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Effects of microfiber and light pollution on coastal ecosystem services provided by the blue mussel, *Mytilus edulis*

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Abstract

Filter-feeding bivalves, such as the blue mussel, *Mytilus edulis*, provide important ecosystem services within coastal ecosystems. Amongst them is the control of phytoplankton abundance and species composition while also contributing to nutrient cycling, which can diminish the impacts of coastal eutrophication and Harmful Algal Blooms (HAB), two major stressors of coastal environments. However, the healthy functioning of mussels and the provisioning of related ecosystem services can in turn be compromised by coastal anthropogenic pressures. This thesis focuses on the effects of two prevailing forms of coastal pollution: microfibers and artificial light at night (ALAN) on coastal ecosystem services provided by mussels.

Microplastics (<5mm) are found at coastal ecosystems around the world and their impact on marine organisms has been investigated by multiple studies therefore an initial review of literature on the effects of microplastic pollution on coastal bivalves was conducted. The review aimed to assess whether the experimental settings employed in laboratory studies are relevant to field observations on microplastics characteristics and concentrations. This investigation revealed that previous studies have used a wide range of shapes, materials, sizes, and concentrations of microplastics; and in many cases, these did not coincide with the characteristics of microplastics found in coastal waters. For instance, the concentrations of microplastics used were frequently orders of magnitude higher than environmental levels. Moreover, 48.5% of studies exposed bivalves to spherical microplastics, whereas in the field, fibres were the prevailing shape. Despite the prevalence of microfibers in the coastal marine environment, the review revealed that research on the effects of microfibers on bivalves is scarce.

To fill this gap in literature and enhance our understanding on the impacts of microfibers on the phytoplankton consumption by bivalves, a short-term experiment was performed simulating acute microfiber concentrations and microalgae bloom conditions. The results showed that microfiber exposure did not cause any immediate effect on the phytoplankton clearance capacity of mussels. However, a 10.5% decrease was observed in the mussel clearance capacity of microfiber-exposed mussels after five days of microfiber-free conditions,

suggesting that even short-term exposure to microfibers can result in long-term interference of the removal of phytoplankton from the water column.

Subsequently, it was imperative to investigate the effects of chronic exposure to microfibers. Hence, a long-term (52days) laboratory experiment followed, where mussels exposed to microfibers (<100 μm) showed a significantly less phytoplankton clearance capacity (-21%) in comparison to mussels in the microfiber free treatment, after 39 days of exposure. This could be attributed to the accumulated microfibers in the digestive gland of the experimental mussels, although the exact mechanism remains to be clarified by further research. Furthermore, at the end of the experiment it was evident that the mussels with the highest filtration accumulated the most microfibers, suggesting that prolonged exposure to microfibers could negatively affect the phytoplankton removal capacity of the mussels with the highest clearance capacity. This can consequently result in a decrease in the ecosystem services provided by mussel populations.

Artificial light at night (ALAN) is another prevalent form of pollution which affects more than 22% of the world's coastlines. This anthropogenic stressor can have negative impacts scaling up from individual to community level as most organisms have adapted their biology to the natural daily, tidal, and seasonal light cycles. Currently, research and conservation efforts in terrestrial ecosystems are focusing on identifying ALAN wavelengths that cause the least disturbance; but similar research on coastal ecosystems and specifically bivalves is still scarce. To test the effect of different ALAN wavelengths on mussels, a controlled laboratory experiment was performed exposing different mussels to green, red, and white LED ALAN wavelengths and a control dark treatment. The results reveal that both activity and clearance capacity, as well as the relationship between them, depended on the wavelength of ALAN. Specifically, mussels exposed to green ALAN had the greatest open/close frequency and lower phytoplankton consumption in comparison to mussels under the red light, however there was no significant difference to the control treatment. The phytoplankton clearance capacity of mussels was also dependent on the season the experimental organisms were collected in, which coincides with the reproductive cycle of the mussels. This suggests a seasonal variation in the phytoplankton removed from the water column and therefore the ecosystem services provided by mussels.

These studies help us understand the severity of the threat which microfibers and light pollution pose on marine bivalves and the ecosystem services they provide. A major step to alleviate the effects of microfibers is the development of appropriate filtration systems within waste treatments plants, capable of retaining microfibers and preventing their introduction in coastal waters. It is also essential to implement appropriate legislation regarding the production and disposal of plastic materials. Furthermore, these results can act as a basis for future experiment on the effect of ALAN wavelengths on bivalves as they suggest that green ALAN may cause more disturbance to mussel population that red ALAN. These effects should also be considered in a broader ecological perspective, even though red ALAN did not impose a negative effect on mussels, when phytoplankton was exposed to red ALAN there was an increase in its abundance and change in community composition which could also lead to harmful algae events.

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Impact of Covid-19

The Covid-19 outbreak affected everyone's life in a different manner. Fortunately, it did not cause any health-related issues to either me or my loved ones. The uncertainty of the situation surely caused some delay in my desk-based work due to stress related reduced productivity. However, the major impact of the lockdown was on the experiment investigating the effects of artificial light at night on the ecosystem services provided by mussels, which had to be paused. This caused a delay in the data collection as the live organisms (mussels and microalgae) involved in the experiment could not be maintained during the lockdown. As a compensation for this delay, I received a three-month extension to my thesis project deadline by the Covid-19 Disruption mitigation plan offered by the University of Glasgow. Although this interruption meant that I had to re-establish all the microalgal cultures and re-set all the setup for performing the remaining replicates of the experiment, this extension was very helpful as it enabled me to carry out and finish this experiment.

Author's Declaration

I, Eleni Christoforou, declare that all the work presented in this thesis is my own work, written in my own words and all the sources used are fully acknowledged and cited. Any collaborations are clearly stated.

Definitions/Abbreviations

Artificial Light at Night (ALAN) – Artificial illumination that facilitates human actives during naturally dark hours. It has been recognised as a major anthropogenic pollutant impacting living organisms hence the term is also known as light pollution.

Biofiltrations – The mechanism of filtering pollutants from the water column using living organisms. In the context of this thesis, biofiltration refers to the filtration mechanism of filter-feeding bivalves used in the removal of excess phytoplankton and nutrients from the water column.

Circadian rhythms – Natural 24-hour cycles that regulate most biological processes of living organisms, mainly determined by light.

Circatidal rhythms – Natural cycles determined by the tidal activity with a period of 12.4 hours.

Diel – Referring to a 24-hour period where semi-diel refers to a 12-hour period.

Ecosystem services – The goods and services provided by ecosystems, and which facilitate human living.

Eutrophication – The enrichment of nutrients usually causing an increase in primary production, frequently leading to (harmful) algal blooms.

Harmful Algal Bloom (HAB) – The excessive growth of toxic (micro) algae which can cause harm to organisms in the community and/or organism in the trophic chain due to their consumption, anoxia, and light-deprivation.

Light Emitting Diodes (LED) – A technology of lighting which has seen increasing use due to its affordability, energy efficiency and capacity to control the intensity and isolate different wavelengths.

Microfibers –Usually used in the textile industry. They can have many different chemical compositions as some can be organic (e.g., cotton, wool), or made from plastic. The latter are categorised as a shape of microplastics (see below).

Microplastics – Plastic particles with diameter smaller than 5mm which could be of different shape (e.g., fibres, fragments, beads) and type (e.g., nylon, polyethene, polystyrene). Microplastics can be either primary (i.e., made with the intention to be used in that size) or secondary (i.e., formed by the degradation of larger plastics).

Phytoplankton clearance capacity/Phytoplankton consumption – Used interchangeably to refer to the phytoplankton removed from the water column by the biofiltration of bivalves.

Wavelength – A measure of light in the visible light spectrum ranging from about 380-760nm. Different wavelengths are visible to the human eye as different colours (see image below)

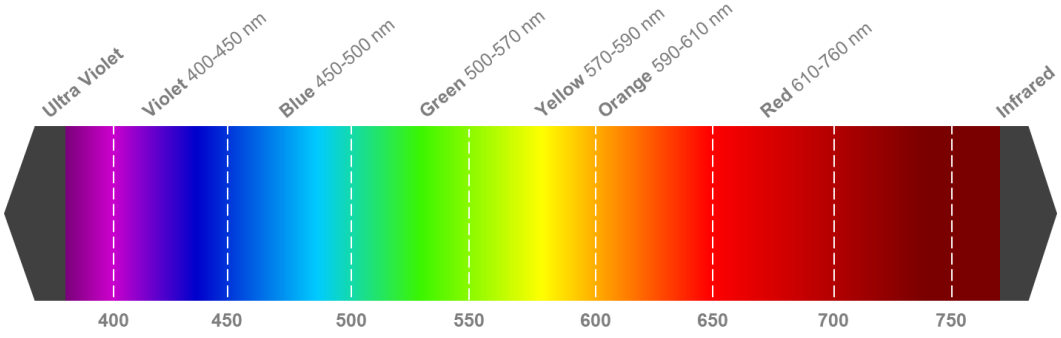

Image credit: analytik.co.uk

General Introduction

Oceans cover about 70% of our planet and have an invaluable role in oxygen production and carbon sequestration (Zehr and Kudela, 2009; Huang *et al.*, 2018) but, marine environments are facing a plethora of pressures that could affect their healthy functioning. Some of these stressors include but are not limited to: changes in temperature, oxygen, salinity, UV-radiation and pH, over-exploitation, eutrophication, invasive species, harmful algal blooms (HAB), plastics, chemical and light pollution, sea-level rise and extreme weather event (Harley *et al.*, 2006; Gissi *et al.*, 2021). Amongst marine environments, the most vulnerable to these unfavourable conditions are coastal ecosystems due to their proximity to anthropogenic activities and riverine inflow which are usually the main sources of pollutants (Adams, 2005).

Coastal ecosystems and the ecosystem services and functions they provide have been crucial for the development and settlement of human populations over the centuries. Coastal areas provide favourable living conditions due to their proximity to food resources, employment opportunities in the fishery, mariculture and maritime industries and recreational activities (Martínez *et al.*, 2007; Liquete *et al.*, 2013). These benefits have led to about one third of the global human population inhabiting coastal areas (Small and Nicholls, 2003) and growing urbanisation has resulted in 54 coastal megacities (population > 1 million) (Kullenberg, 2001). The anthropogenic impacts on coastal ecosystems are vast, including alterations to the shoreline, mortality events, habitat degradation, changes to organisms' behaviour and physiology, introduction of invasive species and diseases and changes to species composition which could result to decrease in the provision of valuable coastal ecosystem services (Martínez *et al.*, 2007; Gissi *et al.*, 2021). Understanding the stressors and investigating their impacts is vital to inform evidence-based environmental policy aimed at the conservation of coastal ecosystems (Prather *et al.*, 2013; Rochman, 2016) and their healthy functioning for more sustainable living. The urgency to act towards more environmentally friendly practices had also been recognised by the United Nations and more than 120 world leaders who are currently gathered here, in Glasgow, at

the climate change conference (COP26) to address some of the most pressing problems caused by climate change.

One of the major stressors faced by coastal ecosystems is eutrophication (Smith and Schindler, 2009; Kellogg *et al.*, 2014; van der Schatte Olivier *et al.*, 2018) which is the over-enrichment of the water column by nutrients like phosphate and nitrate in the water column leading to an increase in primary production (Le Goffe, 1995; Le Moal *et al.*, 2019). These nutrients usually originate from agricultural sources (Dupas *et al.*, 2015; Le Moal *et al.*, 2019), finding their way to marine systems through groundwater as well as sewage inflow (Burton and Armitage, 2005; Conde *et al.*, 2020). The frequency and magnitude of such inflows can become more pronounced by changes associated with climate change and acute weather conditions. For example, high precipitation in inland areas may increase the nutrient inflow to coastal ecosystems (De Carlo *et al.*, 2007; Hoover and MacKenzie, 2009; X. Li *et al.*, 2015), while storms can re-suspend nutrients from the sediment back into the water column, making them available to microalgae and thus promoting their growth (Rabalais *et al.*, 2009; Chen *et al.*, 2018). Furthermore, seasonal fluctuations in water temperature and nutrient cycling create favourable conditions for opportunistic microalgae species, forming what are known as summer or spring blooms (Spatharis *et al.*, 2007). As a result, algal blooms, which are many times formed by toxic microalgal species, can have detrimental effect on the coastal ecosystem due to direct effects on the health of primary consumers (Galimany *et al.*, 2008), oxygen depletion, light deprivation (Rabalais *et al.*, 2009; Le Moal *et al.*, 2019), and overall decrease in water quality (Rabalais *et al.*, 2009), causing changes to the structure and functioning of the local communities (Le Moal *et al.*, 2019).

Some of these impacts caused by eutrophication and algal blooms can be dampened by the ecosystem services provided by coastal bivalves (Kellogg *et al.*, 2014; van der Schatte Olivier *et al.*, 2018). These organisms feed on phytoplankton and through biofiltration, they remove excess microalgal biomass from the water column and control the microalgae species composition (Prins *et al.*, 1998; Tantanasarit *et al.*, 2013; van der Schatte Olivier *et al.*, 2018). Furthermore, they facilitate the nutrient cycle and settlement by biodeposition (Prather *et al.*, 2013;

Kellogg *et al.*, 2014; Kent *et al.*, 2017); hence reducing the nutrients available to primary producers. In a comparison between bivalve groups including scallops, oysters, clams and mussels, the latter have the highest capacity to remove nitrogen and phosphorus from the water column, per tonne of shellfish produced in aquaculture (van der Schatte Olivier *et al.*, 2018). Furthermore, mussels of the genus *Mytilus* are globally distributed (MacDonald and Ward, 2009), with high distribution of the blue mussel *Mytilus edulis* in the North Atlantic (Sukhotin *et al.*, 2007). Mussels are often found in polluted waters (Beyer *et al.*, 2017; J. Li *et al.*, 2019), for instance near sewage discharge areas (Conde *et al.*, 2020) where the problem of eutrophication and algal blooms is most severe.

The effectiveness of mussels in the removal of phytoplankton from the water column can vary based on the concentration and quality of suspended particles (Møhlenberg and Riisgård, 1978; Beecham, 2008). Previous studied have reported that the optimum filtration rate of mussels is at particle concentrations between $2x10^6$cells/L and $6x10^6$cells/L (Riisgård, 1991; Riisgård *et al.*, 2011). Concentrations above that threshold, which can occur during severe algal blooms (Anderson *et al.*, 2002; Spatharis *et al.*, 2007), would decrease the filtration rate of mussels, promote faeces and pseudofaeces production (Riisgård *et al.*, 2011), and therefore affect the energy expenditure of individuals (Navarro and Winter, 1982). However, algal blooms might not be the only coastal anthropogenic stressor that could interfere with the phytoplankton removal capacity of mussels. This work focuses on the potential impacts of two prevailing forms of marine pollution that have recently gained widespread recognition: namely microplastic pollution and artificial light at night (ALAN) (Gaston *et al.*, 2013; Davies and Smyth, 2018; Zapata *et al.*, 2018; Davies *et al.*, 2020).

Marine microplastic pollution is a topic of great concern, both in the scientific community (Ivar Do Sul and Costa, 2014; Andrady, 2017; Xu *et al.*, 2020; Alimi *et al.*, 2021) and in terms of policy making (Gago *et al.*, 2016; Creecy *et al.*, 2020). This is mainly due to the accumulating evidence revealing the thread microplastics are imposing on terrestrial and marine organisms (Haegerbaeumer *et al.*, 2019; Al-Thawadi, 2020); especially when considering the continuously rising production and distribution of plastic and microplastic worldwide (plastic particles with

diameter <5mm) (Kosior and Mitchell, 2020). Also, UV radiation escalates the degradation of large plastics into microplastics (Moore, 2008), inevitably increasing their numbers.

These particles find their way to coastal marine ecosystems through the sewage system inflow (Magnusson and Norén, 2014; Akarsu *et al.*, 2020), wind transport (Li *et al.*, 2018), coastal landfill (Kazour *et al.*, 2019), river inflow (Lebreton *et al.*, 2017; Wang *et al.*, 2019) and coastal activities (Dowarah and Devipriya, 2019). Coastal organisms are especially vulnerable to long-term exposure to microplastics, particularly in semi-enclosed seas where water re-circulation can be limited (Li *et al.*, 2018; Gomiero *et al.*, 2019). However, acute weather conditions can accelerate the inflow of microplastics from terrestrial sources and resuspend microplastic that had settled in aquatic systems (Chen *et al.*, 2018; Suckling and Richard, 2020). These temporary conditions would increase the concentration of microplastics that are readily available to filter feeding organisms. Studies testing the effects of microplastics on bivalves used a range of exposure periods ranging from hours to months. Short-term experiments have been performed within hours or days (Setälä *et al.*, 2016; Sendra *et al.*, 2020; Suckling and Richard, 2020); that is because the duration of extreme conditions and availability, dispersion or settlement of microplastics in the wild would depend on many abiotic conditions like wind action, tides, water turbidity and precipitation. Long-term laboratory experiments duration has ranged from weeks to months (Sussarellu *et al.*, 2016; Gardon *et al.*, 2018; E. Christoforou *et al.*, 2020), however these studies are limited due to difficulties in maintaining the experimental setting for a long period of time.

Accumulating evidence has shown that microplastics are ingested by bivalves (Ward, Rosa, *et al.*, 2019), leading to negative effect on their respiration (Rist *et al.*, 2016), energy budget (Xu *et al.*, 2017), filtration rates (Hu *et al.*, 2016; Rist *et al.*, 2016; Woods *et al.*, 2018) and fecundity (Sussarellu *et al.*, 2016; Gardon *et al.*, 2018), amongst others. Most of our understanding of the impacts of these pollutants on bivalves comes from laboratory based experimental studies and it is therefore crucial to consider the relevance of these laboratory settings to the conditions found in the field. A good example of this disparity is the shape of

microplastic used in laboratory experiment compared to those found in the field: most laboratory studies have focused on microbeads and fragments (Hu *et al.*, 2016; Rist *et al.*, 2016; Sussarellu *et al.*, 2016; Gardon *et al.*, 2018). Little attention has been devoted to microfibers (L. Li *et al.*, 2019; Alnajar *et al.*, 2021); however these are the most dominant microplastic shape in marine systems (Barrows *et al.*, 2018; Gavigan *et al.*, 2020) and one that is most likely to be ingested by filter feeders. This is due to the elongated shape of fibres as, their small diameter can deceive the particle selection mechanism of bivalves (Christoforou *et al.*, 2020); suggesting the entrance of a particle within their feeding range (<50µm, (Newell *et al.*, 1989; Beecham, 2008)) while the fibre would have a greater length. Microfiber pollution is mostly attributed to the growing synthetic clothing industry and the shedding of fibres after domestic washing of synthetic fabrics (Hernandez *et al.*, 2017). These fibres enter the sewage system and then inflow into riverine and coastal ecosystems due to the lack of suitable filtration systems at wastewater treatments (Murphy *et al.*, 2016; Cesa *et al.*, 2017; Henry *et al.*, 2019). Additionally, the recent COVID-19 pandemic has endorsed the increasing use of disposable personal protective equipment like gloves, masks, and wet wipes which can exacerbate the global problem of microplastic and microfiber pollution as most of these products are fibre based (Aragaw, 2020; Fadare and Okoffo, 2020; Shruti *et al.*, 2020, 2021; Shen *et al.*, 2021). Therefore, it is topical and important, to investigate and understand the effects of microfibers on marine bivalves and their phytoplankton removal capacity.

The second form of marine pollution that has recently gained the attention of researchers and conservationists is artificial light at night (ALAN) (Davies and Smyth, 2018). ALAN distribution and intensity is directly related the growing urbanisation, human populations and population densities (Neumann *et al.*, 2015) resulting in the reduction of the natural dark night sky (Altermatt and Ebert, 2016; Falchi *et al.*, 2016). Studies have indicated that changes to natural night lighting interferes with biochemical, physiological and behavioural processes of organisms that are evolutionary adapted to rely on natural day and night cycles (Häfker *et al.*, 2017; Knop *et al.*, 2017; Falcón *et al.*, 2020; Singhal *et al.*, 2021). New opportunities to lessen these impacts have arisen with the development of light-

emitting diode (LED), which is a flexible technology that allows to easily modify the intensity and wavelengths (i.e., colour) of light installations. Thus, interest has been growing on identifying the wavelengths causing the least disturbance to wildlife. The most tested light colours are red, green, blue and/or white. These provide the flexibility of colour vision in humans but allow the possibility of reducing the impact of ALAN based on the spectral sensitivity of the organisms of conservation interest (Spoelstra *et al.*, 2015). For example, bats and turtles are sensitive to low wavelengths (i.e. green and blue) (Miller and Bretschneider, 2006; Spoelstra *et al.*, 2015) but birds are more sensitive to high wavelengths (i.e. red) (Poot *et al.*, 2008). It is evident though that most studies investigating the effect of ALAN have focused on terrestrial organisms (Bruce-white and Shardlow, 2011; Bennie *et al.*, 2015); with coastal ecosystems receiving comparatively little attention, although ALAN has been reported to reach biotic communities inhabiting intertidal zones (Underwood *et al.*, 2017), sandy beaches (Luarte *et al.*, 2016) and marine protected areas (Davies *et al.*, 2016).

Coastal bivalves are potentially susceptible to coastal ALAN as they can be found within all aforementioned coastal areas and it is known that they possess photoreceptors (Morton, 2008; Von Salvini-Plawen, 2008; Audino *et al.*, 2020). Bivalve photoreceptors are involved in the detection of sudden light intensity changes enabling anti-predatory responses (Wilkens, 2008), while they also help regulate circadian rhythms (Ortmann and Grieshaber, 2003; Garcı *et al.*, 2008; Gnyubkin, 2010), such as increased activity and filtration at night (Gnyubkin, 2010; Robson, Garcia De Leaniz, *et al.*, 2010; Hills *et al.*, 2020). Given this information, it is anticipated that ALAN would interfere with the circadian rhythms of bivalves as well as with the ecosystem services provided by coastal bivalves. However, such ALAN impacts have not yet received much attention and are yet to be tested. Furthermore, research on the effects of different wavelengths on the filtration and activity of bivalves is lacking despite being crucial for the implementation of environmentally friendly coastal illumination.

The main aim for this PhD project was to investigate the effects of microfiber and ALAN pollution on the phytoplankton clearance capacity and activity of mussels

which could give an indication on the effects of these stressors on the ecosystem services provided.

Objective 1: Conduct a literature review of the studies investigating the effects of microplastics on bivalves and identify gaps in literature.

This was necessary due to the accumulating volume of research on the topic. It was important to (a) compare the conditions used in these studies with the microplastic conditions in the field and (b) to determine if our understanding of the effect of microplastics is representative of realistic exposures.

Objective 2: Examine any synergistic effects of acute short-term microfiber pollution and algae bloom on the phytoplankton clearance by mussels.

This was achieved by (a) exposing mussels to acute microfiber and phytoplankton concentrations for 24 hours and quantifying their phytoplankton consumption and (b) testing for any post-exposure effects on the phytoplankton consumption of microfiber-exposed mussels after five days of microfiber-free conditions.

Objective 3: Determine any chronic effects of microfiber exposure on the phytoplankton clearance capacity of mussels and to identify any relationship between the phytoplankton clearance capacity and any accumulated microplastics.

Hence, I performed a long-term (52 days) experiment exposing mussels to microfiber where I quantified (a) their phytoplankton consumption and (b) the microfibers accumulated in the digestive system of the exposed mussels.

Objective 4: Investigate possible effects of ALAN wavelengths on the phytoplankton clearance capacity and the activity of mussels.

Here, there was no need for a literature review due to the scarcity of studies investigating the effects of ALAN. Therefore, I performed a laboratory experiment exposing mussels to green, red, and white LED ALAN wavelengths, at relevant coastal illumination irradiance and a control dark treatment. For this experiment

I measured (a) the valve activity of mussels including the duration of open gape (i.e., the time valves of the mussel were open) and the open/close frequency (i.e., the number of time the valve close from an open position and vice versa) per hour, by the use of a custom made valvometry system and (b) their phytoplankton clearance capacity as responses to the ALAN treatments and (c) I tested if ALAN interferes with the relationship between the amount of activity and the clearance capacity of mussels.

To address the above aims and objective it was necessary to refine the methodology for counting the main response variable, namely the phytoplankton clearance capacity (used interchangeably with phytoplankton consumption) of mussels. This can be regarded as a proxy of the biofiltration of mussels and therefore the ecosystem service of removing excess phytoplankton from the water column. It was also crucial to perform some pilot experiments to identify the most suitable experimental design and optimise the experimental settings that would enable valid statistical analysis. Details of these preparatory work can be found in the Appendix 2. At the end of the thesis, I have included the published research paper that we conducted as a team, in parallel to my PhD project, investigating the effects of different ALAN wavelengths on the growth of a green microalgal species and the biomass, diversity, and composition of a diatom assemblage. Results from this work were also incorporated in the discussion of Chapter 4 and the general discussion.

Chapter 1

Effects of microplastics on bivalves: are experimental settings reflecting conditions in the field?

A version of this chapter was published in the Marine Pollution Bulletin in July 2021:

Baroja, E., Christoforou E., Lindström, J. & Spatharis S., (2021) 'Effects of microplastics on bivalves: Are experimental settings reflecting conditions in the field?', Marine Pollution Bulletin, doi: 10.1016/j.marpolbul.2021.112696.

My contributions to this publication were: Conceptualization, Methodology, Writing-Original Draft, Writing-Review & Editing, Visualization and Funding acquisition.

In addition to the published manuscript and to ensure co-authorship by equal contribution, in the current chapter I have conducted further analysis answering and discussing the following questions:

Is there a preference on the microplastic size based on the bivalve taxonomic group used in exposure studies? - Figure 1-6B

What is the duration of exposure used by studies? – Figure 1-7A

Is there a preference in the duration of exposure based on the bivalve response group tested in exposure studies? - Figure 1-7B

Is there a preference in the duration of exposure based on the concentration of microplastics used in exposure studies? - Figure 1-8B

Abstract

Bivalves are the focus of experimental research as they can filtrate a broad size range of microplastics (MPs) with negative consequences on their physiology. Studies use a range of MP shapes, materials, sizes, concentrations, and durations of exposure raising the question: do these reflect environmental observations? Here, we review experimental studies on MPs effects on marine bivalves and contrast the MP characteristics used with corresponding data from the environment. Mussels were the most common bivalve across experiments which reflects their high abundance and broad distribution in the field. Although fibres are the dominant shape of MPs in coastal systems, most experimental studies focus on spherules and beads instead. Most exposure experiments lasted between 1-7 days and MP concentrations are often orders of magnitude higher than environmental levels. For higher relevance of experimental findings, we recommend that exposure experiments run over longer periods, maximum experimental concentrations of MPs are in the range of 100-1000 particles/L, more focus given on microfibers and concentrations are reported in particles/volume.

1.1 Introduction

Mainly due to their small size (<5mm) (Barnes *et al.*, 2009), widespread use and improper disposal or leakage, microplastics (MPs) are currently regarded as one of the most widespread forms of pollution (Napper and Thompson, 2019). Aquatic environments, and coastal ecosystems in particular, are especially prone to MP pollution due to intense anthropogenic activities, e.g., inflows from wastewater treatment plants, coastal landfills, industrial outfall and coastal fisheries (Garcia-Garin *et al.*, 2019; Kazour *et al.*, 2019; Xue *et al.*, 2020). Investigating the effects of MP pollution on marine biota in situ poses many challenges associated with the effects of confounding factors that are difficult to control in the field (Lebreton *et al.*, 2017). For this reason, the bulk of evidence on the impacts of MPs on marine organisms comes from experimental studies under controlled laboratory conditions. A focal group for such investigations are marine bivalves due to their susceptibility in ingesting MPs while filter feeding (Ward, Rosa, *et al.*, 2019; Ward, Zhao, *et al.*, 2019) and because of their importance in coastal ecosystem goods and services (van der Schatte Olivier *et al.*, 2018). However, to assess the impact of MP pollution on coastal marine bivalves, it is imperative to understand how relevant the conditions used in laboratory MP exposure studies are to the conditions and species observed in marine systems.

