10.1016/j.jembe.2017.09.023.

Timms, P., 2019. "Wiped out overnight": the marine heatwaves threatening our oyster industry. ABC News. https://www.abc.net.au/news/2019-11-25/marine-heatwaves-threaten-oyster-industry-great-barrier-reef/11726630?utm_campaign=abc_news_web&utm_content=link&utm_medium=content_shared&utm_source=abc_news_web. (Accessed 14 June 2022).

Troost, K., 2018. Extreme Cockle Mortality on Tidal Flats of Dutch Coastal Waters. Wageningen University & Research. https://www.wur.nl/en/newsarticle/Extreme-cockle-mortality-on-tidal-flats-of-Dutch-coastal-waters.htm. (Accessed 14 June 2022).

Türkoğlu, M., 2010. Temporal variations of surface phytoplankton, nutrients and chlorophyll a in the Dardanelles (Turkish Straits System): a coastal station sample in weekly time intervals. Turk. J. Biol. 34, 319–333. https://doi.org/10.3906/biy-0810-17.

Türkoğlu, M., 2016. First harmful algal bloom record of tycoplanktonic dinoflagellate *Prorocentrum lima* (Ehrenberg) F. Stein, 1878 in the Dardanelles (Turkish Straits System, Turkey). J. Coast. Life Med. 4, 765–774.

Türkoğlu, M., Erdoğan, Y., 2010. Diurnal variations of summer phytoplankton and interactions with some physicochemical characteristics under eutrophication of surface water in the Dardanelles (Çanakkale Strait, Turkey). Turk. J. Biol. 34, 211–225. https://doi.org/10.3906/biy-0807-7.

Turner, L.M., Havenhand, J.N., Alsterberg, C., Turner, A.D., Girisha, S.K., Rai, A., Venugopal, M.N., Karunasagar, I., Godhe, A., 2019. Toxic algae silence physiological responses to multiple climate drivers in a tropical marine food chain. Front. Physiol. 10, 373. https://doi.org/10.3389/fphys.2019.00373.

Turner, A.D., Lewis, A.M., Bradley, K., Maskrey, B.H., 2021. Marine invertebrate interactions with harmful algal blooms - implications for one health. J. Invertebr. Pathol. 186, 107555. https://doi.org/10.1016/j.jip.2021.107555.

Wang, J., Wu, J., 2009. Occurrence and potential risks of harmful algal blooms in the East China Sea. Sci. Total Environ., Thematic Papers: Selected Papers From the 2007 Wetland Pollutant Dynamics and Control Symposium 407, pp. 4012–4021 https://doi.org/10.1016/j.scitotenv.2009.02.040.

Wells, M.L., Trainer, V.L., Smayda, T.J., Karlson, B.S.O., Trick, C.G., Kudela, R.M., Ishikawa, A., Bernard, S., Wulff, A., Anderson, D.M., Cochlan, W.P., 2015. Harmful algal blooms and climate change: learning from the past and present to forecast the future. Harmful Algae 49, 68–93. https://doi.org/10.1016/j.hal.2015.07.009.

Whyte, C., Swan, S., Davidson, K., 2014. Changing wind patterns linked to unusually high *Dinophysis* blooms around the Shetland Islands, Scotland. Harmful Algae 39, 365–373. https://doi.org/10.1016/j.hal.2014.09.00.

Zhao, C., Li, X., Lou, S., Chang, Y., 2011. Assessments of lysosomal membrane responses to stresses with neutral red retention assay and its potential application in the improvement of bivalve aquaculture. Afr. J. Biotechnol. 10 (64), 13968–13973.