Bivalves play an essential role in ecosystem function (Dame, 1993) providing invaluable services including carbon sequestration, nutrient remediation and coastal defence (van der Schatte Olivier *et al.*, 2018). However, different taxonomic groups can have different contribution to ecosystem goods and services. For example, mussels have the greatest potential for bioremediation as they remove the most nitrogen and phosphorus per tonne of shellfish produced (van der Schatte Olivier *et al.*, 2018). On the other hand, clams, oysters and scallops amount to the highest percentages of the global marine bivalve aquaculture (31%, 27% and 23% respectively) (FAO/ICAC, 2018). It is thus essential to understand whether the choice of species in MP exposure studies and the responses studied, reflects their contribution to ecosystem goods and services, or is instead based on ease of accessibility and experimentation in the lab.

The relevance of experimental MP exposure studies should also be assessed with respect to the characteristics of the MPs used. Field studies have shown that certain MP shapes and types tend to be more dominant in marine systems (Ekvall *et al.*, 2019; Baldwin *et al.*, 2020; Galaiduk *et al.*, 2020; O'Connor *et al.*, 2020). For instance, although a range of shapes can be found in marine ecosystems (e.g. spherical, pellets, grains and irregular fragments), fibres are the most common (Henry *et al.*, 2019) accounting for up to 91% of all MPs in the water (Barrows et al., 2018). This is concerning as fibres are longer than they are wide, therefore, they can enter the digestive system of bivalves when penetrating through the narrower width, avoiding the mechanisms of the bivalves to filter out particles larger than ~100µm (Ward, Rosa, *et al.*, 2019; Sendra *et al.*, 2021). Establishing whether fibres are well represented in experimental studies on the effects on bivalves is thus crucial in our understanding of the effects of microfiber ingestion by bivalves.

It has been shown that certain bivalve like scallops and mussels can distinguish, in the pre-ingestion level, between particle density, physicochemical properties and size (Brillant and MacDonald, 2000, 2002; Rosa *et al.*, 2017). Similarly, oysters and mussels can reject particles depending on their surface properties for example in the presence of aluminium oxide (Rosa *et al.*, 2013). In the case of MP pollution, such selective mechanisms could moderate the potential ingestion of MPs by bivalves. Therefore, for experimental inference to be environmentally relevant, it is imperative that the MP material used in bivalve exposure studies is related to what is encountered as the dominant material in the field. This is particularly important because, although some polymer types seem to be dominant globally, (e.g., polypropylene and polyethylene), others have a more localised presence. For instance, additional to polypropylene and polyethylene, polystyrene is dominant in the Mediterranean whereas nylon is dominant in the North-Western Pacific (Pan *et al.*, 2019). Bivalves preferentially select and feed on particles between 1µm and 40µm in diameter (could reach up to 400µm in length) (Beecham, 2008). As a result, particles of different sizes have a different ingestion and retention rate (Møhlenberg and Riisgård, 1978), consequently resulting in a differential behavioural or physiological response by bivalves.

Another vital information on the design of environmentally relevant MP exposure studies on bivalves is the duration of MP exposure and concentration. The duration of exposure might depend on the response tested (De Ruijter *et al.*, 2020) for instance, it was suggested that the filtration capacity of mussels only decreased after a long-term exposure to MP (E. Christoforou *et al.*, 2020) but in many cases, short-term experiments are conducted with higher than realistic MP concentrations aiming at simulating the chronic exposure in the wild (Raimondo *et al.*, 2007; Connors *et al.*, 2017). However, bivalves display sensitivity to the suspended particle concentration for determining their filtration rate and pseudofaeces production (Riisgård, 2001), which could also lead to differences in their behaviour and particle selection capacity (Rosa *et al.*, 2018). Thus, the use of environmentally relevant duration of exposure and concentrations in experimental studies is critical in understanding plausible impacts of MPs on coastal bivalves.

The aim of the present review is to assess whether experimental settings reflect realistic exposure conditions faced by bivalves in the field. This will help determine whether our understanding of the effects of MP pollution is biased by potentially unrealistic study designs. To address this issue, we performed a systematic review of experimental studies assessing the impact of MPs on bivalves and extracted data on the species of bivalves used, the responses monitored during exposure, the characteristics of MP tested, and the duration of exposure and MP concentrations used. Furthermore, we carried out a meta-review (review of review papers) of the MP characteristics observed in aquatic systems; the findings were compared with the MPs used in experimental designs and the relevance of the later is discussed. Findings and recommendations from this study provide a framework for driving future work on the environmental consequences of MPs, towards settings that are more relevant to the actual exposure risks to organisms, including both bivalves and other aquatic organisms.

1.2 Methods

1.2.1 Extracting information from experimental MP exposure studies on bivalves

To conduct a comprehensive review on the effects of MPs on bivalves, databases and repositories were searched for relevant studies published in the period between 1989 to 2021 using keywords and applying the Boolean logic. The electronic databases used were Web of Science, ScienceDirect and Dissertation Abstracts Online. The search was carried out by using the following progression of terms: TS= (Microplastic*OR microfiber* OR nanoplastic* OR polystyrene*) AND TS= (effect* OR impact*) AND TS= (mussel OR bivalve* OR filter feeder OR mollusc OR scallop OR clam OR oyster) AND TS= (marine system OR marine environment*) (Figure 1-1). The search was conducted in March 2020 and updated in September 2020 and February 2021. While we are aware of the potential publication bias towards statistically significant findings in any area of science (Olson *et al.*, 2002), extending the literature search to unpublished research in a systematic fashion is challenging and not attempted here.

The search resulted in 378 publications on effects of MPs on bivalves which were filtered in different phases, following inclusion criteria (schematically illustrated in Figure 1-1). In the first phase, after dropping duplicates, titles and abstracts were screened fulfilling the first three relevance criteria (see criteria 1-3, Figure 1-1). The screening procedure was carried out by using the R metagear package (Lajeunesse, 2016). In the second phase, publications selected were thoroughly read and analysed, ensuring that there was a comparator and inclusion criteria 4-5 were met (see criteria 4-5, Figure 1-1). In the end, 68 studies which fulfilled all selection criteria were included in this review (Table S 1-1). For each publication included in the review, the following data were extracted: year of publication (11 years), journal ID (68), organism (4 taxonomic groups), species (22), responses tested (217), MP shape (7 levels), MP type (12 levels), MP size (classified in four groups), duration of exposure (classified in 6 groups) and MP concentration (classified in 5 groups). In the case where studies investigated the combined effects of MPs with other pollutants, only the main effect of MP was included, omitting interactions with other pollutants.

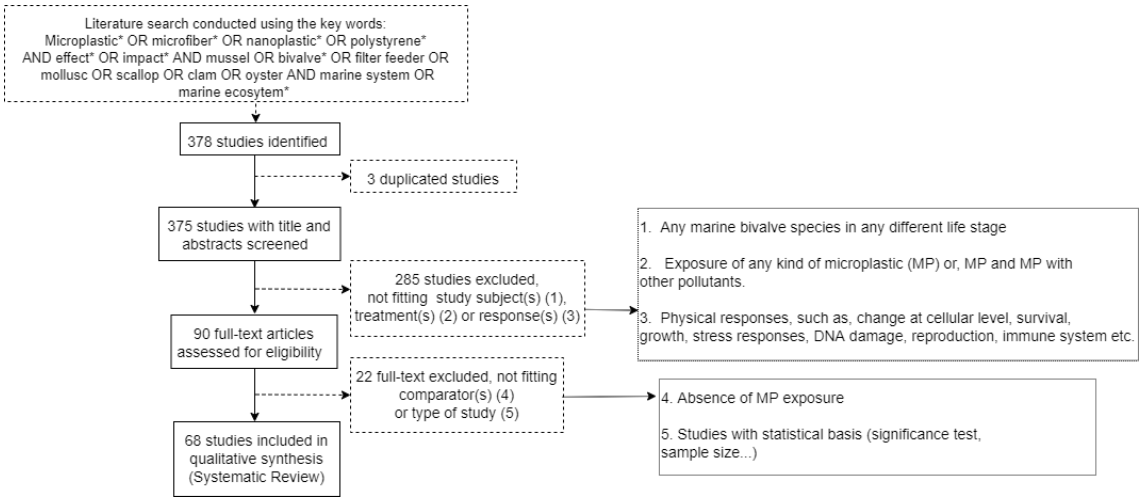

Figure 1-1: Schematic representation of the selection process. Numbers in brackets represent the requirements for inclusion criterion summarized on the right.

Upon extraction of the information from the selected studies, the reported 217 responses were categorised in 18 broader groups for clarity: oxidative damage, immunotoxicity, antioxidant capacity, feeding behaviour, genotoxicity, structural damage, growth, neurotoxicity, apoptosis, mortality, bioaccumulation, larval development, metabolism, fecundity, behaviour, homeostasis, microbiota, and malformations (Table S 1-2). As MP concentrations were reported in particles/volume or weight/volume, a comparison between the two units of measure was not possible. However, to enable interpretation of findings, the MP concentrations of each unit type were transformed to MP/L and mg/L as appropriate and were classified in five groups (<1; 1-100; 100 to 10^4; 10^4 to 10^6; > 10^6).

This review presents the number of publications for each focal variable: bivalve species, the responses recorded after exposure, the characteristics of microplastics tested (i.e., shape, type, and size), the duration of exposure and the MP concentration. Some studies tested the impact of MPs using multiple species, responses, MP characteristics and duration of exposure. As a result, the mentioned publications include multiple data entries identified with the same code.

1.2.2 Meta-review on environmental MPs

To enable a representative comparison and discussion on the relevance of MPs in experimental studies and MPs observed in the marine environment we conducted a literature search on review studies reporting environmental MPs published during the period 2016-2021. The reason we focused on a meta-review of existing reviews on environmental MPs was due to the high number of original research papers on the topic as well as the fact that these have already been summarised by a high number of reviews in a manner that provided useful baseline info on MP characteristics for our study (e.g., info on shape, size, material, concentration). The search was carried out in Web of Science using the following progression of search terms: TS= (Microplastic*) AND TS= (size OR shape OR abundance* OR composition*) AND TS= (marine environment). The search resulted in 196 reviews, from which 13 were selected according to the following criteria: (1) reviews needed to include marine surface or seawater studies and (2) reviews had to include studies on marine systems reporting MP abundance values in particles per volume. After selecting the reviews, the following data were extracted: number of publications per review, number of publications per environment within a given review, highest MP concentration (MP/L) and corresponding geographical location reported within a review, dominant polymers, shape, and size range (μm) reported within a review. It has been demonstrated that most of the marine MPs are coming from riverine inflows (Lebreton *et al.*, 2017; Schmidt *et al.*, 2017; Meijer *et al.*, 2019). In reviews on environmental MPs, using studies from both marine and freshwater systems, the highest concentrations reported are often from rivers. We have reported these concentrations in our meta-review table since they provide an indication of the upper threshold that MP concentration can reach in the coastal environment.

1.3 Results

1.3.1 Data from MP exposure studies on bivalves

The analysis of the responses of bivalves to MP exposure was assessed with a group of 68 publications out of which 67 were published from 2008 onwards (Figure 1-2).

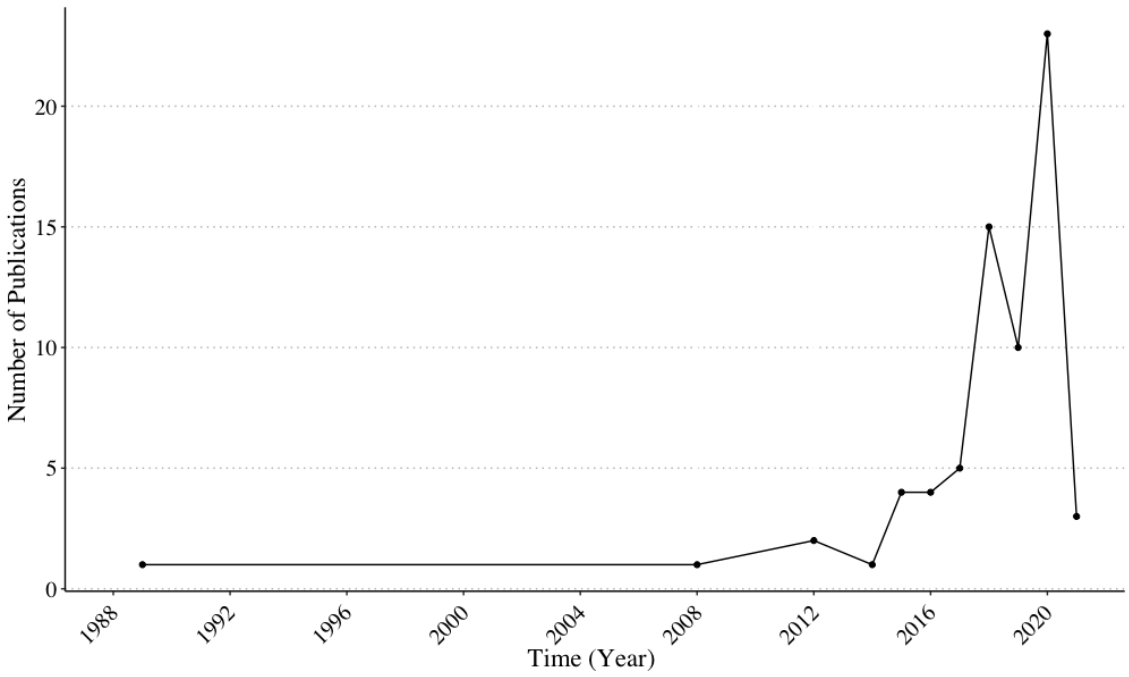

Figure 1-2: Number of publications included in this review that investigate the effects of MPs on bivalves in laboratory settings from 1989 to 2021.

The most frequently used taxonomic group in experimental studies were mussels (n=42, 62%) followed by oysters (n=13, 19%), clams (n=11, 16%) and scallops (n=3, 3%). Overall, 22 species, from the four taxonomic groups were used in the experiments. Specifically, 36.4% of species were mussels, 31.8% clams, 18.2% oysters and 13.6% scallops (Figure 1-3). The most abundant species within each group were the mussel *Mytilus galloprovincialis*, oyster *Crassostrea gigas*, clam *Tegillarca granosa*, and scallops *Chlamys farreri*, *Argopecten irradians* and *Pecten maximus*.

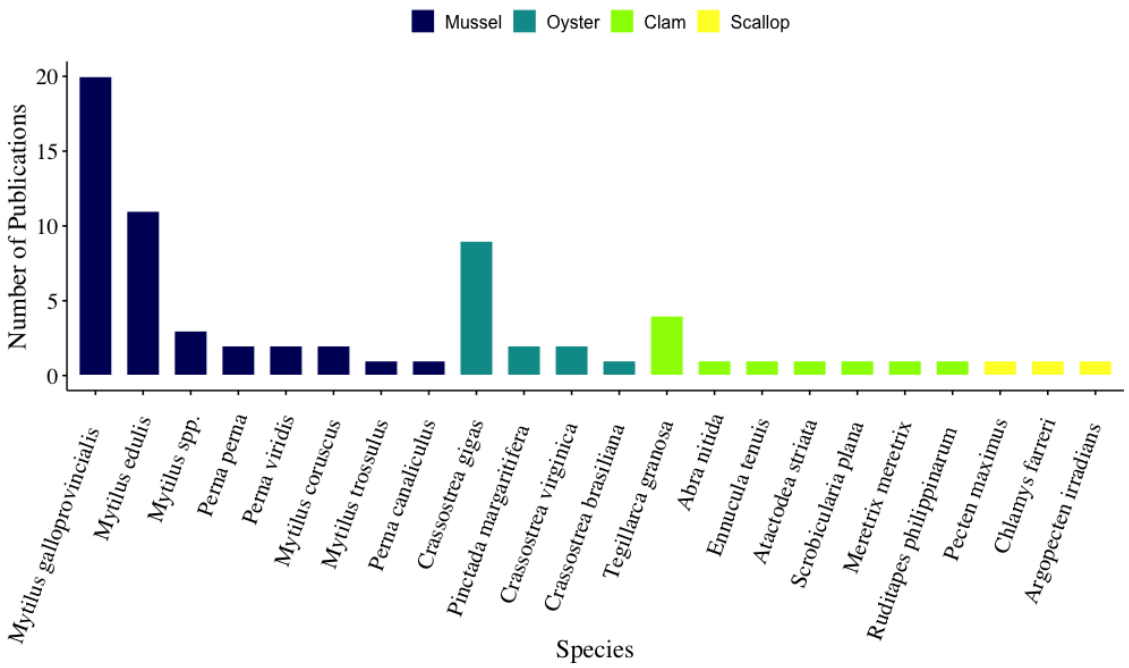

Figure 1-3: Number of publications, investigating the effects of MPs on bivalves in laboratory settings across the different bivalve species used in the experiments.

Bivalve responses to MP exposure, that were more frequently investigated, included immunotoxicity (n=25), oxidative damage (n=23), genotoxicity (n=21), structural damage (n=19) and antioxidant capacity (n=19). Mussels were the most dominant taxonomic group across the different response groups (Figure 1-4). Regarding the other bivalve taxonomic groups, clams were used to study 12, oysters 13 and scallops seven out of the 18 response groups (Figure 1-4). Six out of the 18 response groups have been tested with all bivalve taxonomic groups and these were growth, genotoxicity, antioxidant capacity, oxidative damage, structural damage and immunotoxicity.

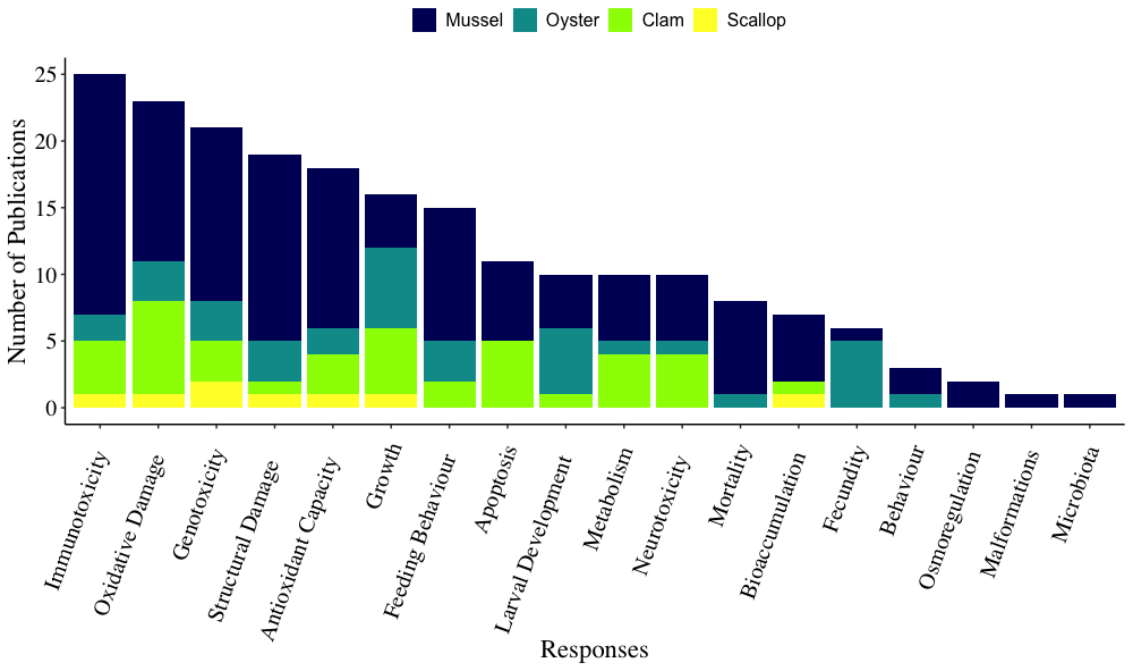

Figure 1-4: Number of publications, investigating the effects of MPs on bivalves in laboratory settings corresponding to the responses of bivalves to MPs, categorised in 18 groups, and the different bivalve taxonomic groups that the responses were studied on.

In terms of the MP used in exposure experiments, bead/spherules (n=33, 48.53%) and unknown shapes (n=23, 33.8%) were the most used, whereas only four trials used fibres (Figure 1-5A). Regarding the composition of the MPs used, polystyrene (PS, n=36) and polyethylene (PE, n=15) were the most common types (Figure 1-5B).

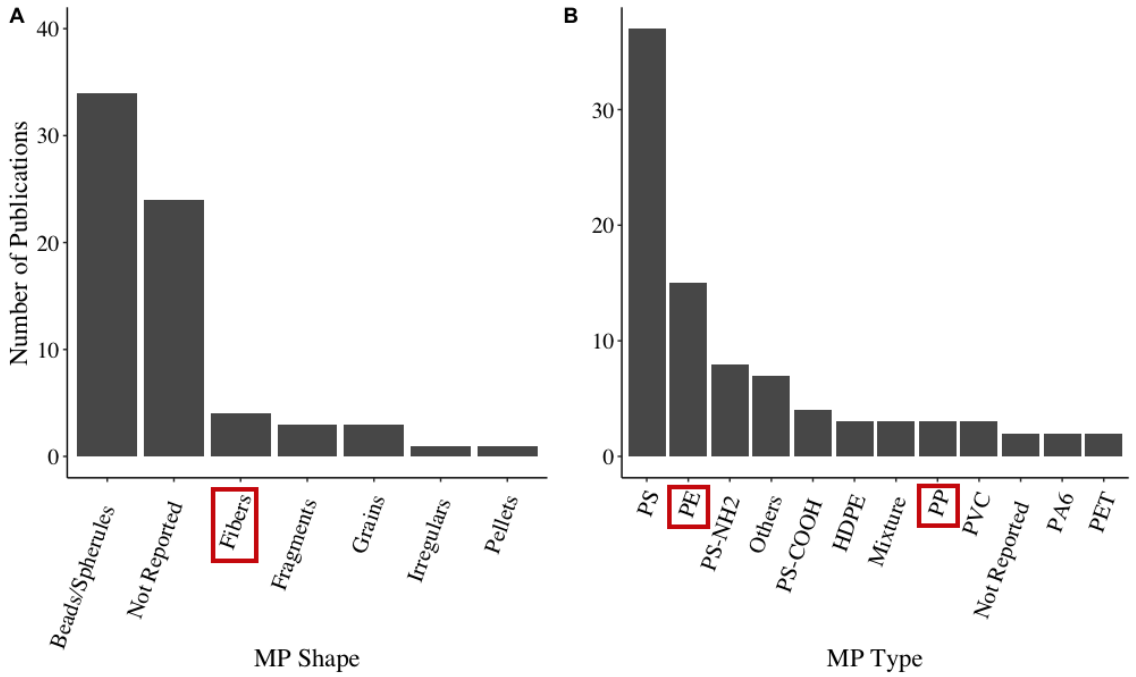

Figure 1-5: Number of publications, investigating the effects of MPs on bivalves in laboratory settings by (A) microplastic shape and (B) type. Red rectangles represent the dominant MP groups in the marine environment based on our meta-review (see Table 1-1). Meaning of acronyms: polystyrene (PS), polyethylene (PE), amino-functionalized polystyrene (PS-NH2), carboxyl-functionalized polystyrene (PS-COOH), high-density polyethylene (HDPE), polypropylene (PP), polyvinyl chloride (PVC), Nylon 6 (PA6) and polyethylene terephthalate (PET).

The most common size group of MPs used in laboratory studies was <10µm (n=40) from which 13 were nanoplastics (0.002-1µm) (Figure 1-6A). A mussel and a scallop exposure study were the only ones using particles >500µm, where in both cases the MPs were 5 000 µm (=5mm) (Figure 1-6B). The largest MP size used in clam and oyster studies were on average 312µm and 400µm respectively.

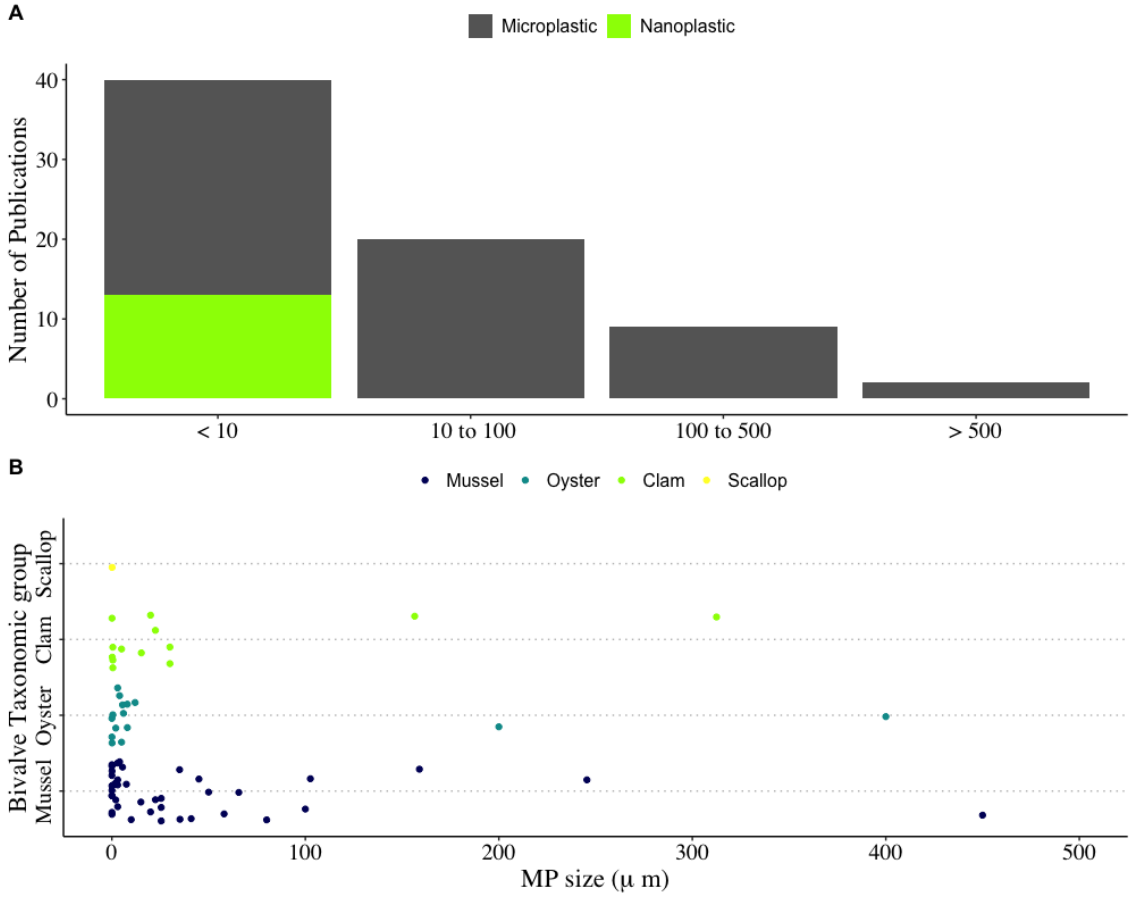

Figure 1-6: Number of publications, investigating the effects of MPs on bivalves in laboratory settings by (A) MP size group (µm) and (B) the MP size (µm) used for each bivalve taxonomic group where two values, one from mussel and one from scallop, of 5000µm, were excluded for easier visualisation.

Many of the studies (39.5%) exposed bivalves to MP for one to seven days (n=34). A bit less than half (n=38, 44.2%) chose an exposure duration longer than a week, from which 11 studies contacted experiments longer than 28 days (Figure 1-7A). These studies, with the longest period of exposure were testing responses within the following groups: immunotoxicity (30 days), feeding behaviour (39, 56, 60 and 90 days), growth (40 and 48 days), microbiota (42 days), genotoxicity (52 and 60 days) and general behaviour (91 days) (Figure 1-7B). The shortest exposure duration was 15mins and was testing the structural damage followed by 1-hour exposures testing fecundity, feeding and general behaviour.

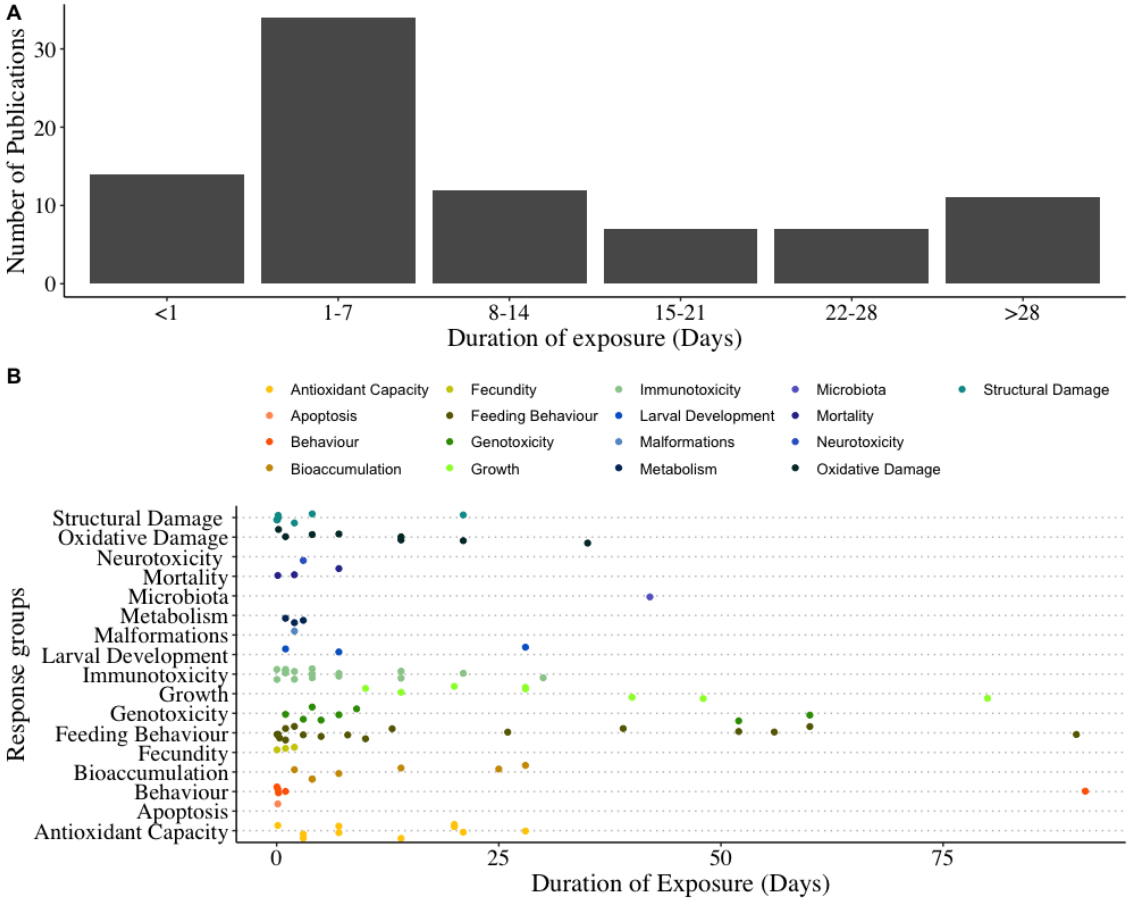

Figure 1-7: Number of publications, investigating the effects of MPs on bivalves in laboratory settings by (A) the duration of exposure and (B) the duration of exposure used for each of the response groups.

Experimental studies reported concentrations of microplastics in two different ways: weight per volume (n=67) or particles per volume (n=27), which prevent direct comparisons between them. The most used concentrations of weight per volume were <1mg/L (n=33) followed by 1 to 100mg/L (n=18) (Figure 1-8A). In relation to particles per volume, most studies used concentrations between 10^4 to 10^6MP/L (n=12) whereas the next most used concentration group was that of the lower range, 10^2–10^4MP/L (n=8). At the highest particles per volume concentration group (>10^6MP/L), the duration of exposure was 1 hour, 1 day and 30 days (Figure 1-8B). The four longest exposure experiments run for 39, 40, 52 and 80 days and were all conducted with concentrations 10^2–10^4MP/L.

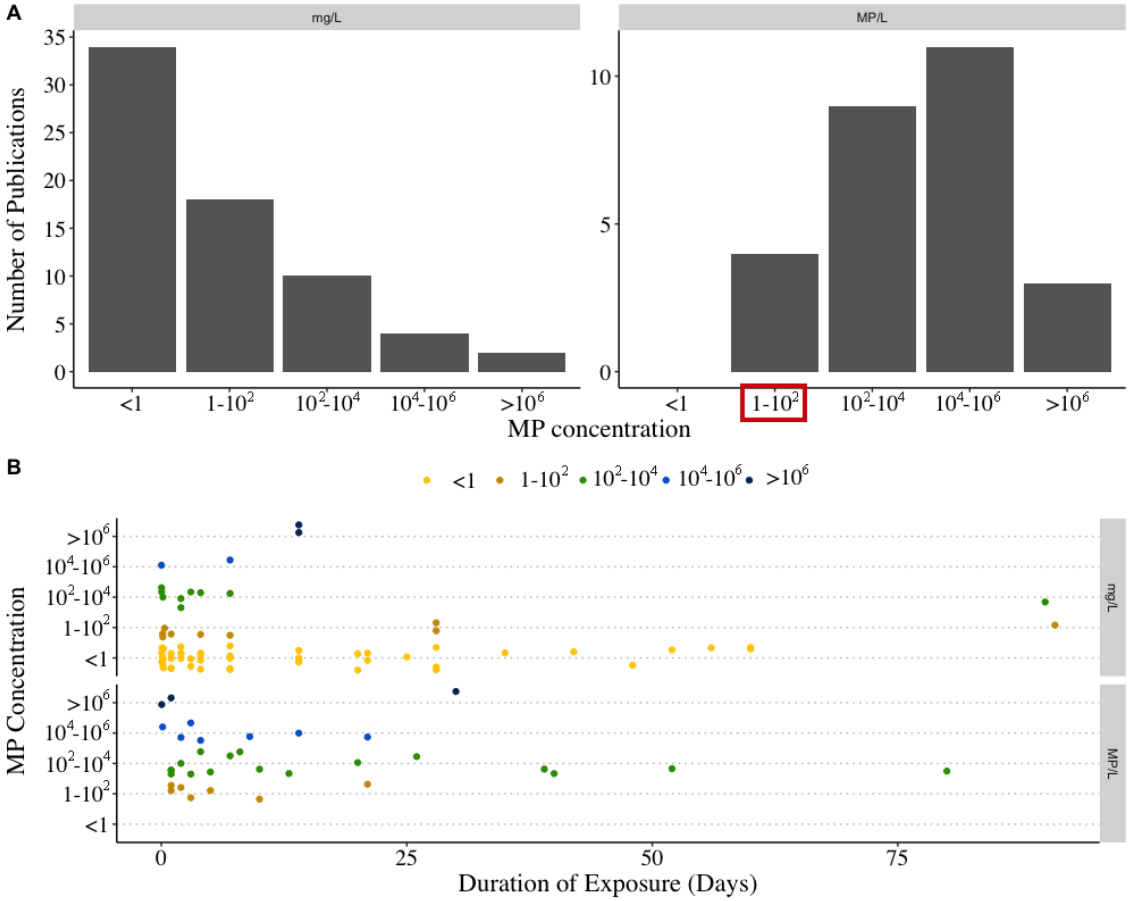

Figure 1-8: Number of publications, investigating the effects of MPs on bivalves in laboratory settings by (A) the MP concentration based on both units (mg/L and MP/L) reported and (B) the duration of exposure used for each of the concentration ranges. Red rectangle in panel A represents the dominant MP concentration range in the marine environment based on data compiled from our meta-review (see Table 1-1).

1.3.2 Data from review papers on environmental MPs

In terms of MPs presence in the natural ecosystems, a summary of the meta-review articles revealed that the most common MP polymers found in marine systems were polypropylene (PP) and polyethylene (PE). Regarding shape, fibres were the dominant group in marine ecosystems (Table 1-1). In terms of the MP dominant size, this varied from 1.2 to 5000μm (Table 1-1). With respect to the abundance of MPs in the environment reported in these reviews, the highest MP concentration was 102 items/L located in Stenungsund Harbour, Sweden (Table 1-1).

Table 1-1: Summary of different MPs characteristics reported within the 13 review papers selected from our systematic meta-review. Location refers to the place where the highest MP concentration [MP] was found. Polymers, shape, and size range refer to the dominant MP group(s) identified by each review. Note that highest concentrations reported for a freshwater system e.g., a canal, indicate the upper threshold of concentration of marine papers examined within the same review.

N° of publications per review	**N° of publications per ecosystem**		**Highest [MP] (MP/L)**	**Geographical location of highest [MP]**	**Polymers**	**Shape**	**Size range (µm)**	**Reference of review paper**
	Marine	**Freshwater**						
59	9	6	1.215	South Easter bays of South Africa			1.2-5000	(Alimi *et al.*, 2021)
18	7	-	0.0035	Bay of Biscay, Spain	PE>PA>PES	Fibres	250-2000	(Mendoza *et al.*, 2020)
12	6	-	6.6	Yangtze Estuary, China		Films>Fibres		(Tang *et al.*, 2020)
>61	23	38	21.839	Rhine-Ruhr, Germany	PP>PE>PVC>PS>PTFE	Fibres		(Xu *et al.*, 2020)
>200	<113	<57	8.369	Lake Yenogoa, Africa	PE>PP>PS		1.2-5000	(Akdogan and Guven, 2019)
180	-	-	100	Canals of Amsterdam				(Cunningham and Sigwart, 2019)
12	7	-	6.6	Yangtze Estuary, China				(Laskar and Kumar, 2019)
38	12	12	102	Skagerrak, Norway/Denmark	PE>PP>PS	Fibres>Fragments		(Wu *et al.*, 2019)
109	58	10	100	Canals of Amsterdam	PE>PET>PA>PP>PS>PVC>PVA	Fibres>Fragments>Beads>Spherules>Films>Foam		(Burns and Boxall, 2018)
52	43	-	2.4	Swedish West Coast	PP>PE		500-1000	(Gago *et al.*, 2018)
>70	-	-	2.8	Hong Kong	PE>PP>PS			(Shahul Hamid *et al.*, 2018)
13	13	-	10.2	Yangtze Estuary, China		Fibres>Fragments>Beads		(Cesa *et al.*, 2017)
-	-	-	102	Stenungsund Harbour, Sweden				(Norén, 2017)

*PE–polyethylene; PA–polyacrylamide; PP–polypropylene; PVC–polyvinylchloride; PS–polystyrene; PET-polyethylene terephthalate; PES-polyester; PVA–vinyl alcohol; PTFE–polytetrafluoroethylene.

1.4 Discussion

This is the first systematic review evaluating the environmental relevance of laboratory studies assessing the effects of MPs on bivalves. Our findings and suggestions draw an outline for future, environmentally meaningful, studies on the impacts of MPs on bivalves. Moreover, this review identifies issues, such as unit disagreement between published works and the lack of reported information when reporting results, which limit our ability to evaluate the extent of the marine bivalves' vulnerability to MP pollution as well as hinder our ability to interpret and compare results between studies.

1.4.1 Relevance of bivalve species and responses tested in exposure studies

Our findings show that 62% of the reviewed studies were focused on assessing the impacts of MPs on mussels. Within this taxonomic group, species from *Mytilus* and *Perna* genera predominated in experimental studies. Species of the genus *Mytilus* predominate in communities with cool water in the northern and southern hemispheres whereas species of the genus *Perna* have a tropical to subtropical distribution in the southern hemisphere (Gosling, 2003). Preference for this group was also evident in the responses to MP exposure tested as mussels were the only bivalve taxonomic group present in all responses. Moreover, 17% of the responses were investigated solely on mussels. We can consider the use of mussels as environmentally relevant since they are the most dominant bivalve group in marine ecosystems globally (E. Gosling, 2003) and were shown to have the greatest potential of bioremediation (van der Schatte Olivier *et al.*, 2018).

After mussels, oysters and clams were the most common bivalve groups in studies, used in 72% and 61% of all responses tested, respectively. Effects of MPs on growth were tested on both organisms, while oysters predominated in larval development and fecundity testing. This is presumably because of their greater economic importance (Gosling, 2003), as in 2018 it was estimated that the aquaculture of marine oysters and clams together was worth $7.27 billion (FAO/ICAC, 2018). Despite the great economic value of scallops - $5.84 billion in 2018 (FAO/ICAC, 2018) - they were the least studied group, present in 39% of all responses tested. A reason for their lower presence could be that most scallop species are found at depths between 10 and 100m in bays and open coast sites (Gosling, 2003) and are thereby harder to access. That fact could also complicate their maintenance in laboratories (Gosling, 2003; Lusher *et al.*, 2017) and therefore causing bias against their selection for experimental studies.

1.4.2 Environmental relevance of MPs used in exposure studies

To evaluate the environmental relevance of experimental studies, we conducted a meta-review of recent reviews assessing the characteristics and concentrations of MPs in nature. Since 48% of the studies included in this review do not report

the MP shape used in experimental trials, drawing conclusions is challenging. Among studies reporting shape, beads/spherules were the most common MP debris. One of the main reasons, for the preference of spherical MPs is their uniform size and shape which facilitates particle identification and quantification compared to irregular debris, such as fibres (Ward, Rosa, *et al.*, 2019). Furthermore, microfibers are not readily available in the market and their generation, within a consistent size range, is strenuous and time-consuming (Cole, 2016; Christoforou *et al.*, 2020). However, there are clear arguments beyond convenience for including different shapes of MPs in experiments, such as, abundance in coastal environments or noxious capacity. According to our meta-review of 13 review papers of environmental MP characteristics, fibres are the most common shape, as concluded in five of the reviews, constituting up to 80-90% of all global water samples (Barrows *et al.*, 2018). A reason for their abundance could be the increase in their demand: the latest World Apparel Fibre Consumption Survey reveals that the use of synthetic fibre for textile industry has increased substantially from 2005 to 2008 (FAO/ICAC 2005). These fibres enter the aquatic environment through garment washing and improper filtration of wastewaters (De Falco *et al.*, 2019). Fibres are not only abundant in aquatic environments but also inside bivalves collected from the wild (Sendra *et al.*, 2021). In addition to their abundance, fibres have proven to be more damaging than other shapes due to their elongated shape and thus their capacity to be ingested by bivalves despite their length (Beecham, 2008). Studies reveal that fibres are more toxic compared to spherical and fragmented MPs (Gray and Weinstein, 2017; Lehtiniemi *et al.*, 2018). Therefore, we emphasise the urgent need for more studies focusing on the effect of microfibers on bivalves.

Apart from the shape, the composition of MPs was another characteristic evaluated for environmental relevance. Our findings showed that 43% and 18% of exposure studies used polystyrene (PS) and polyethylene (PE), respectively. Yet, according to the environmental MP reviews, PE and polypropylene (PP) have been considered as major sources of MP pollution in the aquatic ecosystems investigated, and these materials constituted 36.4% of the European plastic demand in 2017 (Association of Plastic Manufacturers, 2017). Nevertheless, a recent meta-analysis of distribution of plastic types suggested that the relative

abundance of polymer types is not uniform across ocean zones. The study points out that low density polymer types, such as PE, are more common in surface waters, whereas PP and polyester are more dominant in intertidal areas and polyamide and acrylic in subtidal areas (Erni-Cassola *et al.*, 2019). With the exception of few deep-sea species, most marine bivalves used in the exposure studies reviewed were shallow estuarine and coastal species (Dame, 1993). Therefore, to enable more relevant inferences of bivalve exposure to the dominant polymer we recommend a shift of focus from PS and PE to PP in experimental studies.

Furthermore, we determined the environmental relevance of MP size and concentration used in bivalve exposure studies. Particles <10μm were used in 69.4% of the studies from which 32% were nanoplastics (<1μm). Importantly, there is currently no consensus on the most common size range of MPs in aquatic habitats and this could be attributed to the limitation in collection and quantification methodologies of smaller MPs (Covernton *et al.*, 2019). However, Cai et al. (2018) observed that MPs smaller than 300μm contributed 92% of the total MPs quantified in South China Sea and last estimates relating to the abundance of MPs in the marine environment suggest that the amount of nanoplastics could be much higher than previously thought (Lindeque *et al.*, 2020). In terms of bivalve retention efficiency, a study on 13 species of bivalves showed that all particles larger than 4μm were ingested but the retention efficiency of any smaller particles was decreasing the smaller the particles were (Møhlenberg and Riisgård, 1978; Rosa *et al.*, 2018). This is attributed to the selection properties of the bivalve gills as small particles can pass through the intrafilamentary gaps, and are thus not retained on the surface of the ctenidium from where particles are usually directed to the food grove (Rosa *et al.*, 2018; Christoforou *et al.*, 2020). However, in this review it was evident that, in 16 studies, nanoplastics were still able to induce responses from the exposed bivalves. Regarding the highest size range, in two separate studies, mussels and scallops were exposed to MPs larger than 500μm, specifically 5000μm (5mm). In bivalves, particles larger than 50μm are usually rejected because they are larger than the area available in the ctenidium food groove but, particles up to 400μm have been previously found in the gut of oysters (Beecham, 2008). Thus, regarding the environmental and biological relevance of the MP sizes used, we

conclude that the focus on nanoplastics is relevant but the use of particles larger than 500μm should be avoided in bivalve exposure studies.

A critical review on microplastic effect studies suggested that the duration of exposure for benthic invertebrates should be longer than 28 days (De Ruijter *et al.*, 2020) however, in the current review only 11 out of 86 studies cohered to that suggestion. On the contrary, most studies exposed bivalves to MPs for one to seven days. It was also suggested that the duration of exposure usually depends on the response tested. In the current review we observed that, with the only exceptions of growth (10-80 days) and microbiota (42 days), that were tested after more than 10 days of exposure, the rest of the responses were tested after a large range, from hours to months. Specifically, feeding behaviour and general behaviour were tested after a minimum time of 1 hour and maximum time of 90 and 91 days respectively. When choosing the duration of the exposure, it is important to consider the particle size, which would determine the particle selection stage they are expected to affect (Christoforou *et al.*, 2020) and hence the response tested, the gut residence time of the particles (Ogonowski *et al.*, 2018) but more importantly, the environmental relevance i.e., if the aim of the study is to investigate the effects of a chronic exposure or an acute event.

During the last years, one of the most controversial topics in MP research (Burns and Boxall, 2018; A. Haegerbaeumer, M.-T. Mueller, *et al.*, 2019) has been the use of high MP dosages in laboratory exposure studies. Our meta-review regarding the environmental levels of MPs, revealed that the highest MP concentration recorded in aquatic environments was 102 particles/L, in Skagerrak, at the coastal waters of Norway and Denmark. Similar values to this level have been used as a reference to environmentally relevant values for MP concentrations in other studies (Bour et al. 2018, Woods et al. 2018). Based on this threshold, 81.5% of the bivalve exposure studies (reported in particles/volume) reviewed here, would be above realistic limits for environmental concentrations. MP concentrations that are orders of magnitude higher than the maximum reported concentrations from the field could exacerbate the MP impacts and have a questionable biological meaning. On the other hand, in bivalve exposure studies, using the maximum concentration reported from the marine environment and perhaps up to one order

of magnitude higher (100-1000 particles/volume) might be sensible given limitations in the enumeration of MPs <300 μm (Cai *et al.*, 2018; Covernton *et al.*, 2019) and the fact that MPs accumulate much faster than they biodegrade in the marine environment thus concentrations in coastal ecosystems are expected to rise. With that in mind, the use of the long-term exposure times of 39-80 days, with the concentration group 10^2–10^4MP/L is reasonable, and more studies should follow that example when testing the chronic exposure effect of MPs.

1.4.3 Consensus regarding MP research

Another issue faced by researchers in the MP research field is the inconsistency in the units for reporting MP concentrations. In exposure studies, the MP concentrations are reported in particles/volume or weight/volume while environmental concentrations are typically reported in items/area or items/volume. Unfortunately, there is no direct conversion between these units, which prevents us from assessing the environmental relevance of studies and prevents the comparison between findings and conclusions. Hence, we highlight the need for consensus in reporting units for future research. Specifically, we recommend using particles/volume as this is the most frequently reported unit and enables – when the material and the size are provided - to calculate the weight/volume but not vice versa.

Furthermore, 23% of bivalve exposure studies did not report critical information on the MP characteristics like shape and type. Due to the now established impact of shape and type on the response of exposed bivalves but also for allowing for replicability of experimental procedures, it is essential that MP characteristics are provided in detail.

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Chapter 2

Effects of short-term, acute microfiber exposure on the ecosystem services provided by mussels under algal bloom conditions.

Abstract

Marine bivalves are vital for the healthy functioning of coastal ecosystems. Through biofiltration, they provide invaluable ecosystem services which can be disrupted by coastal anthropogenic stressors such as microplastic pollution and algal blooms. Previous studies have shown that ingestion of microplastics can negatively affect the physiology, feeding behaviour, metabolism, and fecundity of bivalves but there is limited literature on the effects of microfibers, which is the most dominant microplastic shape in the marine environment. Furthermore, studies suggest that high phytoplankton concentrations, many times occurring under eutrophication conditions, reduce the phytoplankton clearance capacity of bivalves. However, there are no studies investigating the effects of microfiber pollution under algal bloom conditions which occur especially after storms and episodic rainfall events. Here we investigate the effects of three microfibers and two phytoplankton concentrations, representing acute events, on the phytoplankton clearance capacity of mussels. Furthermore, we test for any post-exposure effect of microfibers on the phytoplankton clearance capacity five days after the exposure. The results indicate no immediate impact of short-term exposure to microfibers. However, after five days of microfiber free conditions, a reduced phytoplankton consumption was detected suggesting a negative post-exposure effect on the ecosystem services.

2.1 Introduction

The healthy functioning of coastal ecosystems as well as their associated goods and services, heavily rely on bivalve populations (Prather *et al.*, 2013; Broszeit *et al.*, 2016; van der Schatte Olivier *et al.*, 2018). Through biofiltration and biodeposition, they facilitate the nutrient cycling by transforming organic nutrients to inorganic ones (Prather *et al.*, 2013; Broszeit *et al.*, 2016; Kent *et al.*, 2017), limit phytoplankton abundance and shape microalgal assemblage composition (Prins *et al.*, 1998; Tantanasarit *et al.*, 2013; van der Schatte Olivier *et al.*, 2018). However, the ability of bivalves to perform these services depends on the water quality, including the concentration and composition of suspended particles (Foster-Smith, 1975; Newell *et al.*, 2001; Saurel *et al.*, 2007). Identifying the water conditions that can interfere with the healthy functioning of bivalve populations is instrumental in policy-making and conservation of coastal ecosystems (Prather *et al.*, 2013; Rochman, 2016).

Coastal ecosystems face a range of stressors, mainly associated with anthropogenic activities, including microplastic pollution (Xu *et al.*, 2020; Alimi *et al.*, 2021) and nutrient inflows leading to eutrophication which in turn can promote Harmful Algal Blooms (Anderson *et al.*, 2002; Landsberg, 2002). In recent years microplastic pollution has been identified as one of the most common forms of marine pollution, interacting with marine biota worldwide (Ivar Do Sul and Costa, 2014; Napper and Thompson, 2020). Experimental studies on bivalves have shown that the ingestion and accumulation of microplastics can impact their physiology, feeding behaviour, metabolism, reproduction and behaviour (Zhang *et al.*, 2020; Baroja *et al.*, 2021). However, only a few studies have related these effects to the provisioning of ecosystem services such as biofiltration and removal of excess microalgal biomass from the water column (Christoforou *et al.*, 2020; Zhang *et al.*, 2020). Evidence also shows that microplastics can persist in the circulatory system of mussels for up to 48 days after exposure with lasting effects on their capacity to biofiltrate (Browne *et al.*, 2008). However, most research has focused on the effects of microbeads (Magni *et al.*, 2018; Baroja *et al.*, 2021) with little attention to microfibers (Christoforou *et al.*, 2020; Alnajar *et al.*, 2021), which are the most dominant microplastics in marine ecosystems (Davidson and

Dudas, 2016; Phuong *et al.*, 2016; Zhang *et al.*, 2020; Baroja *et al.*, 2021). Microfiber pollution has also been exacerbated by the recent COVID-19 pandemic due to the increased use and improper discarding of fibre-based face masks and wet wipes (Aragaw, 2020; Fadare and Okoffo, 2020; Shruti *et al.*, 2020, 2021; Shen *et al.*, 2021). Additionally to the impacts of microplastic, it has been suggested that the threshold for optimum filtration rate by mussels is between 2-6x10^6cells/L, phytoplankton concentrations higher than 5x10^6-1.15x10^7cells/L induce a reduction in their filtration rate, and trigger pseudofeces production at (Riisgård, 1991; Riisgård *et al.*, 2011). Concentrations of phytoplankton at 10^6cells/L and above also commensurate with eutrophication conditions (Anderson *et al.*, 2002; Spatharis *et al.*, 2007) hence, at acute algal bloom conditions, the phytoplankton capacity of mussels could be reduced.

To the best of our knowledge, there is no literature investigating the effects of microplastic pollution under algal bloom conditions, on the ecosystem functioning and services provided my bivalves. This is particularly important after storms and/or sever precipitation, leading to acute microplastic (Moore, 2008; Suckling and Richard, 2020) and algal bloom events (Spatharis *et al.*, 2007) in marine coastal areas. Specifically, water turbidity can re-suspend settled microplastics (Suckling and Richard, 2020) and nutrients from the sediment (Chen *et al.*, 2018) which in turn can enhance microalgae growth, high winds can transport microplastics from land and bloom forming microalgae cells from surface water to enclosed coastal areas (Davidson *et al.*, 2009; Li *et al.*, 2018) and increased rainfall would increase inflow from land sources like rivers, sewage systems and agricultural discharge (De Carlo *et al.*, 2007; Hoover and MacKenzie, 2009). These could lead to about a 97%, increase in the concentration of suspended microplastics (Hitchcock, 2020), that would become readily available to filter feeders. Therefore, if microplastic pollution indeed affects ecosystem services provided by bivalves, this is expected to exacerbate the effects of eutrophication and algal blooms on coastal bivalve populations and the associated ecosystem services they contribute to.

To address the knowledge gaps identified above, this study investigates how the phytoplankton clearance capacity of mussels is affected by short-term exposure

to microfibers under algal bloom conditions. A laboratory experiment, using a crossed design, was performed exposing mussels, for 24 hours, to three microfiber and two phytoplankton concentrations: one within the mussel optimal feeding concentration range and one above the threshold for reduction of the clearance capacity (Riisgård, 1991; Riisgård *et al.*, 2011), but both representing algal bloom conditions. We hypothesize that the phytoplankton clearance capacity of mussels would be lower at the high suboptimal phytoplankton concentration and decrease as the microfiber concentration increase. The high suboptimal phytoplankton and the highest microfiber concentration are expected to be the most impactful due to a synergistic effect of the two stressors. In a subsequent experiment, we tested for potential post-exposure effects of microfibers on the phytoplankton clearance capacity of mussels. With the assumption that microfibers would accumulate in the mussels during the 24-hour exposure period, we hypothesize that after five days of microfiber-free conditions, the mussels exposed to microfibers would have a decreased clearance capacity, in comparison to non-exposed ones.

2.2 Methods

2.2.1 Experimental design

Experiment 1: Short-term effects of microfibers

With the first experiment we wanted to investigate how the phytoplankton clearance capacity of mussels was affected by acute microfiber concentrations during eutrophication events. To achieve this, the experiment was a crossed design of three microfiber (0, 12000 and 110000 mf/L) and two *Tetraselmis* sp. phytoplankton monoculture (see S2.1) concentrations ($1.87x10^6$ and $4.68x10^7$cells/L), where the lower concentration ($1.87x10^6$cells/L) was within mussels' optimal feeding concentration range while the higher concentration ($4.68x10^7$cells/L) was above the threshold at which clearance capacity is reduced (Riisgård, 1991; Riisgård *et al.*, 2011), but both are resembling eutrophication conditions (Figure 2-1). To account for potential variability in the phytoplankton clearance capacity between mussels collected from different sites and size groups, ten replicates of small (4.02cm±0.42S.D.) and ten replicates of large (5.85cm±0.45S.D.) mussels, from two collection sites, were tested. The total

sample size was thus 240 mussels (3 microfiber concentrations x 2 phytoplankton concentrations x 2 collection sites x 2 size groups x 10 replicates). Towards the end of the experiment, the *Tetraselmis* monoculture became contaminated, and the continuation with the last five small groups of mussels from the second collection site was deemed impossible. Therefore, the final sample size was 210. Each of the 210 experimental mussels was exposed to phytoplankton with/without microfibers for 24 hours and the phytoplankton consumed by mussels within that time was calculated (see experimental settings (2.2.3) and sample analysis (2.2.4) further below).

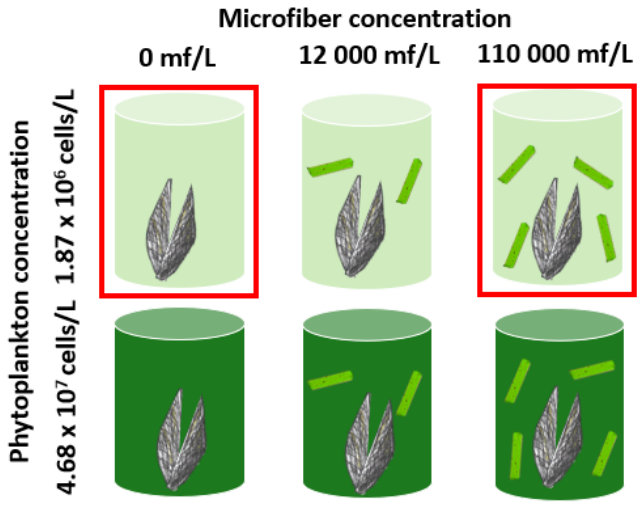

Figure 2-1:Illustration of the experimental design. For the first experiment, the microfiber concentrations of 0, 12 000 and 110 000mf/L are indicated by 0, 2 and 4 small rectangles, respectively, in the experimental vessels. The light and dark green represent the phytoplankton concentrations of $1.87x10^6$ and $4.68x10^7$ cells/L resembling eutrophication conditions, respectively. Each set of six treatments was repeated with large and small mussels collected from two sites to account for any variability between them. Red rectangles indicate the treatments from which mussels were used in experiment 2.

Experiment 2: Post-exposure effects of microfibers

To test for possible post-exposure effect of microfibers during the first experiment, a second experiment was performed with mussels from the lower optimal phytoplankton concentration ($1.87x10^6$cells/L) at the no microfibers (0mf/L) and highest microfiber concentration (110 000mf/L) treatments (see red rectangles in Figure 2-1). It has been reported that microplastics accumulated in

mussels significantly reduce after 93 hours (~four days) of microplastic-free conditions (Birnstiel *et al.*, 2019) therefore mussels remained in their experimental vessels after the first experiment, their water was changed daily for the next three days, and $3x10^6$cells/L *Tetraselmis* sp. monoculture was added daily. On the fourth day mussels were starved and on the fifth day mussels were provided with $1.87x10^6$ cells/L *Tetraselmis* sp. monoculture for 24hours after which their phytoplankton consumption was calculated. In this experiment no microfibers were added.

2.2.2 Study species, field collection and laboratory acclimation

The study was performed using the mussel *Mytilus edulis*, an economically important (van der Schatte Olivier et al., 2018) and globally abundant bivalve, that usually inhabits intertidal environments. Mussels were collected in Scotland, from Arrochar (56.199716, -4.747824) and Millport (55.750768, -4.931442) coastal sites on September 12th (Water temperature: 13 ^oC, salinity: 20 ppm) and October 29th (Water temperature: 12^oC, salinity: 21 ppm), 2018, respectively. In both locations mussels were intertidal hence the collection occurred at low tide when the mussels were not submerged.

The two locations are situated within the Firth of Clyde, Scotland (McIntyre *et al.*, 2012) with Arrochar being at the edge of Loch Long toward the mainland and Millport being situated closer to the open ocean. Due to their geographic position, Arrochar is characterised as a 'litter sink' because of the high levels of marine litter accumulated in the area (McIntyre *et al.*, 2012; Turrell, 2018) while algal bloom events have been reported at Millport (McIntyre *et al.*, 2012). Therefore, the two locations were deemed appropriate for the current study to determine any possible variation in the effects of microfibers on the clearance capacity between individual mussels from populations that were previously exposed to microplastics pollution or algae bloom conditions. It is however recognised that various other abiotic factors could vary between the two sites which could inhibit the direct association of the results to any pre-exposure to the conditions.

Upon collection, epibionts such as algal biofilm, macroalgae and barnacles were scraped off mussel shells and mussels were then transported to our laboratory at

the University of Glasgow. Previous studied have suggested that the filtration rate of mussels can vary based on their size (Riisgård and Møhlenberg, 1979; Tantanasarit *et al.*, 2013) hence the collected mussels were separated in the small and large size groups in order to detect any possible variation between the two size groups. The organisms were then placed in constantly aerated artificial sea water (salinity: 32ppm) rectangular aquariums (5L). Every day a new set of six mussels was randomly selected from the intended size group and individually placed in experimental vessels where they acclimated for three days. The water was changed daily, and a concentration of $3x10^6$cells/L (Riisgård, 1991) Tetraselmis monoculture was added. On the fourth day, acclimated mussels were starved and experiment 1 was initiated. This process was repeated with a new set of six mussels for a total of 35 experimental days, lasting 24hours each. Both the aquariums and experimental vessels were under the same conditions as described in the experimental settings.

2.2.3 Experimental setting

In both experiments, each mussel was placed in a glass cylindrical vessel (800ml, 7.3cm diameter x 25cm height) equipped with a mesh stand (4cm height) to support the mussels above the bottom of the vessels for better water circulation. The experimental vessels were in a wooden box and water baths, maintaining a constant temperature of 13^oC and a 12:12 hour photoperiod. For each experimental vessel, 825ml of artificial salt water (32ppm) was prepared as per the treatment requirements i.e., microfibers and phytoplankton concentrations. Water samples (25ml) were collected from the prepared solution, and the rest was added to the experimental vessels. Each vessel was constantly aerated by an air stone and mixed by a stirring magnet (2.5cm, 320rpm, 10-position magnetic stirrer IKA®RO10) which was located below the mesh stand. The exposure duration was 24 hours, after which another 25ml water sample was collected. These samples were used to quantify the phytoplankton concentration at the beginning (0h) and end (24h) of the experiment.

In the first experiment the microfiber concentrations (12 000 and 110 000mf/L) used in the treatments were higher than observed concentrations in the field

(Baroja et al., 2021); however, they were chosen to represent acute events of microplastic pollution which could potentially affect the organisms after a short-term exposure (Suckling and Richard, 2020) and they are in accordance with other microplastic experimental studies (Browne *et al.*, 2008; Van Cauwenberghe, Devriese, *et al.*, 2015; Woods *et al.*, 2018). The microfibers used were polyamide-6 (PA), commonly known as nylon, which is found in nets and ropes (Ryan and Turra, 2019) used in fishery and shellfish aquaculture industries. Furthermore, nylon is found in synthetic textiles (Cesa *et al.*, 2017) from which shredded fibres, after wash, find their way to marine environments through the sewage system (Cesa *et al.*, 2017; Lebreton *et al.*, 2017). Moreover, nylon has similar buoyancy to seawater allowing its widespread distribution along the water column (Cole *et al.*, 2011) and accessibility to filter feeders of the marine environment. Nylon microfibres are not available for purchase, therefore the microfibers used were prepared in the laboratory according to Cole (2016). Use of this method allowed cutting the fibres to a size close to the mussel feeding range (Defossez and Hawkins, 1997; Christoforou *et al.*, 2020) which was 10μm in diameter and <100μm in length (S2.2). Their unique shape and size also deemed them distinguishable from air-born microfibers (S2.3).

Furthermore, the concentrations of *Tetraselmis* used in experiment 1 were determined by a dose response curve established by pilot experiments (Appendix 1: A2.1). Both concentrations are within the range of algal concentration found in areas subjected to eutrophication in the field (Anderson et al., 2002; Davidson et al., 2009; Spatharis et al., 2007). The lowest concentration ($1.87x10^6$ cells/L) was at the mussel optimum filtration threshold of $x10^6$cells/L (Riisgård, 1991; Riisgård *et al.*, 2011) while the highest concentration ($4.68x10^7$ cells/L) is above that threshold where clearance capacity is expected to decrease and pseudofeces production is triggered.

2.2.4 Sample Analysis

The phytoplankton concentration, at each sampling point, was quantified as per Christoforou et al. (2020). In summary, the samples were filtered using 0.45μm SartoriusTM Cellulose Nitrate Filters. The filters were then dried, made

transparent with the addition of immersion oil, and placed under a light microscope equipped with a microscope camera. Then the phytoplankton cells were enumerated, and the percentage of phytoplankton concentration change was determined by the following equation:

$$Phytoplankton\ Consumption = \frac{Concentration\ (0h) - Concentration\ (24h)}{Concentration\ (0h)} * 100.$$

2.2.5 Data Analysis

Generalised linear models (GLMs) were used to address the objectives of this study. The response variable tested was the percentage of phytoplankton consumed by mussels, which is used as a proxy of the ecosystem services provided. This response variable consists of proportional data constrained between zero and one, hence beta distribution was applied. Since this distribution does not allow exact values of zero and one, the following equation was used to transform the data:

$$Ytransformed = \frac{[Y * (N - 1) + 0.5]}{N},$$

where *Ytransformed* is the transformed value of the proportion of phytoplankton consumed, Y, and N is the sample size (Smithson and Verkuilen, 2006).

To address the first objective, we included the microfiber treatment (0, 12000 and 110000 mf/L), the phytoplankton treatment ($1.87x10^6$ and $4.68x10^7$cells/L), the collection site (binomial variable), the size group (binomial variable) and the two-way interactions between all these explanatory variables as fixed effects in the GLM. To address the second objective, we include as fixed effects the presence or absence of microfibers at the first experiment (binomial variable), the collection site (binomial variable), the size group (binomial variable), and two-way interactions between all variables. All data were included in the models as no outliers were detected by the interquartile range techniques (Vinutha *et al.*, 2018). Model selection was conducted using model AIC comparisons and the selection was also verified by Likelihood Ratio Test (LRT). The analysis was

performed in R v.4.0.4 (R Core Team, 2021) and the Tukey method, in the emmeans package v1.5.2-1 (Lenth, 2018), was utilized to perform pairwise comparisons in the event that a variable was significant.

2.3 Results

2.3.1 Experiment 1: Short-term effects of microfibers

There were no significant differences in the mussel phytoplankton consumption between the microfiber nor the phytoplankton treatments (Table 2-1, Figure 2-2). Furthermore, neither collection site nor the size group had a significant effect on the phytoplankton consumption.

Table 2-1: Summary of the best-supported model explaining the variation in phytoplankton consumption by mussels. ΔAIC and LRT indicate the increase in AIC and difference in the log likelihood of the model given the data, respectively, if the variable was dropped. Asterisks signify the significance level (<0.05*).

	Estimate	**Std. Error**	**Z value**	**P value**	**Δ AIC if dropped**	**LRT if dropped**
Experiment 1: Short-term effects of microfibers						
Intercept	0.0676	0.0845	0.8	0.424		
Experiment 2: Post-exposure effects of microfibers						
Intercept	-0.1288	0.1509	-0.854	0.3933		
Fibre (Y/N)	0.5353	0.2181	2.454	0.0141*	3.892	Df=1, 0.0152*

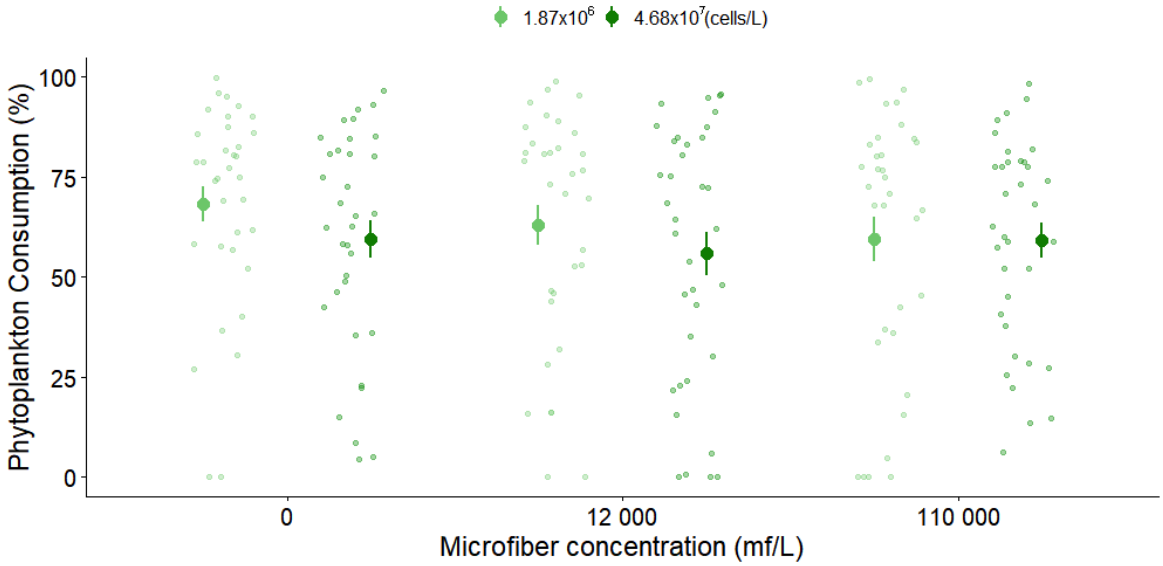

Figure 2-2: The percentage (Mean ± SE) of phytoplankton consumed by mussels at the three microfiber treatments (0, 12 000 and 110 000mf/L) within the two phytoplankton concentrations (Light green – 1.87x106cell/L and Dark green – 4.68x107cell/L). No significant difference of observed between the microfiber or the phytoplankton treatment.

2.3.2 Experiment 2: Post-exposure effects of microfibers

Microfibers had a post-exposure negative effect on the phytoplankton consumed by mussels (Table 2-1). Specifically, mussels that were exposed to microfibers five days before (see experiment 1) had a significantly lower phytoplankton consumption by 10.49%, in comparison to mussels of the microfiber-free treatment (Tukey, p=0.0168,

Figure 2-3). Eight mussels that were exposed to microfibers and only one from the microfiber-free treatment did not consume any phytoplankton during this experiment, but no mortality was observed. The collection site or size group had no effect on the phytoplankton consumed in this experiment.

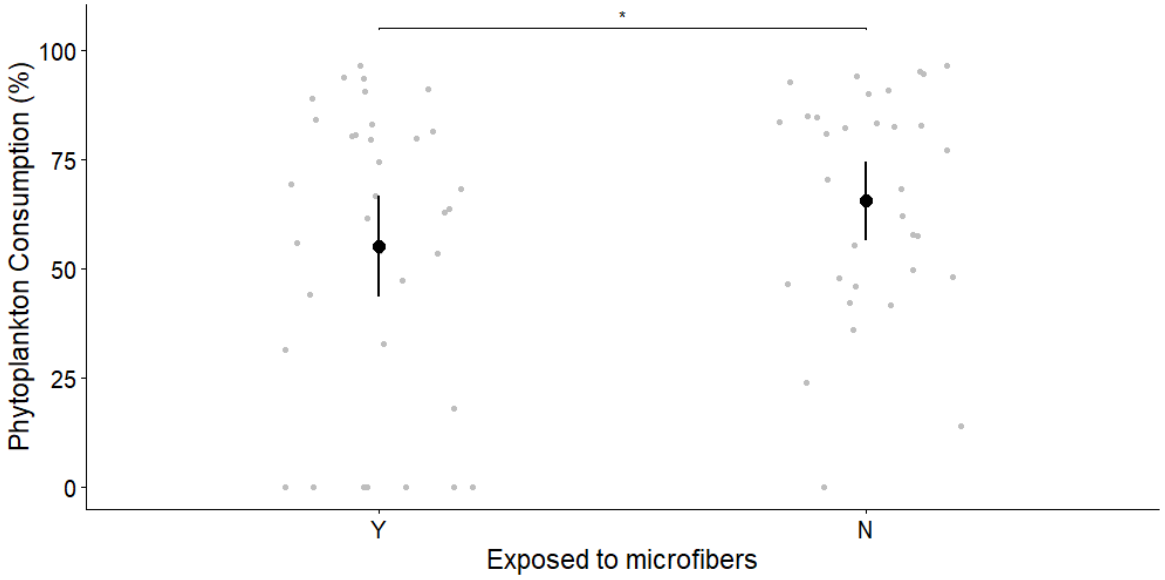

Figure 2-3: The percentage (Mean ± SE) of phytoplankton consumed by mussels five days after a 24-hour exposure to $1.87x10^6$cells/L phytoplankton concentration, in the presence (Y) and absence (N) of microfibers (110 000mf/L). The asterisk (*) indicated the significant difference of $p<0.05$ where the microfiber exposed mussels had a lower phytoplankton consumption than the mussels in the microfiber-free conditions.

2.4 Discussion

The results of this study suggest that short-term exposure to microfibers does not have an immediate impact on the phytoplankton clearance by the blue mussel *Mytilus edulis*. However, after five days of microfiber-free conditions, a reduced phytoplankton consumption was observed in mussels that had been exposed to microfibers, suggesting a post-exposure negative effect on the provisioning of ecosystem services.

The microfiber exposure did not immediately impact the mussel's phytoplankton consumption despite the high concentrations used. Thus, the results do not confirm our hypothesis that microfiber exposure would have a negative effect on the mussels' performance. Lack of change on the feeding activity, at the presence of polystyrene microspheres, was also observed by Browne et al., (2008). As in our experiment, Browne et al., (2008) also performed a short-term experiment, with only 3 hour exposure duration. These results indicate that short-term exposures might not lead to measurable effect on foraging intake, stressing the need for long-term exposure experiments. On the other hand, Harris and Carrington, (2020)

did show a decrease in the clearance capacity of mussels after only 1 hour of submersion in a microplastic treatment. This difference could be explained by the fact that the latter study used $2.5x10^6$microplastics/L, which is one to two orders of magnitude higher than the former studies and four orders of magnitude higher than realistic microplastic concentrations (Baroja *et al.*, 2021). Hence, the results from short-term studies could assist in anticipating the response of bivalves after acute and extreme microplastic pollution events. However, long-term microplastic exposure studies, at realistic concentrations, are vital in understanding the chronic impacts of these stressors on bivalve populations occurring in polluted waters.

Mussels in the current study did not show any difference in their clearance capacity when given the concentration within the optimal range ($1.87x10^6$cells/L) or the above-optimal concentration ($4.68x10^7$cells/L) of *Tetraselmis* sp. On the contrary, in the first experiment, mussels consumed on average $1.19x10^6$ and $2.72x10^7$cells/individual where the latter is 41.91% higher than the upper saturation threshold of $4.3x10^6$–$1.14x10^7$cells/individual (Riisgård, 1991; Riisgård *et al.*, 2011). Wegner et al. (2012) also detected no change in the bivalve gape, used as a proxy for consumption, at phytoplankton concentrations of $6x10^7$ and $1.2x10^8$ cells/L. These results show that mussel populations have the capacity to provide ecosystem services, even at concentrations above the optimal filtration concentrations that had been previously suggested, emphasising the importance of bivalve populations in alleviating the effects of eutrophication and algal blooms of non-toxic microalgal species. Further investigation into the synergistic effect of microfibers and blooms of toxic microalgal species would be an interesting topic of research as previous work has identified a range of effects of toxic algae on bivalves (Matsuyama *et al.*, 1997; Galimany *et al.*, 2008; Peperzak and Poelman, 2008; Detree *et al.*, 2016).

A post-exposure negative effect of microfiber pollution was identified even five days after the 24-hour microfiber exposure. Mussels that had been exposed to the microfiber treatment consumed on average less phytoplankton than the ones in the microfiber treatment. These results might be influenced by seven mussels, which had been exposed to microfibers, that had not consumed any phytoplankton

in the second experiment even though no mortality was identified and none of the data points were determined as outliers. An assumption for this outcome would be the possible accumulation of microfibers into the mussels' digestive gland (Christoforou *et al.*, 2020). This could result in alternations to the gut microbiome of mussels (Li *et al.*, 2020), excess energy expenditure in the attempt to digest non-nutritious particles (Harris and Carrington, 2020) and to the feeling of fullness which would eventually lead to the organism's starvation (Gall and Thompson, 2015). However, further investigation would be necessary to clarify the variation identified, with focus on the ingestion, accumulation, and translocation of microfibers in different bivalve organs, the duration of their persistence and their potential effects. Another possible explanation of this result could be an initial low condition index of those individual which may have prevented them from feeding. Therefore, to detangle between the fitness of the individuals and the treatment effects, the measurement of the condition index of the organisms before and after the experiment could be a valuable co-variable in future studies.

This study also investigated the possible variation in the response of mussels from two different locations and size groups. None of these co-variables was statistically significant in the experiments performed however, the effect of microfiber pollution and algal bloom conditions was only tested for a short-term, resembling only acute environmental conditions. Further investigation would be necessary to identify any effects of chronic exposure due to stressors like microplastic pollution and algal blooms at the mussel population site. It is also important for future studies to investigate and take into consideration the associated environmental conditions and the characteristics of the collection site as these biotic and abiotic factors may affect the response of the experimental organisms.

2.5 Conclusion

The results of this study suggest that short-term exposure to acute microfibers concentrations under eutrophication conditions does not have an immediate impact on the mussels' clearance capacity. There was a post-exposure negative effect on the phytoplankton clearance capacity of mussels, five days after the

microfiber exposure. Therefore, even 24-hours of acute microfiber and eutrophication conditions could negatively affect the ecosystem services provided by mussels which can exacerbate the effect of algal blooms in marine coastal ecosystems.

Acknowledgements

For this work we would like to thank Nissad Development Company and The A.G. Leventis Foundation (sponsors had not further involvement in this research). We would also like to thank the IBAHCM Aquaria Staff for their technical support as well as Alexandra Sliva, Eliska Kosova and Emma Teelucksingh for their help in implementing the experiment.

Chapter 3

Effects of long-term exposure to microfibers on ecosystem services provided by coastal mussels

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Abstract

The biofiltration capacity of bivalve populations is known to alleviate the effects of coastal eutrophication. However, this important ecosystem service could potentially be impaired by the increasing microplastic abundance in near shore environments. It is known that relatively large microplastics (~500μm) impair the filtration capacity of bivalves, however, the effect of smaller microplastics, and specifically microfibers, is not known even though they are more common in many natural systems and similar in size to phytoplankton, the main food source of mussels. Here, we investigated the effects of long-term exposure to microfibers (MFs), which are smaller than 100μm, on the biofiltration capacity of the blue mussel, *Mytilus edulis*. Our findings show that long-term exposure (here 39 days) to microfibers significantly reduced (-21%) the clearance of phytoplankton (*Tetraselmis* sp). While previous studies have shown that larger microplastics can decrease the filtration capacity of mussels after short-term exposure, our findings suggest that, for smaller MFs, mussel's clearance capacity is significantly affected after long-term exposure (39 days in this study). This may be due to the accumulation of MFs in the digestive system. In addition, the most efficient phytoplankton consumers were more susceptible to MF accumulation in the digestive system. This suggests that prolonged exposure to MF of coastal mussels could negatively impact the biofiltration of more potent individuals, thus decreasing the ecosystem service potential of the population.

3.1 Introduction

The intensification of anthropogenic activities along the coastline poses critical environmental pressures on coastal ecosystems. Specifically, coastal eutrophication and Harmful Algal Blooms (HABs) are currently ranked as the most critical stressors of marine ecosystems (Anderson *et al.*, 2002; Kellogg *et al.*, 2014; van der Schatte Olivier *et al.*, 2018), with important implications on both ecosystem and public health (Landsberg, 2002). These effects can be remediated by the ecosystem services provided by filter-feeding organisms, such as bivalves, that remove excess microalgal biomass from the water column (Prins *et al.*, 1998; Tantanasarit *et al.*, 2013; van der Schatte Olivier *et al.*, 2018) and make nutrients available to bottom feeders by biodeposition on the sediment (Kellogg *et al.*, 2014; van der Schatte Olivier *et al.*, 2018). However, coastal ecosystems are also subject to a variety of environmental stressors, such as plastic pollution, which could impact the ability of bivalves to perform these services. Investigating the potential effect of such stressors on the ability of bivalves to perform ecosystem services is thus of fundamental importance for our understanding of coastal ecosystems (Fisher *et al.*, 2008) and is necessary for informing evidence-based environmental policies (Rochman, 2016).

Microplastic (<5mm) pollution has been recently identified as a major environmental stressor in coastal systems and associated biological communities (Mathalon and Hill, 2014; Ryan and Turra, 2019). Previous studies have shown that the ingestion of microplastics by bivalves can result in reduced filtration rates (Rist *et al.*, 2016; Xu *et al.*, 2017; Woods *et al.*, 2018), decreased respiration (Rist *et al.*, 2016), lower energy intake (Xu *et al.*, 2017), inflammation of cell tissue (Von Moos *et al.*, 2012), damaged gills (Cheung and Shin, 2005) and reduced fecundity (Sussarellu *et al.*, 2016; Gardon *et al.*, 2018). While most of these studies have focused on microplastic fragments and beads (Von Moos *et al.*, 2012; Rist *et al.*, 2016; Sussarellu *et al.*, 2016; Xu *et al.*, 2017; Gardon *et al.*, 2018), very little is known about the effect of microfibers (MFs), which is the dominant form of microplastics in the marine environment (Davidson and Dudas, 2016; Qu *et al.*, 2018; Railo *et al.*, 2018; Covernton *et al.*, 2019). The underrepresentation of MFs in studies is mainly due to fact that MFs are not available for commercial

purchase and their preparation in the lab is tedious, thus experimentations especially with specific size ranges are scarce (Wagner *et al.*, 2017). As MFs within the 10-40µm size range are both within the preferred feeding size range of mussels (Ruppert *et al.*, 2004; Strohmeier *et al.*, 2012; Van Cauwenberghe, Claessens, *et al.*, 2015; Willer and Aldridge, 2017; Fernandez *et al.*, 2019) and represent the majority of MFs in the water column (Thompson *et al.*, 2004; Doyle *et al.*, 2011; Covernton *et al.*, 2019) more information about their ecosystem effects is urgently needed.

Browne *et al.*, (2008) showed that ingested polystyrene microspheres (3 and 10µm) were translocated to the circulatory system of the marine bivalve *M. edulis* and remain there for more than 48 days. Furthermore, Von Moos *et al.*, (2012) demonstrated that small plastic particles (0-80µm) were taken up into epithelial cells of the digestive system of mussels, where they induced a strong inflammatory response. Hence, smaller particles are more likely to be ingested and seem to undergo translocation more readily than particles larger than 100µm (Kolandhasamy *et al.*, 2018; Ward, Zhao, *et al.*, 2019). However, most of these studies have focused on short-term exposure and the subsequent acute effects, while little is known about chronic consequences of continuous long-term exposure to MFs. Here, we hypothesize that the translocation and long-term presence of particles <100µm into the digestive system and tissue of organisms, will negatively affect the ecosystem service of phytoplankton clearance by coastal mussel populations.

To test this hypothesis, we investigated, in a lab experiment, the impact of long-term exposure to MFs on the ability of mussels to remove excess biomass of microalgae from the water column. The main objectives of this study were (a) to investigate the phytoplankton removal capacity of individual mussels throughout a period of continuous exposure under pristine and MF-polluted conditions and (b) to identify any relationship between phytoplankton removal capacity and amount of MFs accumulated in the digestive system of mussels.

3.2 Materials and Methods

3.2.1 Microfiber preparation

We used nylon as the material for our MFs as this is one of the most common materials of MFs found in the environment. The abundance of these MFs can be attributed to nylon's extensive use in aquaculture and fisheries (e.g., nets and ropes) (Cole *et al.*, 2011; Davidson and Dudas, 2016; Ryan and Turra, 2019) as well as the clothing industry (e.g., synthetic textile fibers released in effluent water from washing machines) (Browne *et al.*, 2011; Magnusson and Norén, 2014; J. Li *et al.*, 2015; Cesa *et al.*, 2017). Additionally, nylon, having neutral buoyancy, can be widely distributed within the water column (Cole *et al.*, 2011) thus being highly bioavailable to filter-feeding organisms.

The microfibers were prepared as per Cole (2016). In summary, nylon (polyamide 6) threads (10µm diameter) were encapsulated within a freezing agent, solidified in dry ice and a cryotome machine was used to cut them in 30µm length. The freezing agent was then melted, and the cylindrical MFs were retrieved. The resulting length was 35.20µm (± 12.9S.D) with only 8% of the MF being >100µm long (S2.2). Although this method is not widely used due to the increased requirement in time and effort, the MFs produced are highly appropriate for experimentation purposes as they have specific shape and structure. This renders them easily distinguishable from other types of MFs potentially encountered in samples due to airborne inputs.

3.2.2 Mussel collection and acclimation

For this experiment, rope-grown, juvenile mussels (33.9mm; ±1.6S.D.) were collected from Loch Sunart (56°41'15.7"N, 5°36'55.0"W) in May 2019. *M. edulis* was selected as a model organism due to its (a) global coastal distribution (MacDonald and Ward, 2009), (b) low position at the trophic chain (Rist *et al.*, 2016), (c) great abundance, particularly near polluted and eutrophic sites (Beyer *et al.*, 2017; J. Li *et al.*, 2019), (d) greater water clearance rate in comparison to other bivalves (MacDonald and Ward, 2009), and (e) economic importance e.g., in shellfish aquaculture (Willer and Aldridge, 2017; van der Schatte Olivier *et al.*, 2018).

In the laboratory, mussels were placed in 5L aquariums containing artificial saltwater (salinity 32ppm) and were allowed to purify for two days with continuous monitoring of water chemistry indicators such as ammonia and nitrates. For acclimation to the experimental conditions, forty-four mussels were individually placed in 800ml glass vessels equipped with cylindrical mesh stands to support the mussels at a standard height of 4cm from the bottom across all vessels. The experimental vessels were under diurnal photoperiod (12:12) and the water temperature was maintained between 12-13°C and was constantly aerated via air pumps to also ensure sufficient mixing of the water column. Mussels were fed $3x10^6$cells/L of *Tetraselmis* sp. monoculture (Riisgård, 1991) once per day for another two days (Defossez and Hawkins, 1997; Browne *et al.*, 2008). On the 5th day the mussels were starved for 24 hours prior to the initiation of the experiment.

3.2.3 Experimental Design

The experiment consisted of a MF exposure treatment and a control treatment that was lacking MFs, and each treatment consisted of 22 replicates (i.e., 22 glass vessels containing a single mussel each). Mussels in both treatments were fed daily, with a single dose of *Tetraselmis* sp. at a concentration of $3x10^6$cells/L (S2.1). A concentration of 24,000MF/L of MFs was also added only to the MF treatment at the time of feeding. The continuous aeration of the water ensured the constant resuspension of MFs and *Tetraselmis* cells in the water column. To avoid airborne microplastics contamination, cotton lab-coat and vinyl gloves were worn at all stages of the study. Furthermore, the experimental vessels were located in a wooden enclosed box minimising the settlement of airborne fibres in our vessels. The ambient conditions were maintained as detailed above and artificial salt-water was changed every second day. The water changes and daily feeding was performed at the end of the light period. The mussels' shell (length) was measured at the beginning and end of the experiment to determine any effect of microfibers on their growth.

The total duration of the experiment was 52 days and water samples for the quantification of phytoplankton consumption were taken every 13 days after day 1, for a total of 5 sampling points (Days 1, 13, 26, 39 and 52). For each sampling

point, the glass vessels were drained and cleaned, then 870ml of water per vessel were prepared with the addition of *Tetraselmis* sp. and MFs, as per treatment requirements. Water samples (70ml) were collected from each experimental vessel at 0 and 24 hours to measure the concentration of algae and MFs. The phytoplankton and MF percentage consumption at each time point was thus estimated as:

$$Phytoplankton\ Consumption = \frac{Concentration\ (0h) - Concentration\ (24h)}{Concentration\ (0h)} * 100.$$

There is no literature, at the moment, focusing on concentrations of <100μm microplastics, probably due to the challenging methodologies involved in the sampling and quantification of such small microplastic fractions in the marine environment. Reports of current marine concentrations of microplastics >100μm are of limited guidance here as different studies have suggested that the ambient concentrations of smaller microplastics are underestimated (Phuong *et al.*, 2016; Barrows *et al.*, 2017; Covernton *et al.*, 2019; Lindeque *et al.*, 2020). Therefore, due to the lack of available published data, we used a concentration of 24,000MF/L, in accordance with other mussel exposure studies. Woods et al. (2018) and Wang et al. (2020) used concentrations in the range of 3,000-30,000particles/L and 10-1,000,000particles/L respectively while higher concentration of 42,000 particles/L and 110,000particles/L were used by Van Cauwenberghe et al. (2015) and Browne et al. (2008) respectively.

Long-term experiments with mussels are subject to contamination with periphyton diatom species that are attached as biofilm to the inner and outer shell of the mussels collected from the field (Pérès *et al.*, 1996; Sweat, 2016). To minimise the impact of these opportunistic diatoms, the surfaces inside the experimental containers (glass and mussels) were cleaned every second day. Moreover, to account for potential effects of diatom contamination in our statistical inference, water samples (50ml) were analysed spectrophotometrically (Parsons *et al.*, 1984) and chlorophyll-c, a proxy for diatom biomass, was accounted for in our models.

3.2.4 Sample Analysis

For the quantification of phytoplankton removal capacity of mussels, 20ml of water, from samples preserved with lugol, were filtered using Sartorius™ Cellulose Nitrate Membrane Filters (0.45μm pore size, 25mm diameter). The filters were then dried for one hour in an incubator at 40°C. Each filter was then made transparent using immersion oil and examined under a light microscope. The phytoplankton cells were counted in 15 randomly selected fields of view (coefficient of variation <0.7), on the surface of the filter paper (MFs were also estimated for our records). Manual counting was preferred to an automated technique to ensure sensitivity of counting at low phytoplankton concentrations (i.e., after 24h of feeding), to enable the distinction of phytoplankton from MFs, and to avoid the overestimation of counts due to the potential presence of other particles such as airborne fibres, mussel faeces, pseudofaeces and gametes.

For the quantification of MFs in the digestive track of mussels, the organisms were individually wrapped and preserved in a -20°C freezer upon the termination of the experiment. Each mussel was defrosted for 30 minutes in room temperature before the soft tissue was removed from the shell and washed under running Milli-Q water for 30 seconds to eliminate any MF possibly attached to the surface of the mussel's tissue (Kolandhasamy *et al.*, 2018). The digestive gland, which surrounds the stomach (Morton and Puljas, 2018), was separated from the rest of the mantle and organs. The digestive gland and stomach were then immerged in a 25ml, 0.31% trypsin solution (Courtene-Jones *et al.*, 2017), and gently stirred for 30mins at 45°C . The solution was then centrifuged at 3,500rpm, 15°C for 15 minutes resulting in the settlement of organic matter and MFs at the bottom of the tube as precipitate. Most of the supernatant was removed, leaving about 1ml to prevent any disturbance to the precipitate layer, which was then homogenised using a pipette. The homogenised mixture was inspected under an optical microscope (x10/0.25) and all laboratory-produced MFs were quantified (smallest MF size detected was 13.8μm).

3.2.5 Data analysis

To test the effect of treatment, sampling day and diatom fouling (chlorophyll-c) on the phytoplankton percentage consumption by mussels, we used a generalised linear mixed model (GLMM). The response variable, comprising of proportions bounded between 0 and 1, was not normally distributed (Shapiro-Wilk normality test, p-value<0.05), thus the beta distribution was used to model the data. Since the beta distribution does not accept exact values of zero and one, data were transformed using the following equation:

$$Ytransformed = \frac{[Y * (N - 1) + 0.5]}{N},$$

where *Ytransformed* is the transformed value of the phytoplankton consumption proportion, Y, and N is the sample size (Smithson and Verkuilen, 2006). Sampling day was included as a continuous variable to account for the long-term effects of the MFs. Mussel ID was included as random effect to account for repeated, non-independent measures taken from the same animal. The possible models were fitted using the R glmmTMB package (Brooks *et al.*, 2017) and model selection was performed based on Likelihood Ratio Tests (LRT). Independent t-tests were used to compare treatment effects within the same sampling day. To determine the effect of MFs on the growth of mussels we used a linear model with the treatment as an explanatory variable.

A linear model was used to test for the effect of phytoplankton consumption, MF consumption and diatom fouling on the MFs accumulated in the digestive gland and stomach. Prior to the analysis, data were log-transformed to eliminate heteroscedasticity in MF counts (0) across the values of phytoplankton consumption.

3.3 Results

3.3.1 Effect of long-term MF exposure on phytoplankton removal capacity

The average consumption of *Tetraselmis* cells across the treatments and sampling points was 85.9% (± 18.8S.D.). Across sampling points, the average *Tetraselmis* consumption in the MF exposure treatment was 83.1% (± 20.6S.D.) whereas in the control treatment was 88.7% (±16.5S.D.). There was a significant interaction between treatment and sampling day (Table 3-1), which suggests that the effect of MFs on the phytoplankton removal capacity by mussels varied in time. After 26 days of exposure, mussels exposed to MFs showed a greater variation in the phytoplankton consumption i.e., clearance capacity (Figure 3-1). On day 39, mussels exposed to MFs had a significantly lower phytoplankton removal capacity by 21.3% compared to the mussels in the control treatment (t-test, p=0.0014, N=44) (Figure 3-1). On the last sampling day (day52) there was no significant difference between the two treatments (t-test, p=0.17, N=44) (Figure 3-1). This coincided with a spark of opportunistic diatoms across all replicates (0), which had a significant negative effect on *Tetraselmis* consumption by mussels No significant difference was observed between the growth of mussels in the control (0.50mm; ±0.22S.D.) and microfiber (0.59mm; ±0.22S.D.) treatments ($F_{1,42}$=1.5728, p=0.2167) upon termination of the experiment.

Table 3-1: Summary of the best-supported model explaining the variation in phytoplankton removal capacity by mussels. ΔAIC and LRT indicate the increase in AIC and difference in the log likelihood of the model given the data, respectively, if the variable was dropped. Dash (-) indicate interaction between the variables and asterisks signify the significance level (<0.001***, <0.01**, <0.05*).

	Estimate	**Std. Error**	**Z value**	**P value**	**Δ AIC if dropped**	**LRT if dropped**
Intercept	1.95	0.229	8.49	<0.001***		
Chlorophyll-C	-2.62	0.723	-3.61	<0.001***	10.4	Df=1, <0.001***
Day-treatment	-0.018	0.007	-2.36	<0.018*	8.3	Df=3, <0.002**

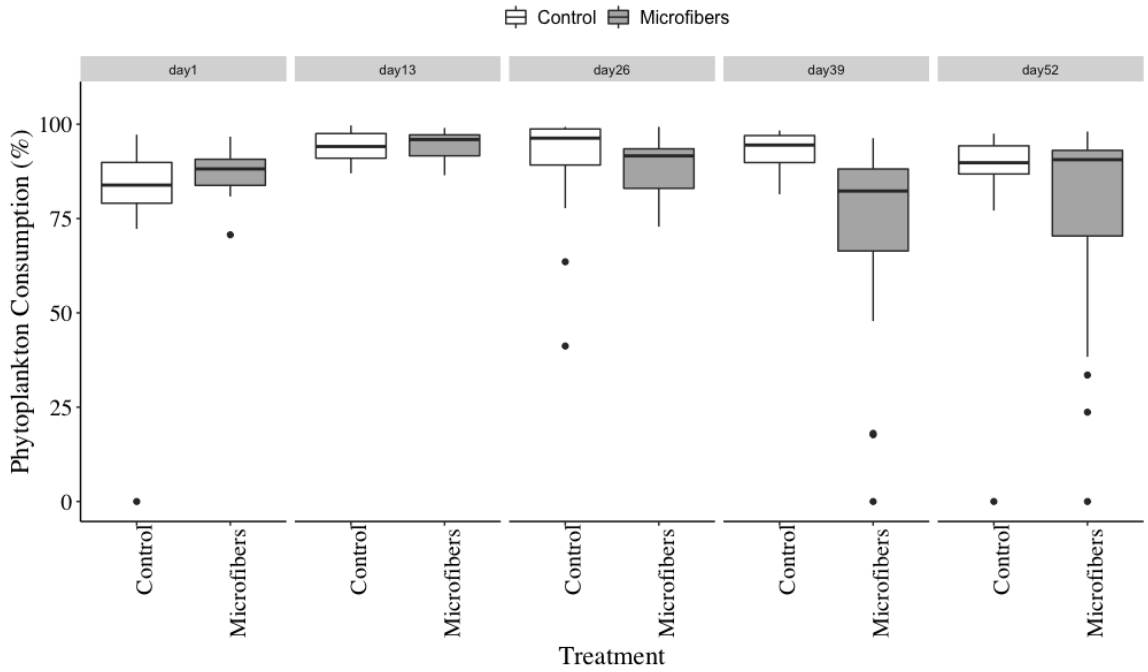

Figure 3-1:The percentage of Tetraselmis sp. consumed by mussels in the Control (white) and Microfiber (gray) Treatments at each sampling day (1, 13, 26, 39 and 52). A significant difference ($p<0.01$) between the two treatments at day 39 is indicated with asterisks (**). Please note that “Day” was included as a continuous variable in the corresponding statistical model.

3.3.2 Accumulation of MFs in the digestive gland and stomach

The number of MFs accumulated in the digestive gland and stomach of the 22 mussels that were subject to the MF treatment, had a high variation ranging between 24 and 3,170 with a mean of 475MF per mussel (± 651S.D.) (0). The MF accumulation varied positively with *Tetraselmis* consumption ($F_{1,18}=9.90$, $p=0.0056$) (Figure 3-2) whereas the MF consumption ($F_{1,18}=0.52$, $p>0.1$) or the presence of diatoms ($F_{1,18}=0.8027$, $p>0.1$) had no influence on the MFs accumulated.

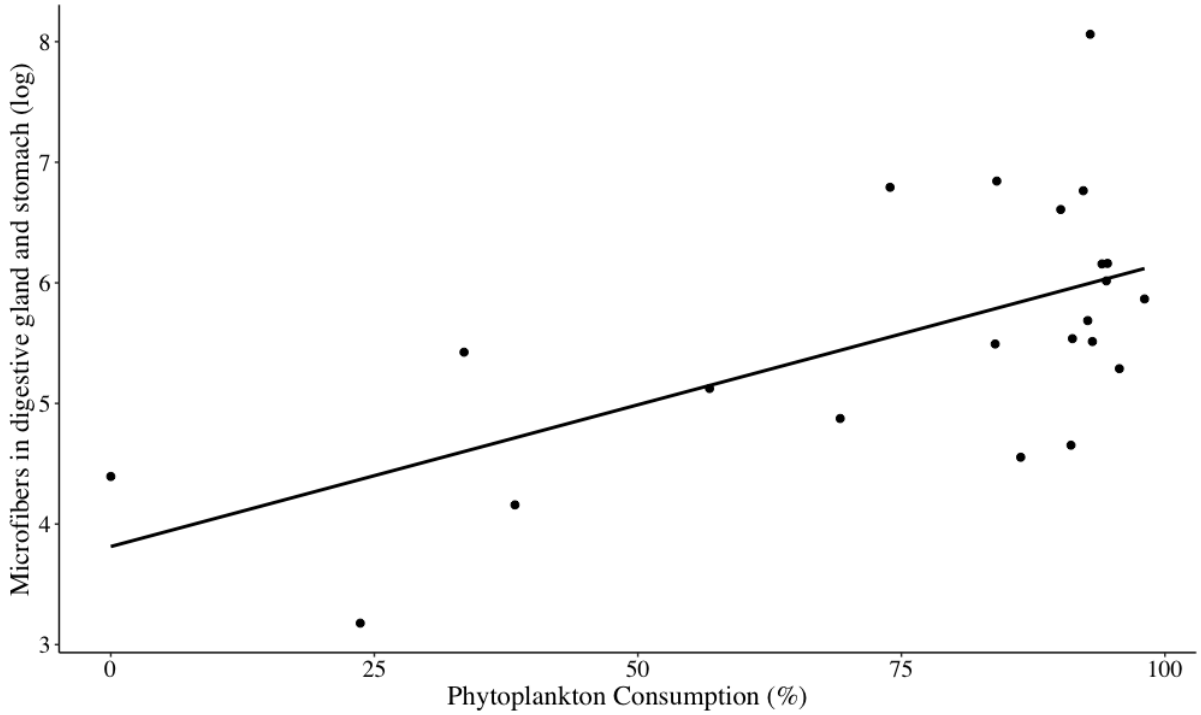

Figure 3-2:Relationship between the microfibers retained in the digestive gland and stomach of mussels, and consumption of *Tetraselmis* cells at the end of the experiment (day52).

3.4 Discussion

Findings from this long-term experiment indicate that the capacity of mussels to remove phytoplankton biomass from the water column can be negatively impacted by long-term exposure to MFs of 10-100µm size range. Specifically, mussels exposed to MFs showed an average decrease of 21.3% in their phytoplankton removal capacity after 39 days of exposure to ambient concentrations of MFs. This finding is important as it indicates that the ecosystem service of mitigating eutrophication and HABs in coastal systems can be impaired by the presence of another dominant stressor such as MFs. Another long-term exposure experiment (44 days) that used PVS particles (1-50µm), at a higher concentration than those used in our study, also showed a decrease in the clearance rate of mussels by 79% (Rist *et al.*, 2016). These findings stress the importance of prolonging experimental duration, a suggestion also stressed by Qu et al. (2018) and Von Moos et al. (2012).

Even though short-term effect of MFs was not observed in our study, mussels exposed to MFs showed higher unpredictability in their clearance capacity from

earlier on (day 26), as indicated by the higher variation in the phytoplankton removal percentages. A short-term study exposing mussels to 3μm and 9.6μm of polystyrene microspheres (15,000particles/treatment) for 3 hours reported a lower clearance rate 48 days after the exposure than after 6 days. This suggests that the effect on the mussel clearance capacity was exacerbated even after the termination of a short-term exposure. Thus, we can only expect that the continuous long-term exposure to microplastics, which is an environmentally plausible condition, will have a more deleterious effect on the ecosystem services provided by coastal mussels. For more realistic and representative measures of future laboratory exposure studies, there is a need for accurate estimates of environmental concentration of microplastics <100μm.

Another important finding of this study was that higher amounts of MFs in the digestive gland of mussels were associated with higher microalgae consumption. This can be explained by considering the physiological feeding mechanism of a mussel, as illustrated in figure 3. After uptake, through the inhalant siphon of the mussel (Figure 3-3, step 1), food particles are sorted by size at the lamellae filaments of the gills (Figure 3-3, step 2). At this pre-ingestion level, particles smaller than 1-6μm pass through the interfilamental gaps and are immediately expelled along with water. Thereafter, the larger particles that have been retained, will be led by the frontal cilia of the gills to the food grooves from where they reach the labial palps for further sorting (Figure 3-3, step 3). There is a literature gap regarding the exact sizes being sorted at the labial palps; however, rejected large and excess particles are released in the mantle cavity to be ejected as pseudofaeces (Rouillon and Navarro, 2003; Ruppert *et al.*, 2004; Ren *et al.*, 2006). The remaining smaller particles are directed to the mouth, oesophagus and stomach (Figure 3-3, step 4) where extracellular digestion is initiated by the rotation of the crystalline style (Morton, 1983; Ward, Rosa, *et al.*, 2019), and the sorting fields will direct particles >100μm (Kolandhasamy *et al.*, 2018) to the rejection truck to be excreted as faeces. Particles <100μm, either enter the digestive ducts or remain suspended in the stomach (Ruppert *et al.*, 2004). Due to the similarity in size of MFs investigated in this study with the food particles (i.e., microalgae) consumed, MFs passed the pre-ingestion sorting and reached the stomach and digestive gland. This was also observed by Fernández and Albentosa

(2019) where mussels showed no difference in the clearance of microalgae and microplastics of similar size and explains our finding that mussels that were high-consumers were more susceptible in accumulating MFs in their gut. This also suggests that the presence of MFs in the water column may specifically impair individuals with the highest clearance capacity, which, in the long-term, can impact the ecosystem service of microalgal removal by mussel populations.

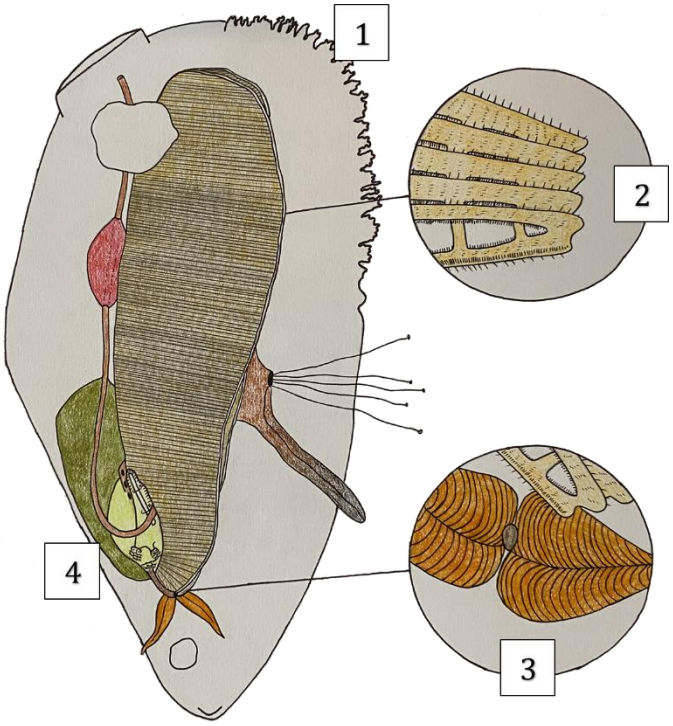

Figure 3-3: Internal anatomical diagram of a mussel displaying the 4 main particle-sorting areas: (1) In the inhalant syphon, particles <5000µm long & <50µm wide enter the mussel (Cucci *et al.*, 1985; Kolandhasamy *et al.*, 2018; Rosa *et al.*, 2018). (2) At the gills, particles >1-6µm are retained (Dral, 1967; Ruppert *et al.*, 2004; Rosa *et al.*, 2018) and transported to the food grooves from where they enter (3) the labial palps for further sorting (rejected particles form pseudofaeces). Accepted particles are lead into (4) the stomach, where particles <100µm can enter the digestive system (Kolandhasamy *et al.*, 2018) (for more details see 0, illustration by: Eleni Christoforou).

Our findings suggest that long-term exposure to even small MFs can negatively impact the ability of mussels to perform ecosystem services. This impairment did not occur as a result of potential disruption of the filtration process due to larger particles damaging the cilia of the gills (Cheung and Shin, 2005), or filtered out at the pre-ingestion phase and the pseudofaeces production (Woods *et al.*, 2018), but rather as a result of accumulation of MFs in the digestive gland. The exact

mechanism by which MF accumulation might have affected the clearance capacity of microalgae in this experiment is unknown. However, earlier work has shown that microbeads of 10µm diameter can penetrate the biological membranes of the digestive gland and translocate into the mussel's tissue (Rist *et al.*, 2016), 3µm and 9.6µm polystyrene particles can reach the circulatory system of mussels (Browne *et al.*, 2008), and that the presence of polyethylene particles can trigger an inflammatory response (Von Moos *et al.*, 2012). Additionally, the presence of MFs in the stomach and digestive gland of mussels could trigger a feeling of satiation making the uptake of more food less likely and eventually leading to the mussel's starvation (Gall and Thompson, 2015). Further research is required to determine the specific mechanism underlying the effect of ingested small MFs on the physiology of the organisms.

An interesting observation from our experiment was that the fouling of our experimental vessels by opportunistic diatoms had a negative impact on the clearance of *Tetraselmis* cells, comparable to that of MFs. More research is required on whether this is linked to the fouling diatom in our experiment showing a structural resemblance in size and shape to our MFs (0) or e.g., to preferential grazing by the mussels of the fouling diatoms compared to the flagellate *Tetraselmis* cells (Cucci *et al.*, 1985; Shumway *et al.*, 1985; Rouillon and Navarro, 2003; Ren *et al.*, 2006; Safi and Hayden, 2010).

3.5 Conclusion

Our findings show that long-term exposure to MF (<100µm) can significantly decrease the clearance capacity of mussels, and thus the ecosystem services they provide. MFs accumulated in the digestive gland and stomach of mussels which was linked to the intensity of phytoplankton consumption. This suggests that individuals with high clearance capacity would be more susceptible to microfiber ingestion. These effects may vary in the presence of different phytoplankton species; thus, we stress that further research is required on this topic.

Acknowledgements

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Chapter 4

The effects of Artificial Light at Night (ALAN) on mussels and the ecosystem services they provide

Abstract

Artificial light at night (ALAN) is one of the most widespread forms of environmental pollution, mostly associated with growing human population and urbanisation. As the biology of most organisms is tuned to the natural day and night cycles, ALAN has the potential to affect multitude of behavioural and physiological processes. Moreover, studies show that the effects of ALAN can depend on the wavelength of the light. However, research so far has mostly focused on terrestrial organisms whereas literature on the response of coastal organisms to different ALAN wavelengths is scarce. This is the first study testing the effect of the most commonly used ALAN wavelengths: green, red, and white, on the activity and phytoplankton clearance capacity of mussels. We performed a laboratory-based exposure experiment where the mussel activity was tracked by a valvometry system, and the phytoplankton clearance capacity was quantified. ALAN did not influence the mussel proportion of open gape but there was a higher open/close frequency in mussels exposed to the green ALAN in comparison to the red and white. The phytoplankton consumption was ~10% lower in mussels under the green ALAN than mussels exposed to red ALAN, but no significant difference was observed in comparison to the dark control. Time of collection also influenced the phytoplankton consumption with winter collected mussels (pre-spawning) consuming ~9% more phytoplankton than autumn collected ones (post-spawning). More experimental work should be contacted on the effects on ALAN on bivalves for accurate recommendation but these results suggest that green ALAN may have a negative impact on coastal mussel populations whereas red ALAN, the least impactful to mussel populations, can induce an increase in phytoplankton abundance, which could lead to harmful algal blooms. As mussels under white ALAN had similar responses to mussels in the dark control, dim white ALAN could be considered but the sensitivity of other organisms in the community should also be investigated.

4.1 Introduction

Artificial light at night (ALAN) is currently one of the most prevailing forms of environmental pollution with an annual increase of 2.2% in lit areas worldwide (Kyba *et al.*, 2017). This is inevitably affecting living organisms which are evolutionarily adapted to rely on circadian rhythms for essential biochemical, physiological and behavioural processes (Pittendrigh, 1960; Naylor, 1999; Sharma, 2003). Impacts induced by alterations to these natural processes can scale up from individual to community level (Maggi and Benedetti-Cecchi, 2018; Garratt *et al.*, 2019; Hölker *et al.*, 2021) and have negative effects on the functioning of ecosystems (Zapata *et al.*, 2018). Recently, the development of light-emitting diode (LED) technology (Cho *et al.*, 2017; Zissis and Bertoldi, 2018) has provided an energy-efficient and more affordable opportunity to shift to ALAN wavelengths that are less stressful to wildlife. Research on this topic is currently largely focused on terrestrial and freshwater ecosystems (Bedrosian *et al.*, 2013; Brüning *et al.*, 2018; Tidau *et al.*, 2021) (Poot *et al.*, 2008; Bedrosian *et al.*, 2013; Brüning *et al.*, 2016; van Dis *et al.*, 2021). It has been shown that about 1.9 million km^2 of shallow (1m) coastal areas are affected by biologically important ALAN which can reach depths up to 50m in clear waters. Therefore more focus should be directed on coastal marine ecosystems where ALAN can be impactful to aquatic communities (Davies *et al.*, 2020; Smyth *et al.*, 2021) and where studies are currently limited.

Bivalves are vital for the healthy functioning of coastal ecosystems (Prather *et al.*, 2013; van der Schatte Olivier *et al.*, 2018) as they are ecosystem engineers (Prather *et al.*, 2013) and, through biofiltration and biodeposition, provide invaluable ecosystem services i.e., facilitate the nutrient cycle (Prather *et al.*, 2013; Kent *et al.*, 2017) and control phytoplankton abundance and composition remediating the impacts of eutrophication and algal blooms (Prins *et al.*, 1998; Tantanasarit *et al.*, 2013; van der Schatte Olivier *et al.*, 2018). It is therefore concerning that sessile invertebrates, like mussels, can be especially vulnerable to light pollution due to direct and continuous exposure (Bolton *et al.*, 2017; Gaston *et al.*, 2017) to street and harbour lighting (Zissis and Bertoldi, 2018). However, studies on the effects of ALAN on bivalves are currently lacking.

Epifaunal bivalves possess photoreceptor organs on their mantle or even light-sensitive multi-cellular eyes (Morton, 2008; Von Salvini-Plawen, 2008; Audino *et al.*, 2020). Their main role is to induce an anti-predatory response when a change is detected in the light intensity of the organisms' immediate environment (Wilkens, 2008). These photoreceptors are also involved in the regulation of circadian rhythms displayed by bivalves (Ortmann and Grieshaber, 2003; Garcı *et al.*, 2008; Gnyubkin, 2010). Bivalves show nocturnal activity with increased valve movement, greater valve gape angles, higher exhalant pumping (Robson, Garcia, *et al.*, 2010), longer duration of open gape (Kobak and Nowacki, 2007; Gnyubkin, 2010) and higher filtration (Hills *et al.*, 2020) and growth rates (Strömgren, 1976; Nielsen and Strömgren, 1985) compared to their diurnal activity. As the regulation of diel cycles in these processes is under strong control by light, we could expect ALAN to disrupt them, possibly also affecting the associated ecosystem services provided.

Previous studies have proposed that the impacts of ALAN on a variety of species could be mitigated by using specific wavelengths of light (Gaston *et al.*, 2017). The light spectra commonly used in exposure studies are red, green, blue and/or white (Spoelstra *et al.*, 2015) as white light is usually broad spectrum and contains blue wavelengths (Bedrosian *et al.*, 2013; Gaston and Holt, 2018; Diamantopoulou *et al.*, 2021). All colours allow full colour vision in humans therefore can still be used as a form of night illumination. Red is suggested to be used near organisms with sensitivity or attraction to short wavelengths like blue and green (Spoelstra *et al.*, 2015). Such organisms are bats (Spoelstra *et al.*, 2015), sea turtles (Miller and Bretschneider, 2006) and corals (Ayalon *et al.*, 2019). Research on migratory seabirds and mice recommend the use of green light to minimise the effects of ALAN (Poot *et al.*, 2008). This variation in the proposed light spectra is attributed to the organisms' diverse sensitivity to light wavelengths due to differences in the evolutionary development of their photoreceptive cells and organs (Von Salvini-Plawen, 2008; Alaasam *et al.*, 2021). Consequently, there is no consensus on the most appropriate ALAN wavelengths for the conservation of coastal wild populations and research should be conducted on all organisms that are exposed to ALAN.

Information on bivalves' spectral photosensitivity is scarce and mostly centred around scallop species, which have the most complex eyes, and show peak absorbance at 480-504nm and 513-549nm (Cronly-Dillon, 1966; Speiser *et al.*, 2011). Scallops species inhabiting deeper waters are more sensitive to longer wavelengths in comparison to coastal scallops (Speiser *et al.*, 2011). Hence, the range in sensitivity seen in different species could be attributed to the light attenuation and wavelength absorption at different depths and water conditions. Comparative studies on the effects of different wavelengths on mussels point to an overall avoidance of coloured light and preference to dark areas (Kobak and Nowacki, 2007). A negative effect of red fluorescent light was expressed as lower growth rate (Nielsen and Strömgren, 1985) and greater sensitivity of the photoreceptor cell responsible for the shadow reflex (Cornwall and Gorman, 1983); however, research on the effect of ALAN wavelengths on the gaping activity and feeding behaviour of bivalves is lacking. It is critical to investigate these responses as they are directly linked to important ecosystem functions provided by bivalves such as the control of phytoplankton abundance and nutrient cycling.

The aim of this study was to determine whether the behaviour and phytoplankton clearance capacity can be impacted by ALAN, and whether these effects are wavelength specific. To achieve this, we performed a laboratory experiment exposing the blue mussel, *Mytilus edulis* to wavelengths relevant for coastal illumination and conservation; namely green, red, and white light, compared to a dark night control. The responses studied included the circadian rhythms of gaping activity (i.e., proportion of time open and the open/close frequency) and the phytoplankton clearance capacity of mussels. Due to the decrease in the duration of dark night by ALAN, we would expect that, the nocturnal activity of mussels would be reduced changing the natural amplitude of diel rhythms of gaping activity. This change in activity would also lower the phytoplankton clearance capacity. Informed predictions on the effect of different spectra is restricted by the limitation in available literature. Based on the results of Nielsen and Strömgren in 1985 we would anticipate that red light would be the most impactful on coastal mussels because of the negative effect it had on their growth, which is usually a sign of suboptimal physiological functioning. The relationship between gaping activity and phytoplankton clearance capacity and how this depends on the

ALAN colour was also investigated. We would not anticipate any changes to this relationship as we would expect both responses to change in the same manner within each colour treatment.

4.2 Methods

4.2.1 Experimental design

We performed a 2-stage exposure experiment to investigate the effect of different wavelengths on (a) the circadian rhythm and the hourly gaping activity (proportion of time open and open/close frequency) and (b) the phytoplankton clearance capacity of mussels. During the first stage, mussels were continuously fed with a peristaltic pump and their gaping activity (open/close status) was recorded every minute for 12 consecutive days. The second stage was performed a day prior and a day after the first stage. There, mussels were fed only once at the beginning of a 24-hour period and both their phytoplankton consumption and activity were measured during that period. The experiment was performed in light-sealed boxes under 12hour day: 12hour night photoperiod. In each box, a white LED light (3702.5lux ± 249.2SD, 496-627nm, peak at 548nm) was used to simulate day light. For the night treatments we used green (505-586nm, peak at 536nm), red (510-664nm, peak at 634nm) and white (503-620nm, peak at 536nm) LED ALAN (19.86lux ± 0.5 SD) (S4.1) or a dark night treatment (0 lux), acting as a control. Mussel body condition, including energy storage, is affected by seasonal variation and food availability which determine the reproductive cycle (Fernández *et al.*, 2015), thus the experiment was repeated twice per each reproductive stage (pre, during and post spawning) to account for any possible variability for a total of 72 mussels (4 treatments x 3 different mussels per treatment x 3 runs each experimenting with a different reproduction stage x 2 replicates per run).

The light sources were 40cm above the water surface and their illuminance was measured with a LI-210R photometric sensor (LI-COR, USA) at the water surface. The lux levels used were environmentally relevant, standardised according to the day-time range of a cloudy day (1000-10,000lux) and the ALAN of the average street illuminance (15lux) (Gaston *et al.*, 2013) and lux levels measured at the water surface of a coastal environment (5-21.6lux) (Davies *et al.*, 2015).

Experimental vessels, containing the mussels, were constantly aerated, and positioned in water baths fed by a common thermoregulated water tank to maintain temperature between 12-14°C.

4.2.2 Collection & acclimation

Rope grown *Mytilus edulis* mussels (6.28cm ± 0.34SD, n=72) were collected from Loch Eil, Scotland in 2020. Mussel energy storage, conditional index and weight is affected by the food availability and the reproductive stage which are correlated with annual seasonality (Okumuş and Stirling, 1998; Fernández *et al.*, 2015). Therefore, to account for this variability in the mussels' fitness, the specimens were collected in winter (January 22nd, water temperature: 7ºC, salinity:23‰), summer (August 23rd, water temperature: 14ºC, salinity:25‰) and autumn (October 4th, water temperature: 12ºC, salinity:23‰), which are representative of the pre, during (including spring) and post spawning stages, respectively.

In the laboratory, epibionts such as barnacles, macroalgae and epiphytic diatoms were scraped off the mussel shells to reduce interferences with gaping activity and feeding. Mussels were then placed in 5L artificial salt water (32ppm) aquariums, located in the light-sealed boxes under 12hour day: 12hour night photoperiod, and were starved for 48 hours to standardise the mussels' hunger level prior to the treatments. After depuration, the valvometry system was attached to 12 experimental mussels which were individually placed in experimental cylindrical glass vessels (height: 25cm, diameter: 7.3cm) filled with 550ml of artificial salt water (32ppm). They were then allowed to acclimate for the first 24 hours. *Tetraselmis* sp. microalgal monoculture (S2.1) was provided through a peristaltic pump for 15 mins every three hours, for a total phytoplankton concentration of 3x10^{6}cells/L. Mussels were then starved for another 24 hours before the initiation of the experiments.

4.2.3 Recording of gaping activity

To investigate the effect of ALAN wavelengths on the circadian rhythm in gaping activity of mussels, the gaping status (open/close) of the mussels was tracked and recorded every minute (Robson *et al.*, 2009) for the duration of the experiment.

This was achieved by a custom-made valvometry system (S4.2), developed by the Bioelectronics Unit of the Institute of Biodiversity, Animal Health & Comparative Medicine, at the University of Glasgow, according to previous bivalve studies (Andrade *et al.*, 2016; Comeau *et al.*, 2018; Clements and Comeau, 2019).

Acclimated mussels were placed into three experimental vessels in each of four light-sealed treatment boxes for 12 experimental days. It has been suggested that mussels' activity and feeding could be affected by the time of feeding in laboratory experiments (Robson, Garcia, *et al.*, 2010). Hence, to eliminate any effects anthropogenic disturbances could have on our measurements of the mussels' activity and circadian rhythm, a peristaltic pump provided *Tetraselmis* sp. in a continuous manner for 15 mins every three hours. This added up to 982ml of artificial saltwater, after 48 hours, with a total phytoplankton concentration of $3x10^6$ cells/L. At the end of each 48-interval, within the last hour of the daytime period (17:00-18:00), the gaping status recording was paused to allow for the renewal of the artificial salt water. During that time, the experimental containers were rotated within their treatment boxes to allow for a more homogeneous light exposure and food provision among replicates. Also, the proper attachment of the valvometry system was assessed; a mussel was considered closed when the valves were in contact at the posterior end, otherwise it was regarded as open (Figure 4-1). When the recorded activity was not consistent with the visual inspection of the mussel gaping status, the data collected for that specific mussel in the latest 48h interval were discarded because there was no way of knowing when the malfunction occurred within those 48 hours.

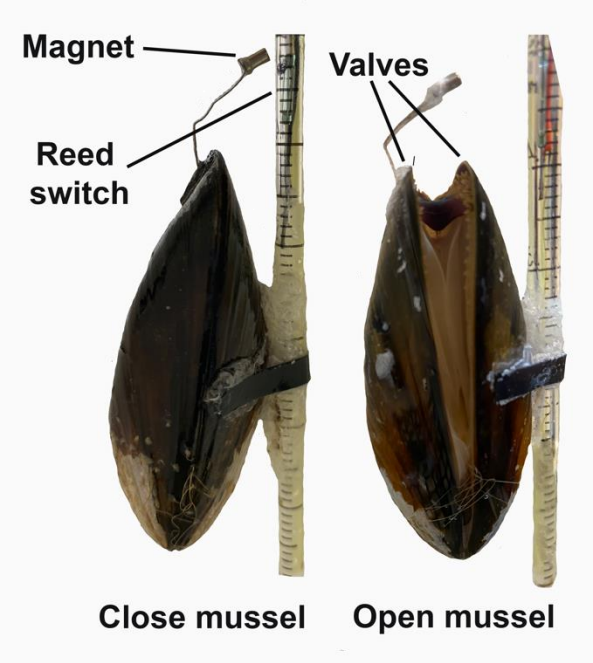

Figure 4-1: Photos of mussels equipped with the valvometry system where the magnet, the reed switch and the valves of the mussels are labelled. The mussel on the left represents a closed mussel where the magnet would trigger the reed switch, close the circuit and a signal would be recorded while the mussel on the right is open and no signal is indicated. For more details regarding the valvometry system see S4.2.

To assess how ALAN affected circadian rhythms of gaping activity, we first calculated the proportion of time that a mussel was open, using the following equation:

$$Proportion\ of\ time = \frac{\Sigma(Minutes\ of\ open\ gape\ in\ 1\ hour)}{\Sigma(Minutes\ recorded\ in\ 1\ hour)}$$

The sum of the minutes recorded per hour were divided by the minutes recorded to account for any incomplete hours which occasionally occurred due to system errors. The value is multiplied by 100 to express the proportion time open as a percentage.

We also calculated the frequency of switches between open and close gaping status per hour, using the following equation:

$$Open/Close\ frequency = \frac{\Sigma(Switches\ in\ 1\ hour)}{\Sigma(Minutes\ recorded\ in\ 1\ hour)} * 60,$$

where switches are the transitions from close to open and vice versa per hour. These are divided by the number of minutes recorded and then multiplied by 60 for a standardized count within an hour. The proportion of time open and the open/close frequency are not correlated (S4.3) hence both are used in the analysis. The activity measurements were categorised by day and night-time to disentangle between the two light regimes (day light and ALAN or dark control).

4.2.4 Phytoplankton clearance capacity

To investigate the effect of ALAN wavelengths on the clearance capacity of mussels, the phytoplankton consumed by mussels in a 24-hour period was quantified. To achieve that, 850ml of artificial salt water and *Tetraselmis* sp. monoculture ($3x10^6$cells/L) was prepared, from which 50ml water sample was collected to measure the initial concentration (0h) and the rest was added to the experimental vessels, each containing a single mussel. After 24 hours, a second 50ml water sample was collected, to measure the final concentration. Thereafter the gaping activity experiment was performed for 12 days, after which the mussels were starved for 24 hours. Then the clearance capacity experimental stage was repeated where *Tetraselmis* solution ($3x10^6$cells/L) was prepared for each experimental vessel and samples were collected at 0 and 24 hours following the same protocol above.

The mussels' clearance capacity was quantified as per Christoforou et. al. (2020). For the estimation of cell concentration within the water, 50ml water samples were preserved in amber glass bottles with lugol iodine solution and filtered using Sartorius™ Cellulose Nitrate Membrane Filters. The filters were then dried, made transparent using immersion oil and the cells were visualised and counted using a light microscope camera at x40 magnification. The percentage of phytoplankton

consumed, proxy of mussels' clearance capacity, at each replicate vessel, was estimated using the following equation:

$$Phytoplankton\ Consumption = \frac{Concentration\ (0h) - Concentration\ (24h)}{Concentration\ (0h)} * 100.$$

4.2.5 Relationship between gaping activity and phytoplankton consumption

To determine whether any change to the phytoplankton consumption was linked to the mussels' activity, the latter was tracked during the phytoplankton clearance capacity experimental stage and was calculated as in section (4.2.3). The relationship between the activity and phytoplankton consumption was only representative of the beginning and end of the experiment as the phytoplankton consumption of mussels in the 12-day activity tracking experimental stage was not quantified.

4.2.6 Data analysis

To determine the variables affecting the circadian rhythm of mussel gaping activity, two General Additive Mixed Models (GAMMs) were fitted to the data using the mgcv package (v1.8-33) (Wood, 2011) in R v.4.0.4 (R Core Team, 2021), using either the proportion of time open or the open/close frequency as response variables. In each of the two models, the explanatory variables included were: the light treatment (green, red, white, and dark), whether it was day or night-time (binomial variable), the mussel collection season (winter, summer and autumn corresponding to pre, during and post spawning respectively), the experimental day (1-12), whether water change was performed on a certain day (as this may affect foraging rate and thereby gaping activity; binomial variable) and the hour of the day which was modelled as a smooth term. An interaction between light treatment and Day-time/Night-time was also included as the effect of light treatment could vary between the two. The proportion of time open was not following the Gaussian distribution (Shapiro-Wilk normality test, p-value <0.001), hence beta distribution was used to model the data of proportion of time open. Since beta distribution does not allow the use of values of observation which

are exactly zero or one, the data were transformed by using the following equation:

$$Ytransformed = \frac{[Y * (N - 1) + 0.5]}{N},$$

where *Ytransformed* is the transformed value of Y, and N is the sample size (Smithson and Verkuilen, 2006), in this case N=72 mussels. To test for zero-inflation in the open/close frequency count data, these models were fitted with Poisson, quasi-Poisson, negative binomial, and zero-inflated Poisson error distributions. The model with Poisson error distribution had the highest deviance explained (22%) and was used to model the open/close frequency data, after they were rounded to the closest integer.

The variation in the phytoplankton consumption was analysed using a generalized linear mixed model (GLMM). As in the previous analysis, the response variable was comprised of proportional data and were transformed using the aforementioned equation. The global model including the light treatment, spawning stage, experimental day, acclimation period and the initial phytoplankton concentration as explanatory variables was fitted using the glmmTMB package (v1.0.2.1)(Brooks *et al.*, 2017) in R v.4.0.4 (R Core Team, 2021).

To determine any effect of gaping activity on the phytoplankton clearance capacity and whether this was affected by the ALAN treatments, we used GLMM. The response variable was the phytoplankton consumption with the explanatory variables being the proportion of time open and the open/close frequency collected during the 24-hour clearance capacity experiment, the light treatment, reproduction stage, experimental day, acclimation period, the initial phytoplankton concentration. An interaction between the two activity variables and light treatment was also included in this model to account for any variation between the light treatments.

In all models, the mussel ID nested within the run number was used as a random effect to account for individual mussel variation within the runs. The analysis was conducted in R v.4.0.4 (R Core Team, 2021) and model selection was performed

using model AIC comparisons. Pairwise comparison tests were performed on significant variables, using the Tukey method in the emmeans package v1.5.2-1 (Lenth, 2018). Outliers detected in the phytoplankton consumption data were omitted from the analysis (S4.4).

4.3 Results

4.3.1 Effects of ALAN wavelengths on circadian rhythms of gaping activity

The circadian activity expressed both as a proportion of time valves were open and frequency of open/close events showed a clear diurnal pattern (Figure 4-2) with hour of the day having a significant effect on both variables (Table 4-1). Specifically, across all light treatments, the proportion of time open showed a clear semi-diurnal periodicity with minimum at 3:00 and 15:00 hours and maximum at 7:00 and 19:00 hours (Figure 4-2A). Actograms of the proportion open of individual mussels during each run of the experiments can be found at S4.5.

The frequency of open/close valve events showed a graphically less pronounced diurnal pattern between the light treatments (Figure 4-2B). Specifically, mussels exposed to the green ALAN showed a diurnal pattern with the least open/close frequency one hour after daylight (7:00) and the greatest one hour after ALAN was on (19:00) (Figure 4-2B). Mussels in the other light treatments had a similar but milder semi-diurnal pattern than the one seen in the proportion of open gape (Figure 4-2B).

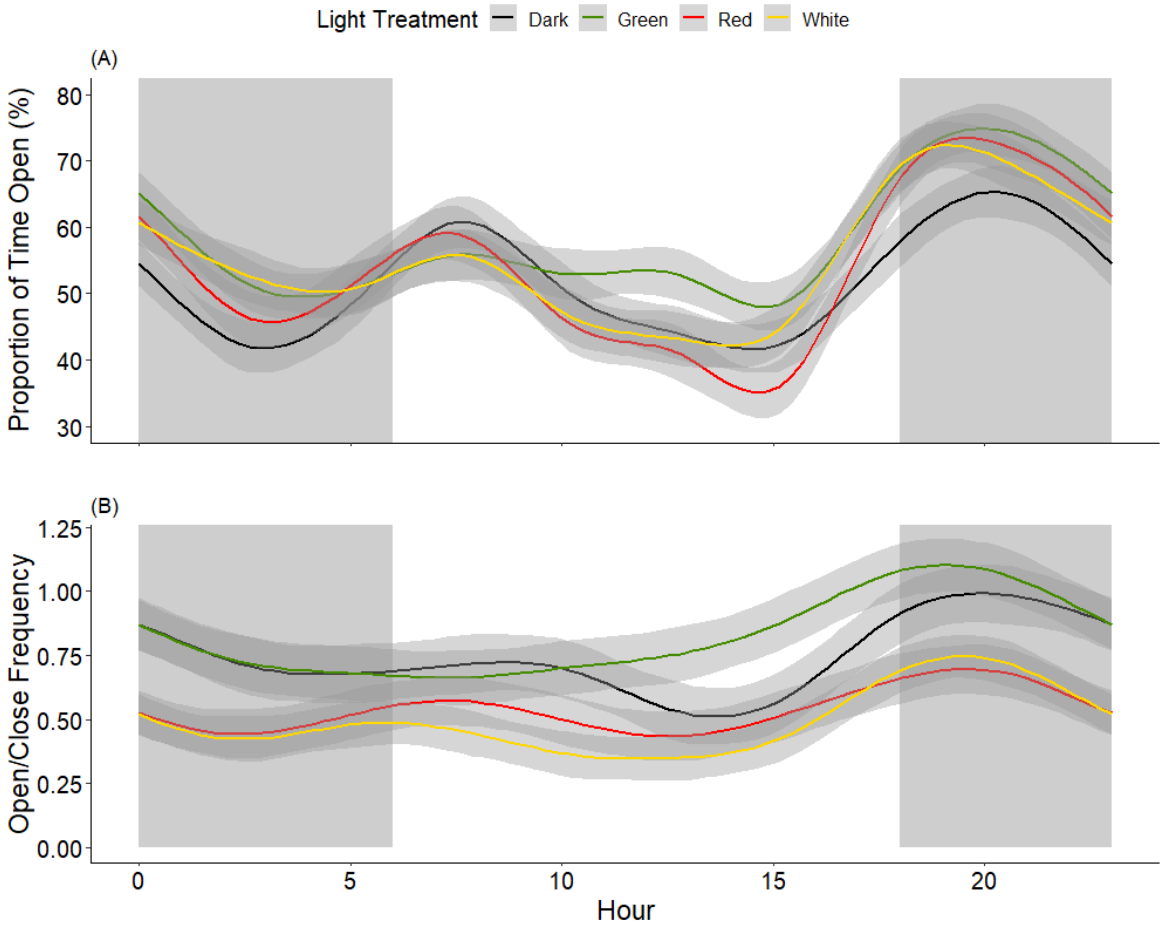

Figure 4-2: The mean (lines) and 95% confidence interval (grey bands) of the (A) proportion of open gape expressed as a percentage and (B) open/close frequency at each light treatment (dark, green, red, and white) through a 24-hour period collected over 12 days of each of six runs. The white and grey background rectangles represent day-time and night-time respectively. In proportion of time open mussels showed a semi-diel circadian rhythm in all treatments tested. Similar but milder patterns are observed in the open/close frequency except from mussels in the green treatment that showed a diel circadian rhythm.

Table 4-1:The explanatory variables of the best-supported models explaining the variation in the proportion of time open, the open/close frequency and the phytoplankton consumption of mussels, after AIC model selection. The ΔAIC indicated the difference in the AIC when the explanatory variable is dropped from the best-supported model. The GAM summary outputs can be seen at S4.6.

Response (bold) & explanatory variables (not bold)	**|Δ AIC|**
Proportion of Time Open	
Experimental day	301.35
Water change	219.46
s (Hour, by=Treatment)	510.86
Open/Close Frequency	
Light treatment * Day-time/Night-time	11.4
Experimental day	196.51
Water change	44.67
s (Hour, by=Treatment)	433.48
Phytoplankton Consumption	
Light treatment	2.06
Spawning stage	3.28
Phytoplankton Consumption (testing the relationship with gaping activity)	
Proportion of time open * Light treatment	5.90

4.3.2 Gaping Activity

The light treatments did not have a statistically significant effect on the proportion of time open, but they did affected the open/close frequency of mussels (Table 4-1). That effect was dependent on whether it was day-time or night-time indicated by an interaction of the two explanatory variables (Table 4-1). The open/close frequency of mussels was significantly higher in the dark treatment in comparison to the red (Tukey, day-time: $p=0.0002$ and night-time: $p=0.0019$) and white (Tukey, day-time & night-time: $p<0.001$) ALAN treatments by 0.12 and 0.22 switches respectively during day-time and 0.30 And 0.29 during night-time (Figure 4-3). Green ALAN had the greatest open/close frequency, which was significantly higher than mussels in the red (Tukey, day-time & night-time: $p<0.001$) and white (Tukey, day-time & night-time: $p<0.001$) ALAN treatments by

0.22 and 0.32 switches respectively during daytime and 0.35 and 0.34 during night-time. There was no significant difference in comparison to the dark treatment (Figure 4-3). Mussels exposed to the red and white ALAN treatment had a significantly higher open/close frequency during the night-time than at day-time (Tukey, red: $p<0.0001$ & white: $p=0.0008$) (Figure 4-3B). The mussel collection season did not influence the open/close frequency of mussels.

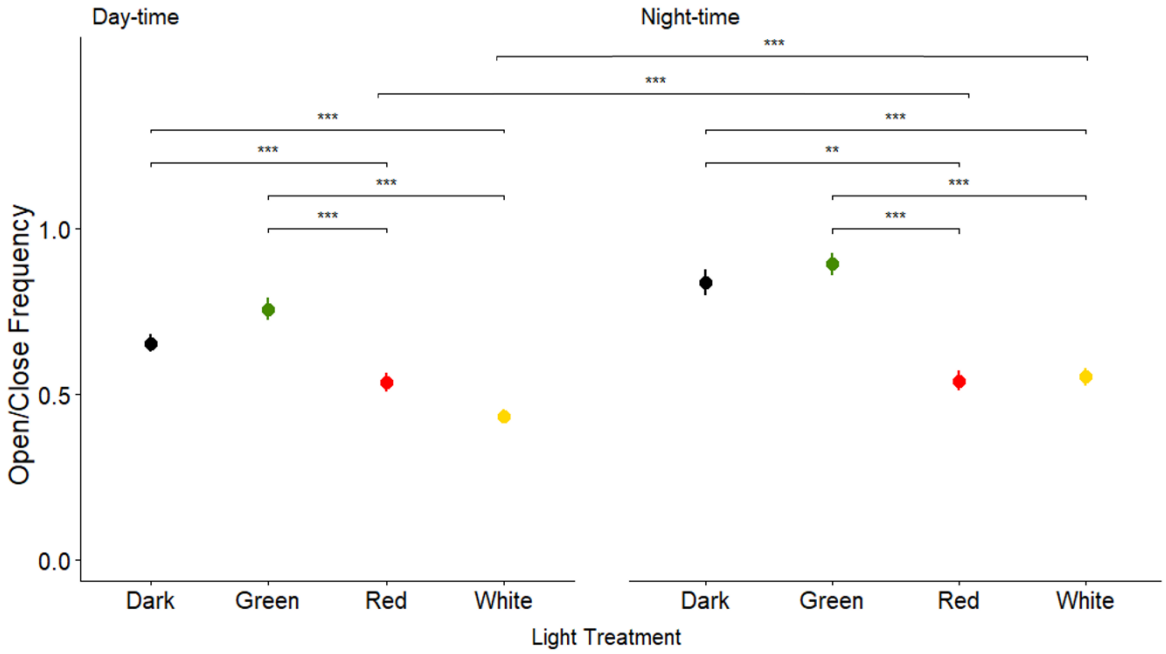

Figure 4-3: The mean ± SE of the open/close frequency per hour at day-time and night-time at each light treatment (dark, green, red, and white). Significant difference between the treatments and between day and night-time is indicated with asterisks ($p<0.001$*** and $p<0.01$**).

4.3.3 Effects of ALAN wavelengths on phytoplankton clearance capacity

Mussel phytoplankton clearance capacity was significantly affected by the light wavelength treatments and mussel collection season which correspond the reproduction stages of mussels (Table 4-1). Experimental day had no significant effect on the mussels' phytoplankton removal capacity thus results from both experimental days were included in the light treatment and mussel collection season pairwise comparisons (Figure 4-4). Mussels exposed to the red ALAN had a significantly higher phytoplankton consumption than mussels exposed to green ALAN by 9.81% (Tukey, $p=0.0269$). There was no significant difference between

the other treatments (Figure 4-4). A significant difference of 9.16% (Tukey, p=0.0038) was identified between the phytoplankton consumption of mussels collected in winter (pre-spawning) and autumn (post-spawning), with the latter being lower.

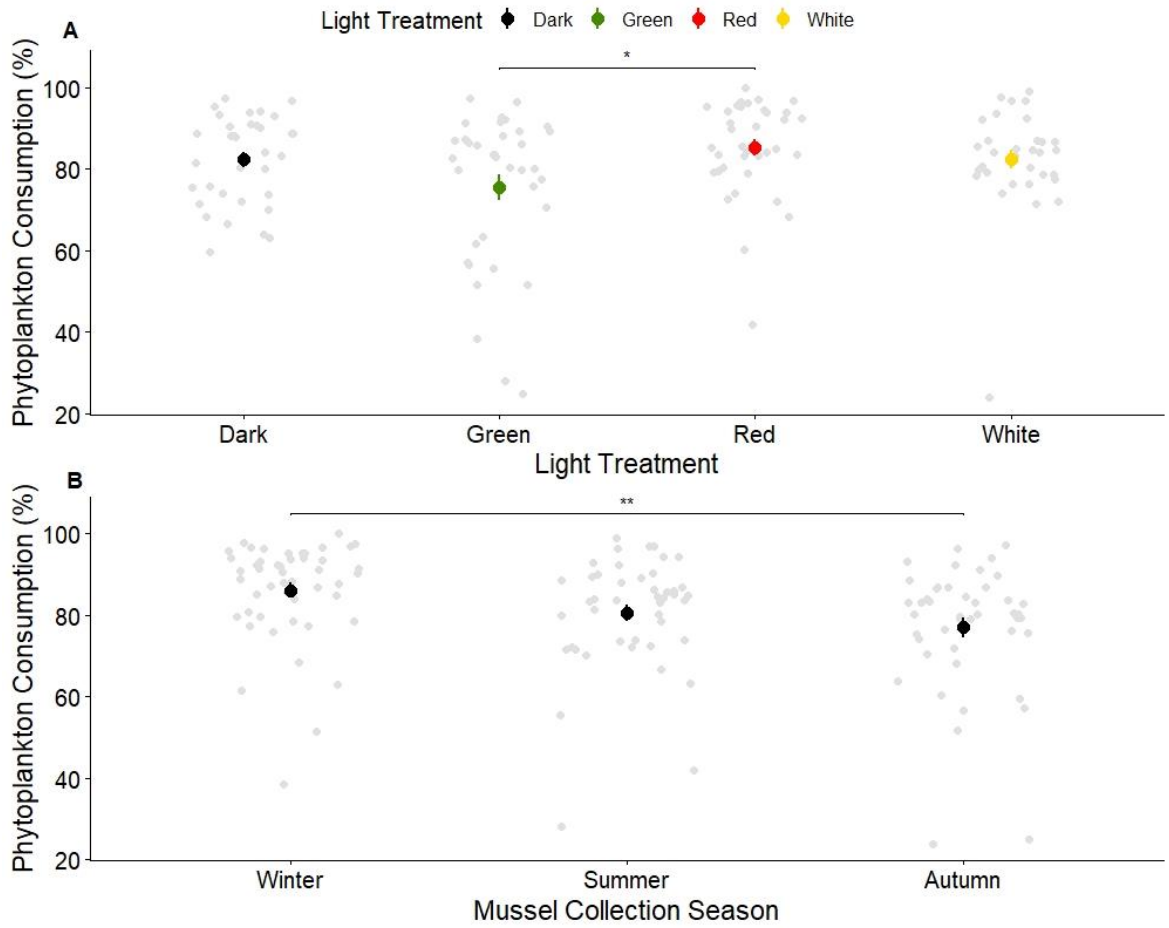

Figure 4-4: The mean ± SE of the phytoplankton consumption within 24 hours by mussels, expressed as a percentage, at each (A) light treatment (dark, green, red and white) and (B) mussel collection season (winter, summer and spring which correspond the reproduction stages of pre, during and post spawning, respectively). Significant difference between the treatments is indicated with asterisks ($p<0.01$** and $p<0.05$*).

4.3.4 Effects of ALAN wavelengths on the relationship between mussel clearance capacity and gaping activity

The phytoplankton consumption was significantly affected by the proportion of time open, however this depended on the light treatments (Table 4-1). While

these variables were positively related in the dark, red, and white treatments, they were negatively related in the green ALAN (Figure 4-5). No relationship was found between the phytoplankton removal capacity and the open/close frequency of mussels (S4.7).

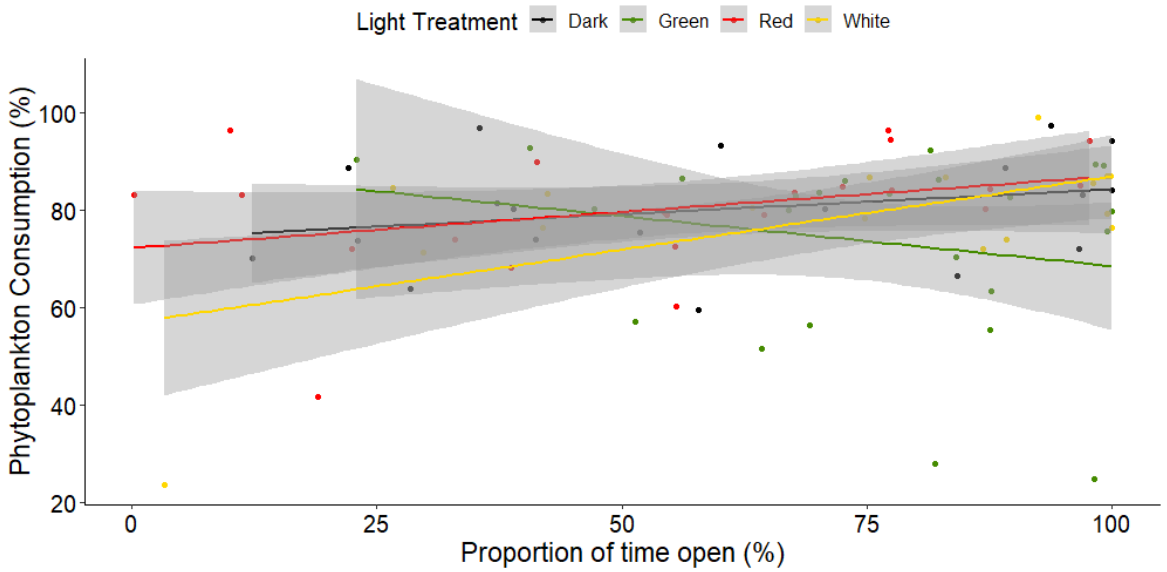

Figure 4-5: The relationship between the phytoplankton consumption and the proportion of time open, both expressed in percentages, under the different light wavelength treatments (dark, green, red, and white) represented by a regression line and the 95% confidence intervals (grey bands).

4.4 Discussion

This is, to our knowledge, the first study investigating the effect of ALAN on bivalves. ALAN did influence the circadian rhythm of mussels gaping activity, the hourly gaping activity, and the phytoplankton clearance capacity of coastal mussels, but that effect was wavelength dependent with not all wavelengths having a significant difference to the dark control. Green ALAN appeared to have the greatest impact with mussels exposed to it having a different circadian rhythm in the open/close frequency, greater open/close frequency, and lower phytoplankton consumption than those exposed to the other light treatments. Additionally, green ALAN induced a differential relationship between the gaping activity and the phytoplankton consumption of mussels in comparison to the relationship identified in the red, white, and dark control treatments. Furthermore, the season that mussels were collected in had an influence on their

phytoplankton consumption capacity without that being affected by the light treatment.

When mussels were exposed to different ALAN wavelengths and a dark control treatment, they showed semi-diel rhythm in the proportion of time open, with two peaks of activity, one early at daytime and one early at night-time. This pattern was different from the circadian behaviour displayed in the field by the Mediterranean bivalve *Pinna nobilis* (Garcı *et al.*, 2008) and the freshwater clam *Corbicula fluminea* (Ortmann and Grieshaber, 2003), which were mostly open at day-time hours. Our pattern was also different to the mussels *Mytilus galloprovincialis* (Gnyubkin, 2010; Comeau *et al.*, 2018) and *Limnoperna secures* (Comeau and Babarro, 2014), which after laboratory based experiments, also showed diel circadian rhythm being mostly open at night. It is not clear why this discrepancy in the circadian rhythm of the proportion of open gape, between our study and previous studies has occurred. However, a possible explanation could arise from the semi-diurnal rhythm pattern observed in our study, which seems to match a circatidal rhythm (S4.8); even though the mussels were fully submerged for the duration of the experiment. This tide-related behavioural pattern was also observed in the valve opening of fully submerged clams (Williams and Pilditch, 1997), oyster (Tran *et al.*, 2020), and mussels (Gnyubkin, 2010), the water propulsion of mussels under continuous dark, light, and natural day and night lighting (Pampapathi, 1954) as well as in the cell renewal cycles of the mussel *Mytilus galloprovincialis* (Zaldibar *et al.*, 2004). As suggested by Ortmann and Grieshaber (2003), the relationship between tidal activity and the fluctuation of phytoplankton levels in the field could determine the gaping activity of bivalves however, in the present study, there was constant phytoplankton provision. Hence, the behaviour observed may be attributed to an entrained behavioural rhythm due to a circadian clock that is dependent on the tidal activity (Tran *et al.*, 2020), and consequently the anticipation of food brought in with the tide (Williams and Pilditch, 1997; Riisgård *et al.*, 2006; Saurel *et al.*, 2007). Though, it would be necessary to confirm this finding in a natural or semi-natural setting.

The open/close frequency of mussels had a less pronounced circadian pattern than the proportion of time open. For this behaviour, the resemblance of the rhythm

to a tidal one is far from clear, with only a mid-day and an evening peak under the control treatment (S4.8). Due to the lack of studies investigating the circadian rhythm of the open/close frequency of mussels, putting our results in context of the available literature is challenging, but it does reveal a clear knowledge gap. Nevertheless, distinct differences between the light treatments were still detectable. As in the proportion of open gape, mussels exposed to the red and white ALAN and the dark control treatments showed a semi-diel rhythm in their open/close frequency while mussels exposed to the green ALAN showed a diel rhythm. Opening and closing more frequently between 10am and mid-night at the green ALAN than the control treatment, where they had almost identical open/close frequency the earlier hours of the day, could indicate that mussels perceive the green ALAN in the same way as darkness; but the green ALAN has a carry-on effect to the mussel's activity during the day and the first hours of night-time. The effect of ALAN on the daytime activity of mussels was also confirmed by the fact that there was no significant difference between the day-time and night-time proportion of time open, even though, mussels under all treatments were exposed to the same day-time lighting conditions. A similar impact of ALAN during the hours of natural light was also observed in the melatonin production by fish (Brüning *et al.*, 2016), the behaviour of birds (Dominoni *et al.*, 2013) and the assemblage of pollinators (Knop *et al.*, 2017) suggesting that ALAN does not only affect night-time biological processes but also day-time ones.

Interestingly, the proportion of time open and the open/close frequency of mussels, when exposed to ALAN conditions, are not correlated (S4.3). Difference between these activity responses was also observed when mussels were exposed to increased CO_2 levels (Hasler *et al.*, 2017) meaning that the two activity responses could expose different impacts of the pollutant in question. For instance, it was suggested that CO_2, serotonin and other compounds induce a relaxation of the adductor mussel (Salánki, 1963; Hasler *et al.*, 2017) hence, the mussel would be open due to the elasticity of the ligaments at the hinge (Elizabeth Gosling, 2003). Other forms of pollutants that would not affect the adductor muscles directly could prompt a higher open/close frequency, in comparison to optimal circumstances, which is considered a stress response (Andrade *et al.*,

2016) as bivalves would open to test the ambient conditions and close at the presence of a disturbance (Curtis *et al.*, 2000; Kobak and Nowacki, 2007).

In the present study, mussels exposed to green ALAN had the highest open/close frequency followed by the mussels in the control treatment and those subjected to red and white ALAN. These imply that green ALAN, in comparison to the other ALAN treatments, may be impactful to the individuals' physiology and fitness, as increased opening and closing is energetically costly, can affect the heart rate of bivalves (Curtis *et al.*, 2000) and increase chances of predation (Kobak and Nowacki, 2007). However, the average open/close frequency under all treatments was <1 h^{-1} which was analogous to the zebra mussel activity (Kobak and Nowacki, 2007) but lower than the *Pinna nobilis* activity in the field (Garcı *et al.*, 2008). Our results are also similar to open/close switches observed in oysters under control condition but minimal in comparison to the >20 h^{-1} at the presence of toxic algae (Nagai *et al.*, 2006). Therefore, even though differences were identified between the treatments, ALAN overall does not provoke an extremely stressful behaviour in the mussels as other toxic algae did in oysters (Nagai *et al.*, 2006).

Mussels exposed to red ALAN consumed the most phytoplankton while mussels exposed to the green ALAN the least. No differences were identified when compared to the white ALAN or the dark control treatment. In terms of the green ALAN, studies on the response of phytoplankton have identified growth in the green algae *Tetraselmis suesica* and the diatom species *Skeletonema* along with increased abundance of other diatom species (Oh *et al.*, 2008; Diamantopoulou *et al.*, 2021) but inhibition of the harmful dinoflagellate *Heterocapsa circularisquama* (Oh *et al.*, 2008). Based on the latter, Oh et al. (2008) recommended the use of green wavelength lights in bivalve aquaculture areas however, the decreased feeding identified in this study might have direct impacts on the cultured bivalves including declined growth (Riisgård, 1991) and meat yield (Riisgård and Randløv, 1981). Despite the high phytoplankton consumption identified here under red ALAN, red light from a fluorescent source has been shown to decrease mussel growth (Strömgren, 1976; Nielsen and Strömgren, 1985) but there is no evidence in literature of any effects LED ALAN sources could have on bivalve growth; a matter that merits investigation. Even though red ALAN

seems to be beneficial to mussel phytoplankton consumption, it has been found to stimulate the abundance and dominance of diatom phytoplankton species (Diamantopoulou *et al.*, 2021), hence red ALAN could stimulate algae bloom events. With both green and red ALAN stimulating algae growth and change in their assemblage towards more toxic species, in addition to the reduced phytoplankton consumption under green ALAN, the anticipated ecological impact would be an increased potential for harmful algal blooms. Sequentially, the open/close frequency of mussels could increase notably in the presence of toxic algae species as seen in oysters (Nagai *et al.*, 2006).

Additional to the ALAN treatments, we have tested for possible effect of the mussels' collection season on the phytoplankton clearance capacity of mussels. This is an important variable as the collection season usually coincides with variation in the food availability (Kautsky, 1982), spawning stages (Okumuş and Stirling, 1998; Fernández *et al.*, 2015) and natural photoperiod. Mussels had a greater phytoplankton removal capacity during winter, which is during the pre-spawning reproductive stage and while the daylength was about 8 hours. The lowest phytoplankton consumption was in Autumn which in turn coincides with the post-spawning stage and approximately 11 hours of day. These outcomes can be explained by previous studies investigating the effects of spawning on the biochemical composition of mussels. Specifically, during the pre-spawning stage mussels exhibit an increase in their meat weight in correlation with to increased glycogen and protein stored in the mantle for gonad development (Okumuş and Stirling, 1998; Fernández *et al.*, 2015). On the contrary, during the post-spawning period there is a decrease in growth, and about 20% weight loss (Smaal and Vonck, 1997) due to gonad regress as well as damage to the reproductive tissue (Kautsky, 1982). Hence, there is increased phytoplankton consumption during the winter pre-spawning period due to increased metabolic requirements for gametogenesis and decrease energy budget during the post-spawning period in early autumn. There is no substantial evidence that the experimental photoperiod (12 hours daylight) may have affected the consumption of phytoplankton as, in that case, winter collected mussels would have spawned if mislead to think that it was summer.

Regarding the relation between the gaping activity and feeding rates of bivalves, past studies have concluded that the valve gape state (open or close) cannot be used as a proxy for the feeding activity (Jørgensen *et al.*, 1988; Frank *et al.*, 2007; Macdonald *et al.*, 2009). However, some studies have shown a linear relationship between the siphon area and the clearance rate (Jørgensen *et al.*, 1988), as well as between the valve gape state and the clearance rate (Frank *et al.*, 2007). In the present study, our results show a significant relationship between the phytoplankton consumption and the proportion of open gape, but the direction of such relationship depended on the ALAN treatment. A positive linear relationship between the proportion of time open and the phytoplankton clearance capacity was observed in the mussels exposed to white and red ALAN and the dark control treatment. However, exposure to green ALAN reverted this relationship i.e., as the proportion of time open decreased, the phytoplankton consumption increased. According to Kobak and Nowacki (2007) zebra mussels avoided red, green, white and blue light by moving to a shaded area hence, a possible explanation could be that mussels in our experiment were open to protrude their foot in an attempt to move away from the green ALAN, even though that was impossible due to the attachment on the valvometry system, and therefore not filtering efficiently during that time. Zebra mussels were exposure to the different wavelengths for 24 hours (Kobak and Nowacki, 2007) while in our experiment we used 12 hours of daylight and 12 hours of ALAN, which are more realistic conditions to the field. The lower duration of exposure in our experiment could justify why this escape response was not shown by mussels exposed to the red and white ALAN while emphasising the possibly impactful effect of green light under any light regime. In an ecological perspective, relocation would have a great energy expenditure especially if green ALAN is extended over a large coastline with limited options for shade, which could also introduce competition for space.

The present study confirms that mussels are sensitive to wavelengths around green colour, like scallops (Cronly-Dillon, 1966; Speiser *et al.*, 2011), possibly resulting in negative effects on the ecosystem services provide by these organisms. This work can be considered the basis for future experimental studies on the effects of ALAN on coastal organism. With the acquired results we would suggest that the use of green ALAN wavelengths would best be avoided in coastal areas. Based on

our findings, red ALAN seems to be the least disturbing to the ecosystem functioning of mussels. These results contradict our hypothesis that red ALAN would be more impactful but, the disparity could be attributed to the lack of literature on the specific long wavelengths that bivalves are sensitive to. Regarding coastal mussel populations, we agree with the suggestions made by studies on sea turtles (Miller and Bretschneider, 2006) and corals (Ayalon *et al.*, 2019), that red ALAN would be the least disturbing ALAN for mussels populations however, the possible increase in phytoplankton abundance under red light would increase the possibility of harmful algal event (Diamantopoulou *et al.*, 2021). The use of white ALAN may be considered at lower intensities as it showed no significant effect on neither mussel nor phytoplankton assemblages but the specific photosensitivity of other organism of conservation interest must be considered. Moreover, local habitat characteristics must also be considered, as cloudy conditions and tidal retreats significantly amplify red light irradiance (Davies *et al.*, 2020). Additionally, more studies need to be employed investigating the effect of ALAN wavelengths on other bivalve species and ecosystem services in conjunction with other pollutants and stressors faced by coastal ecosystems.

Acknowledgements

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General Discussion

This is the first work investigating separately the independent effects of microfiber pollution and artificial light at night (ALAN), two of the most prevailing stressors of coastal ecosystems, on mussels. After conducting a literature review of microplastic exposure studies on bivalves, I identified the need for more environmentally relevant experimental work with a focus on microfibers and using realistic concentrations. Thereafter, I conducted a short-term experiment investigating the effect of acute microfiber concentrations and eutrophication conditions on the filtration capacity of mussel. I found no immediate impacts imposed by the stressor, but a post-exposure decrease in the phytoplankton clearance capacity of mussels was observed after five days of microfiber-free conditions. A subsequent long-term exposure experiment, investigating the effects of chronic microfiber exposure, confirmed the lack of immediate short-term effects but indicated a negative result on the ecosystem services provided by mussels after 39 days of exposure. This effect was likely due to microfiber accumulation in the digestive system of mussels. It was also evident that the most potent individuals accumulated the greatest quantity of microfibers in their digestive system. Lastly, ALAN had a wavelength-dependent effect on the activity and phytoplankton clearance capacity of mussels. Mussels exposed to green ALAN showed the greatest gaping activity and the least phytoplankton consumption in comparison to red ALAN while no statistical differences were observed between the green ALAN and the dark control treatment.

The high volume of research investigating the effects of microplastics on bivalves called for a systematic literature review assessing their experimental settings and relevance to the conditions in the field. Nearly all studies have used mussels for their experiments and more than 217 types of responses were tested. The focus was mainly on the effects of spherical micro/nanoplastics, despite the high abundance of microfibers in the field (Barrows et al. 2018), and polystyrene (PS) even though the distributions of different types of microplastics in the field is not uniform across the world's coastal ecosystems (Erni-Cassola *et al.*, 2019). Regarding the microplastic size, most studies used particles smaller than 10μm however, there is no consensus in the microplastic size in the field due to

limitations in the sampling methodology (Covernton *et al.*, 2019). The duration of exposure was usually 1-7 days but, many factors should be considered before making that choice including the bivalve particle selection stage that is expected to be affected (Christoforou *et al.*, 2020), the response tested, the residence time of the particles (Ogonowski *et al.*, 2018) and the environmental relevance. One of the most controversial topics in microplastic research is the microplastic concentration (Burns & Boxall 2018, Haegerbaeumer et al. 2019) as extreme dosages are used, in comparison to field conditions, which could hinder our understanding of the actual impact of microplastics in nature. Furthermore, this review unravelled problems like the deficiency of reported microplastic characteristics and discrepancy in the units, especially about the microplastics concentrations used. Therefore, I recommend that future exposure studies aiming at investigating the vulnerability of bivalves to microplastic pollution should focus on microfibers between 1-500μm at concentrations ranging from 100particles/L to 1000particles/L. The microplastic type and duration of exposure would be dependent on the geographical location of interest which the conditions would be simulating. Lastly, studies should report the concentration in particles/L and provide all the details regarding the experimental setting to allow transparency, replicability, and comparison between them for better interpretation of the effects identified.

Acute weather conditions can be responsible for the extended inflow and resuspension of microplastics and nutrients from terrestrial sources and marine sediment respectively (Spatharis *et al.*, 2007; Chen *et al.*, 2018; Suckling and Richard, 2020). In that case, the concentrations of microplastics available to filter feeders is increased and eutrophication in the area would be exacerbated for a short-term. To simulate these acute microfiber concentrations and eutrophication conditions I performed a short-term laboratory based cross-design experiment. The results did not show any immediate effect on the phytoplankton clearance capacity of mussels after a 24hour exposure. There was however a reduction in the phytoplankton clearance capacity of microfiber exposed mussels after five days of microfiber free conditions. This study highlights the importance of conducting long-term experiments when investigating the effects of chronic microfiber exposure, but short-term exposure could assist in predicting the

responses of bivalves to acute events. It was also important to find that even short-term microfiber exposure can have lasting post-exposure effects interfering with the ability of mussels to reduce the phytoplankton abundance and therefore effectively provide their ecosystem services especially under eutrophication conditions.

As previously identified, long-term exposure studies are necessary in investigating the chronic effects of microfiber pollution on the ecosystem services provided by mussels. Hence, I conducted a 52-day long-term experiment using a microfiber (<100μm) and a control, microfiber-free treatment. The results confirm the absence of any immediate effects after a short-term exposure but increased variability in the phytoplankton consumption by mussels after 26 days of exposure show higher unpredictability in their clearance capacity. After 39 days, there was a significantly lower phytoplankton consumption, by 21.3%, by microfiber-exposed mussels than the control treatment revealing a long-term negative effect. This effect was hindered on day 52 by the presence of fouling diatoms which interfered with the consumption of the flagellate *Tetraselmis* sp. used in the quantification of the phytoplankton clearance capacity of mussels. This occurrence suggests that the effects of microfiber pollution on bivalves may vary in the presence of different microalgae species. Another important finding of this study was that the abundance of microfibers accumulated in the digestive system of mussels was positively related with their clearance capacity indicating that the most efficient phytoplankton consumers are also the most vulnerable to microfiber accumulating. The latter suggests that microfiber pollution may have the greatest impact on the individuals with highest clearance capacity which would considerably impact the ecosystem services provided by the mussel populations.

Artificial light at night is also one of the most widespread forms of environmental pollution. With the majority of research in the field focused on terrestrial organisms (Bennie *et al.*, 2015), this is the first study investigating the effects of ALAN on bivalves. The results on the circadian rhythm of the proportion of time open showed a resemblance to a circatidal rhythm which could be related to the anticipation of food in nature (Williams and Pilditch, 1997; Riisgård *et al.*, 2006; Saurel *et al.*, 2007) despite the absence of any tidal activity simulation in the

laboratory. Even though mussels in all treatments were exposed to the same daytime conditions but different ALAN treatments, there was a great similarity of the responses between day and night-time. These suggest that ALAN does not only affect night-time biological processes but also day-time ones worsening the effects of this sensory pollutant.

The ALAN exposure experiment was conducted with different ALAN wavelengths: green, red, white, and dark as a control. These could give information regarding the least stressful ALAN wavelength for coastal illumination near mussel populations. The results suggest that green ALAN was the most impactful from the wavelengths tested. It induced an increase in the open/close frequency of the mussels' valves which could lead to higher predation risks (Robson, Garcia De Leaniz, *et al.*, 2010) and energy expenditure (Curtis *et al.*, 2000; Kobak and Nowacki, 2007). Additionally, green ALAN caused a decline in the mussels' clearance capacity and it also encourages growth of green microalgae and diatoms (Oh *et al.*, 2008; Diamantopoulou *et al.*, 2021). Hence, increase in phytoplankton abundance and decrease in the bivalve phytoplankton consumption could have detrimental effects on coastal ecosystems due to the possibility of eutrophication and harmful algal bloom events. Red ALAN showed the opposite effects with a lower open/close frequency and an improvement in the phytoplankton clearance capacity indicating that it might be the least impactful option for mussel populations. These results agree with suggestions made by studies on sea turtles (Miller and Bretschneider, 2006) and corals (Ayalon *et al.*, 2019) but, red ALAN could also increase the abundance of diatom phytoplankton species which could also trigger harmful algal bloom events (Diamantopoulou *et al.*, 2021). Additional to the effects of ALAN, mussels collected in different seasons showed a variation in their phytoplankton clearance capacity with winter-collected mussels consuming more than the autumn-collected ones. This difference could be attributed to the mussel seasonal reproductive cycle as, in winter mussels are feeding more to meet the metabolic requirements related to gonad development including gametogenesis (Smaal and Vonck, 1997) while in autumn, mussels had already spawned, losing weight and suffering from gonad damage (Kautsky, 1982; Smaal and Vonck, 1997).

Conclusions and future recommendations

The systematic review conducted on studies investigating the effects of microplastics on bivalves suggest that our knowledge and understanding of the extent of the microplastic pollution problem might be biased due to the high concentrations of microplastics used. This work sets a framework for future microplastic exposure studies which should focus more on microfibers and use longer duration of exposure and lower concentrations. Additionally, all the required information on the microplastic characteristics and concentration should be provided in detail to allow for comparability between the studies. Information and results from these studies, on the impacts of realistic microplastic conditions on bivalves and future projections, would be valuable in providing evidence-based information for stakeholders to act upon the current plastic production and disposal practices which are the main causes of the microplastic pollution problem.

Through the microfiber exposure experiments it was apparent that short-term exposure did not affect the ecosystem services provided by mussels but there was a negative effect both post-exposure and after a long-term exposure. It was therefore evident that microfiber pollution does impair the capacity of mussels to control the phytoplankton abundance and therefore remediate the effects of eutrophication. These negative impacts of microfibers are expected to increase because of the continuous production of fiber-based materials, especially due to the COVID-19 pandemic, and their degradation, which would increase the abundance of microfibers in coastal ecosystems. Therefore, these results should be considered by policy makers and waste treatment plants who should implement solutions, like appropriate filtration systems to reduce the inflow of microfibers in coastal ecosystems. As these experiments were conducted with the mussel *Mytilus edulis*, the phytoplankton *Tetraselmis* sp. and nylon microfibers, it would be interesting for future work to repeat the same experimental designs with different bivalve species, phytoplankton species, including toxic bloom forming microalgae species, and/or different microfiber types. These would account for the differences between the filtration mechanism of bivalve taxonomic groups, the difference observed in the long-term experiment regarding the clearance of

Tetraselmis sp. at the presence of fouling diatom and the differences in the composition and characteristics of microfiber types.

Regarding the artificial light at night, it was apparent that green light might not be the most appropriate ALAN for coastal illumination near mussel populations especially because not only it has the potential to reduce the ecosystem services provided by mussels through reduced feeding and higher energy expenditure, but it also increased the phytoplankton abundance and changed the relative abundance of the microalgae assemblage which can amplify the problem of eutrophication and harmful algal blooms. Considering the results conducted in the current study and by Diamantopoulou *et al.*, (2021), where mussels and phytoplankton had similar response to white ALAN and the dark control, I would recommends the use of dim white ALAN near coastal population, especially in the absence of turtle nests and coral colonies and only if artificial illumination is absolutely necessary. Further research on the sensitivity and possible implications to other coastal organisms in the community should be conducted before these lights are installed. In addition, more research is required on the sensitivity of other mussel species and bivalve taxonomic groups to ALAN wavelengths and their effect on responses like growth, fecundity, and mortality as well as any implication on the provision of ecosystem services.

Furthermore, the exact role of mussels in ecosystem functioning should be quantified under different environmental conditions such as water turbidity, water renewal times and nutrient remineralisation. It would also be interesting to investigate any potential new ecosystem services that mussels could provide like the filtration and removal of microplastics from the water column. The meat of those mussels, may not be suitable for consumption but they could then have a plethora of uses as summarised by Naik and Hayes, (2019) including the utilisation of the shells as ingredients for livestock feed, the creation of adhesives from the byssus thread produced and other biotechnological applications.

Lastly, I suggest that particular attention should be given by future studies on the synergistic effects of marine stressors like microfiber pollution, ALAN, and harmful algal blooms as well as other stressors associated with climate change like water

acidification, increase in water temperature and anoxia. Knowledge and understanding of the impacts these stressors impose on coastal ecosystem would inform evidence based environmental policy making and conservation efforts which, in their turn, should be implemented to reduce these pressures and form healthier coastal ecosystems. Healthy coastal ecosystems are directly related to undisrupted and continuous provision of their goods and services which are so vital for the environment and humanity.

Appendix 1

Chapter 1 - Supplementary Material

Table S 1-1: Reference list of the studies included in this review assessing effects of MP on marine bivalves.

Publication number	Reference
1.	Alvarez MR, Friedl FE, Johnson JS and Hinsch GW 1989 Factors affecting in vitro phagocytosis by oyster haemocytes. Journal of Invertebrate Pathology 54: 233–241.
2.	Browne MA, Dissanayake A, Galloway TS, Lowe DM and Thompson RC 2008 Ingested microscopic plastic translocate to the circulatory system of the mussel, Mytilus edulis (L.). Environmental Science and Technology 42: 5026–5031.
3.	Wegner A, Besseling E, Foekema EM, Kamermans P and Koelmans AA 2012 Effects of nanopolystyrene on the feeding behavior of the blue mussel (Mytilus edulis L.). Environmental Toxicology and Chemistry 31: 2490–2497.
4.	Von Moos N, Burkhardt-Holm P and Köhler A 2012 Uptake and effects of microplastics on cells and tissue of the blue mussel Mytilus edulis L. after an experimental exposure. Environmental Science and Technology 46: 11327–11335.
5.	Garcia-valtanen P, Figueras A, Novoa B and Estepa A 2013 Mussel Mytilus galloprovincialis 33: 2328–2346.
6.	Van Cauwenberghe L, Claessens M, Vandegehuchte MB and Janssen CR 2015 Microplastics are taken up by mussels (Mytilus edulis) and lugworms (Arenicola marina) living in natural habitats. Environmental Pollution 199: 10–17.
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Table S 1-2: Recompilation and classification of responses tested in laboratory studies assessing the effects of MP on marine bivalves.

Responses	Grouped responses
Absorption efficiency	Feeding behaviour
Pseudo faeces and faeces production	
Filtering activity	
Clearance rate	
MP in faeces	
MP in pseudo faeces	
Filtration rate	
Ingestion rate	
Consumption	
Oxygen consumption	Metabolism
Assimilation efficiency	
Respiration rate	
IDH (Isocitrate dehydrogenase) activity	
SDH (Succinate dehydrogenase) activity	
Pyruvate kinase activity	
CYP1A1 content	
GABA (Aminobutyric acid) content	
PK (Pyruvate kinase) activity	
Isoleucine level	

Leucine level	
Valine level	
Alanine level	
Dimethylglycine level	
Glycine level	
Tyrosine level	
Lactate level	
Acetoacetate level	
Succinate level	
Malonate level	
Glycogen level	
Glucose level	
CYP1A1 content	
Cortisol levels	
% depolarised mitochondrial membrane	
EROD (Ethoxy resorufin-O-dethylase) activity	
MDHF (Malate-dehydrogenase-fumarate) activity	
CS (Citrate synthase) activity	
TMRE (Mitochondrial Membrane Potential Assay)	
NAO (Nonyl acridine orange) assay	
Hurst exponent (Loss of NADH in time)	
Protein amount	Growth
Lectin content	
Lipid amount	
Carbohydrate amount	
Total energy	
Energy reserves	
Scope of growth	
ATP content	
% of cell size	
Development arrest	
Shell length	

Condition index	
Red granulocyte %	Immunotoxicity
Basophil %	
Hyalinocyte %	
Hyalinocyte size	
Granulocyte size	
Phagocytic cells	
(THC) Total haemocyte count	
Haemocyte infiltration	
Phagocytosis of haemocytes	
Haemocyte viability	
Haemocyte in diapedesis	
Haemocyte mortality	
Phagocytosis capacity	
Haemocyte concentration	
Granulocyte concentration	
Lysozyme activity	
Immunotoxicity	
Expression of MYTLB	
Oxidative activity	Oxidative Damage
LPO (Lipid Peroxidation)	
PCC (Protein Carbonyl Content)	
Vacuolation	
AOX (Alternative Oxidase) activity	
Oxidative stress	
Oxidative damage	
Oxygen species concentration	
MDA (Malondialdehyde) levels	
H2O2 concentration	
O2 concentration	
NOS (Nitric toxic radicals) amount	
Extracellular ROS production	

Nitrite accumulation	
Fecundity	Fecundity
Death spermatozoa	
VAP (velocity of the average path)	
Oocyte diameter	
Total oocyte count	
Fertilization success	
Maximum swimming speed	
Sperm velocity	
Gonad growth	
Gametogenesis	
Larval growth	Larval Development
Development rate	
Shell height	
Hatchling rate	
Larvae malformations	
Shell biogenesis	
Metamorphosis success	
Development arrest	
Embryotoxicity	
Expression of EP	
Expression of CA	
Expression of CS	
D-larval Yield	
Lysosomal damage	Structural Damage
Lysosomal integrity	
Lysosomal membrane stability	
Anisotropy	
NRRT (Neural red retention time)	
F-actin level	
Viscosity	
Protein aggregates	

Lysozyme release	
Expression of GUSB	
Expression of HEX	
Expression of CTSL	
Histological damage	
Digestive tubule atrophy	
Intestinal inflammation	
Intracellular damage	
Apoptosis	Apoptosis
% apoptotic cells	
Number of apoptotic cells	
% non-valid cells	
Necrosis	
Caspase-3-activity	
Caspase 3/7 activity	
Cell viability	
Expression of P53	
FADD protein production	
Percentage of haemocytes positive to FITC Annexin V binding	
Percentage of haemocytes positive to both ANX and PI staining	
Fluoranthene accumulation	Bioaccumulation
BDE-209 accumulation	
MP accumulation	
Rho 123 accumulation	
BaP accumulation	
Xenobiotic accumulation	
Neurotoxicity	Neurotoxicity
DOP (Dopamine)	
SER (Serine)	
GLU (Glutamate)	
AChE (Acetylcholinesterase)	

MAO (Monoamine oxidase)	
Expression of 5-HT1	
Expression of MeER1	
Expression of MeER2	
Neuroendocrine signalling	
Stress-related protein production	Osmoregulation
Hypotaurine level	
Betaine level	
Taurine level	
Homarine level	
ROS (Reactive oxygen species) production	Antioxidant Capacity
SOD (Superoxide Dismutase) activity	
GST (Glutathione S-transferase) activity	
Glutathione reductase	
Glutathione level	
Expression of MT10	
Expression of MT20	
CAT (Catalase) activity	
GPx (Glutathione peroxidase) activity	
Expression of genes related to CAT	
DBF (Dioscorea batatas flesh) activity	
GSH (Glutathione) activity	
TBARS (Thiobarbituric acid reactive substances)	
Ferric reducing antioxidant potential	
DNA damage	Genotoxicity
Expression of MYTC	
Expression of LYC	
ABCB transcripts	
ABCC transcripts	
Gene expression	
Tail DNA	
OTM (Olive tail moment)	

DNA tail	
Nuclear alteration	
Expression of genes related to RNA protein	
Expression of genes related to protein binding	
Expression of genes related to nucleotide binding	
Expression of genes related to metal ion binding	
Expression level of CYPIA2	
Expression level of NFkB	
Expression level of IKKa	
Expression level of Caspase 3	
Relative expression for CAT gene	
Relative expression for GST gene	
Relative expression for SOD gene	
GPx relative gene expression	
Downregulation of AcP (Acyl Carrier Protein) gene	
Protein related to DNA binding	
DNA reparation rate	
Downregulation of genes	
DNA strands break	
SOD (Superoxide Dismutase) mRNA	
CAT (Catalase) mRNA	
% of low DNA content	
Glutathione- S-transferase 1 (gst1)	
Toll-like receptor 13 (tlr13)	
Myeloid differentiation primary response 88 (myd88)	
Heat shock protein 70 (hsp70)	
Micronuclei per 1000 cells	
MN (Micronucleus assay)	
Putative heavy metal binding protein	
Protein related to neurogenesis	
Nuclear alteration	
Cellular viability	Mortality

Death individuals	
Valve closure	Behaviour
Byssus production	
Production of vitellogenin	
Mussel activity	
Maximum swimming speed	
Swimming trajectory: circular, rectilinear, motionless	
Malformation rate	Malformations
Number of OUT (Operational Taxonomic Group)	Microbiota

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S2.1 Tetraselmis sp. monoculture

The monoculture of the green microalgae *Tetraselmis* was cultivated from an inoculum taken from microalgae disks of live *Tetraselmis* sp. (Florida Aqua Farms) and supplemented with F/2 media according to Guillard R.L. Robert (1975). The cultures were maintained at a constant illumination under fluorescent lights, temperature of 21ºC and were renewed bi-weekly. The concentration of the dense culture was calculated by using Fast-Read 102® counting chambers.

S2.2 Microfiber size distribution

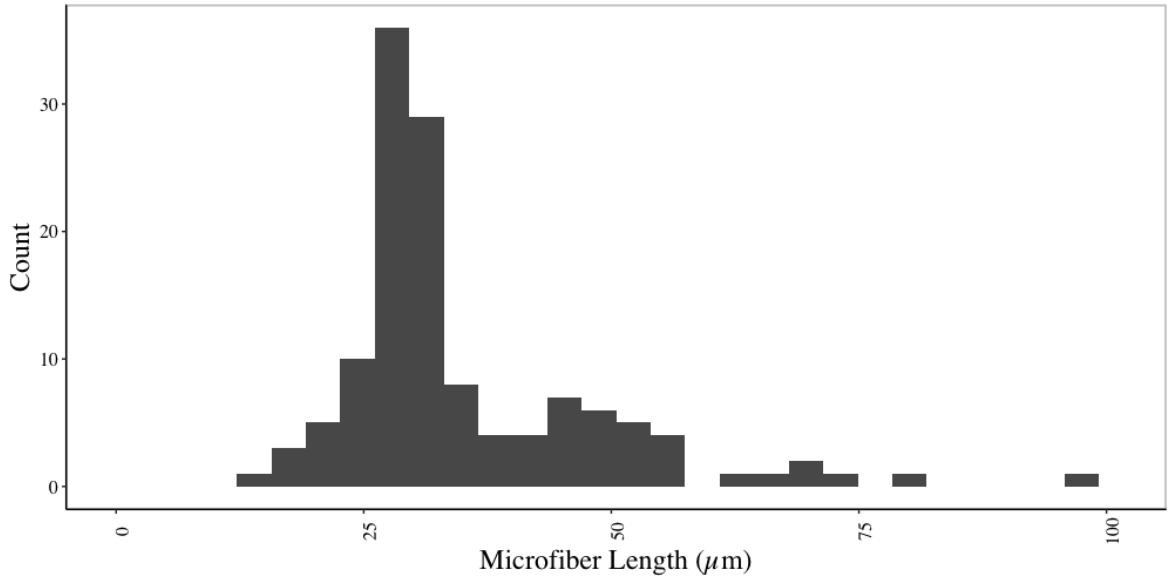

Figure S 2-1: Frequency distribution of the lengths of microfibers used in this experiment. From this graph, 8.78% of the microfibers measured were >100µm and excluded from this diagram.

S2.3 Microfibers use in comparison to air-borne microfibers

Figure S 4-2: (A) Nylon microfiber prepared in the laboratory and used in the experiment, (B) Other air-borne microfibers found in the water samples (images not to scale but attention is drawn on the variation in colour and shape).

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Microfibers accumulated in the mussels

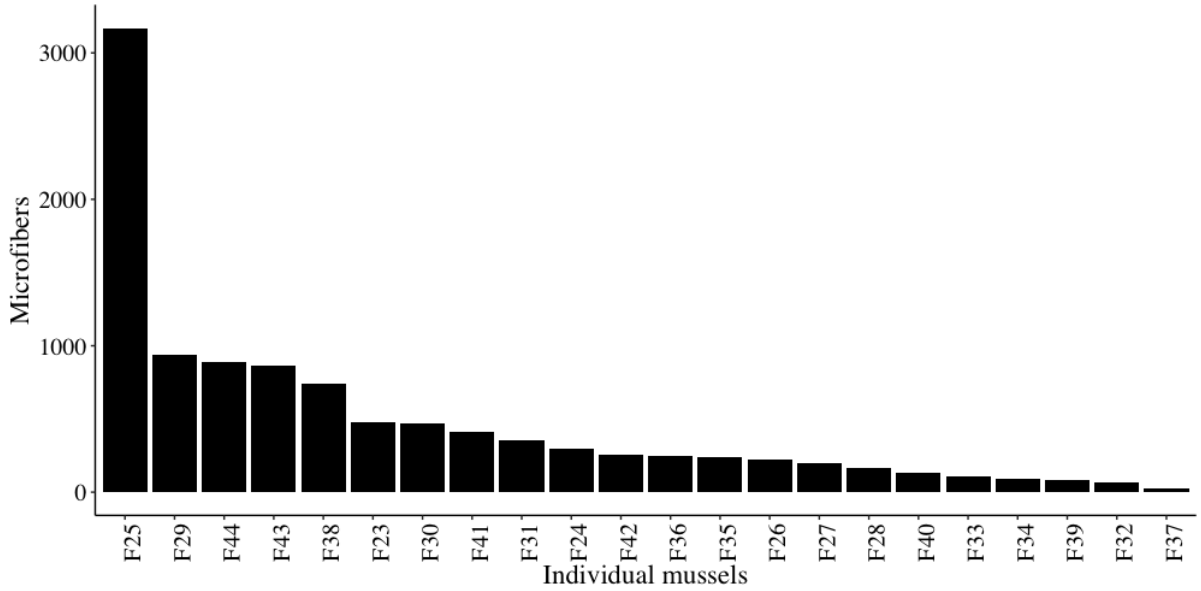

Figure S 4-1: The number of microfibers found in the digestive gland and stomach of individual mussels after enzymic digestion.

Chlorophyll-C concentration

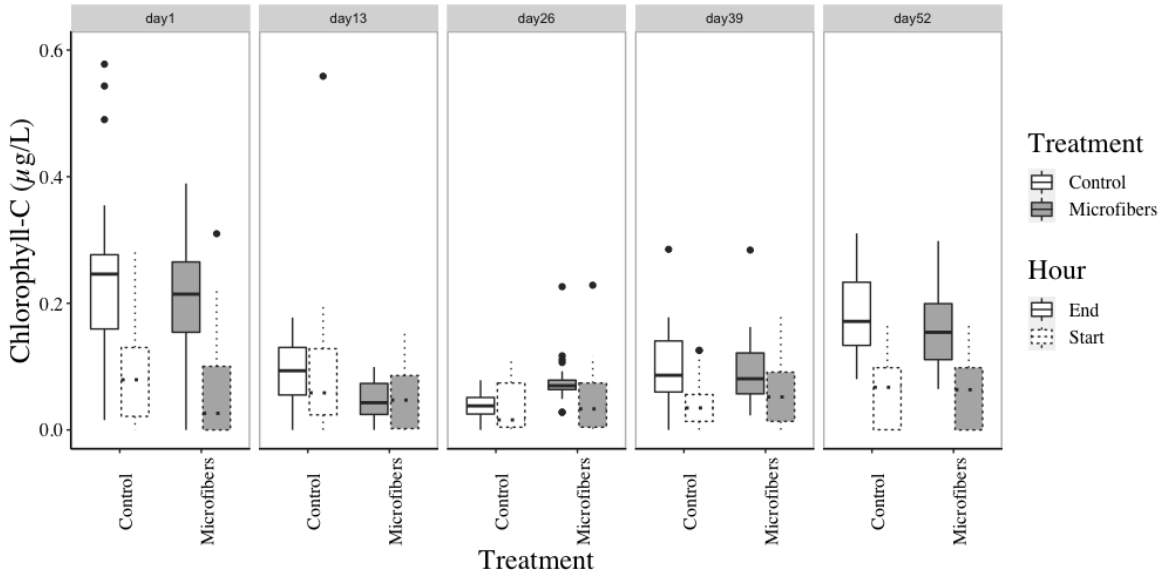

Figure S 3-2: Chlorophyll-C concentration expressing diatom contamination, during the experiment at the Start-0h and End-24h of each of the five sampling points for both Control and Microfiber treatments.

Anatomical particle selection pathway in mussels

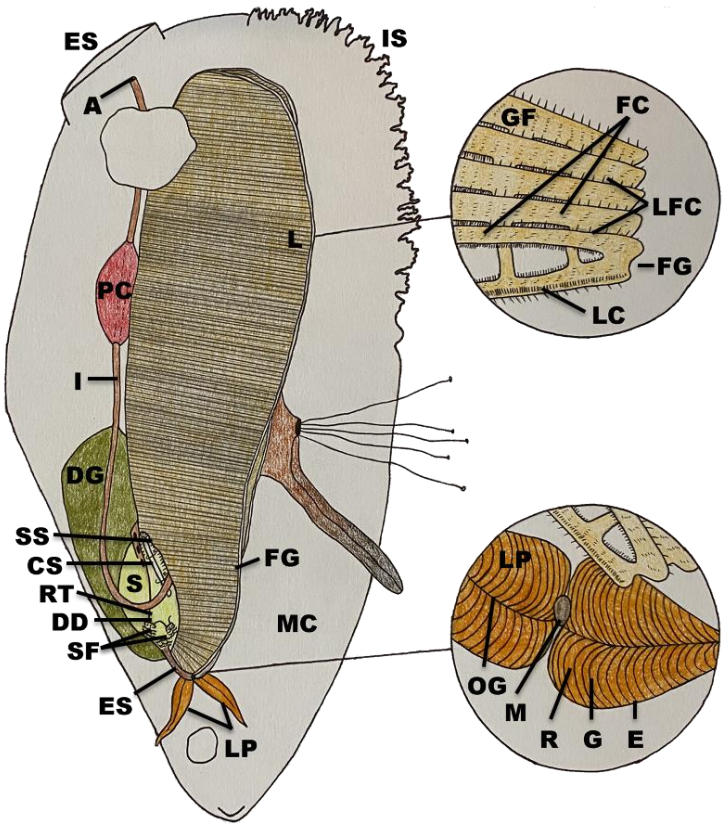

Figure S 3-3: Inflow water currying organic and inorganic particles (<500µm long (Newell *et al.*, 1989; Rosa *et al.*, 2018), <50µm wide (Newell *et al.*, 1989)) enter the inhalant chamber through the Inhalant siphon (IS). Lamellae (L) formed by the combined Gill Filaments (GF). Lateral Cilia (LC) create the inflow feeding and respiratory current. Laterofrontal Cilia (LFC) at the edge of the GF overlap each other forming a sticky mesh over the intrafilamentary spaces (ostia) and retain large particles (>1-6µm (Dral, 1967; Ruppert *et al.*, 2004; Rosa *et al.*, 2018)). Water and small particles pass through the ostia the Exhalant Chamber (EC) where they will get ejected through the Exhalant Siphon (ES). Frontal Cirri (FC) entangle larger particles in mucus and transport them over the surface of the L to the ciliated longitudinal Food Groove (FG) which transport particles anteriorly to the Labial Palps (LP). Ciliary current carry unwanted particles in the Grooves (G) of the LP to the Edge (E) where they are released in the Mantle Cavity (MC) to then be ejected as pseudofaeces. Food particles pass across the Ridges (R) of the LP to the Oral Groove (OG) leading to the Mouth (M) (Morton and Puljas, 2018). Ciliary action at the Style Sac (SS) rotate the Crystalline Style (CS) forming a current pulling the food particle from the Esophagus (ES) into the Stomach (S) where grinding of the particles may occur. Ciliated Sorting Fields (SF) move smaller particles (<100µm (Kolandhasamy *et al.*, 2018)) to the Digestive Gland (DG) (also known as digestive diverticula) through the Digestive Ducts (DD) for intracellular digestion or remain suspended in the stomach. Large particles enter the Rejection Track (RT) leading to the Intestine (I) to be ejected as faeces from the Anus (A) (Morton, 1983; Elizabeth Gosling, 2003). Illustration by: Eleni Christoforou.

Diatoms in comparison to microfibers used

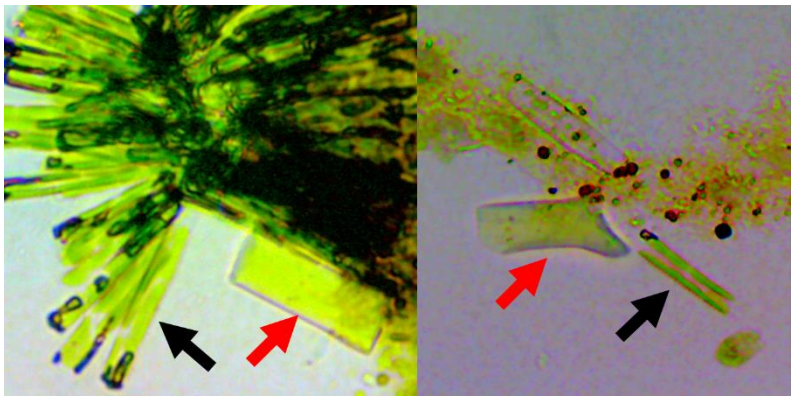

Figure S 3-4: Images depicting similarity in size and shape of the diatoms (black arrow) found in the samples in comparison to the microfibers (red arrow) used in the experiment.

Chapter 4 - Supplementary Material

S4.1 LED Light spectra

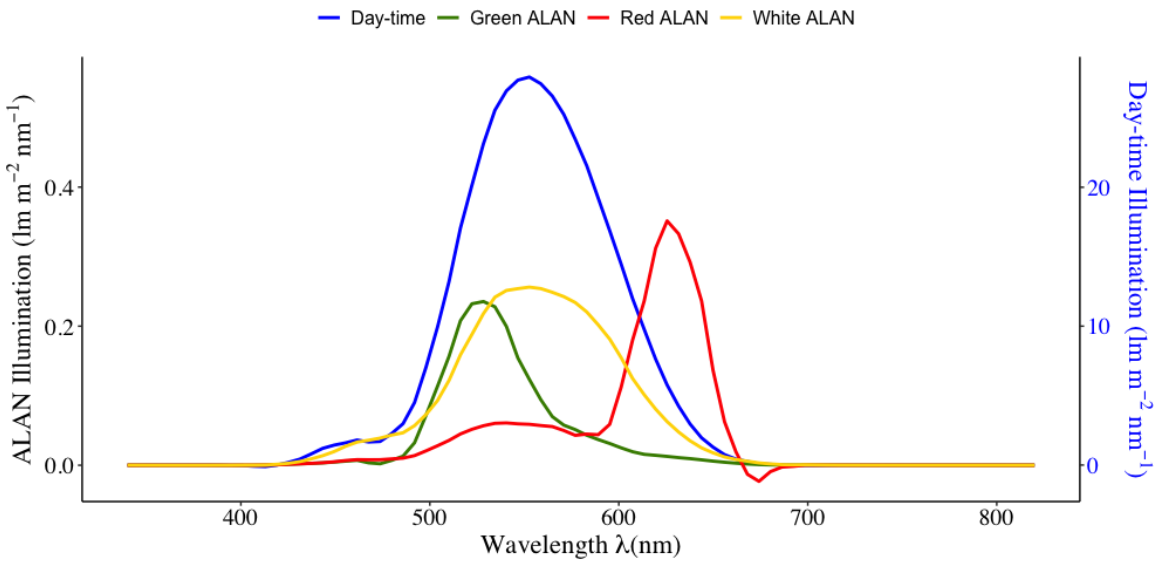

Figure S 4-1: Spectra profile of the day-time (blue - right y-axis) and ALAN (green, red and white – left y-axis) measured with an Apogee® SS-110 Field Spectroradiometer. The illumination level of daylight was standardised at ~3700Lux and all ALAN treatments were standardised at ~20Lux.

S4.2 Valvometry system

For each run of the experiment, 12 independent reed switches were inserted in 15ml plastic serological pipettes which were sealed to prevent water damage and connected to three 4-channel boxes. These boxes were equipped with a potentiometer which attenuated the voltage from 5 Volt-DC to 2.5 volts, as required by the data acquisition devise. For this purpose, a Picolog1216 USB Voltage Data Logger (Pico Technology®), was connected through an external terminal board, which would send the signal from the 12 reed switches. Any active reed switch supplied 2.5 volts to the Picolog devise indicating the presence of physiological activity of the mussel's shell. The Picolog6® dedicated software was used to record the signal.

Each switch could be set at close circuit position by using a small Neodynium (N42) rod magnet (Magnet Experi ®, F214, 2mm diameter x 4mm long). A magnet was attached to a thin stainless-steel strip which in turn was attached to the posterior end of the left valve of the mussel via an aquarium safe instant adhesive gel (JBL

PRO HARY®). The stainless-steel strip was bent to avoid any contact with the protruding mantle at the exhalant valve. The serological pipette was secured at the right valve of the mussel via a thin strip of a rubber waterproof tape (Flex Tape®, FTB501). When the mussel was at a closed position (i.e., the magnet was at a close proximity causing the reed switch to close), 2.5 volts would be applied to one of the inputs of the PicoLog1216. When the mussel was open, there would be no output hence zero volts would be recorded. A simplified schematic representation of the mussel gape tracking system can be seen in Figure S4-2.

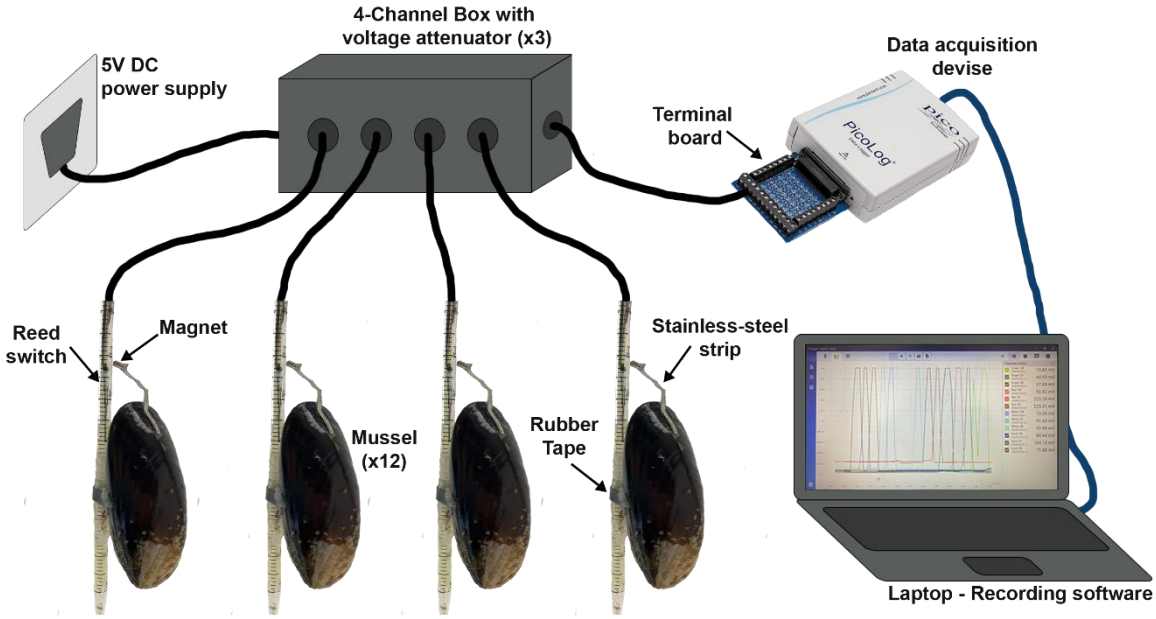

Figure S 4-2: Simplified schematic representation of the mussel valvometry system. Only one out of the 3-in-total 4-chanel boxes are presented hence only four out of the 12 mussels tracked per run.

S4.3 Relation between proportion of time open and open/close frequency

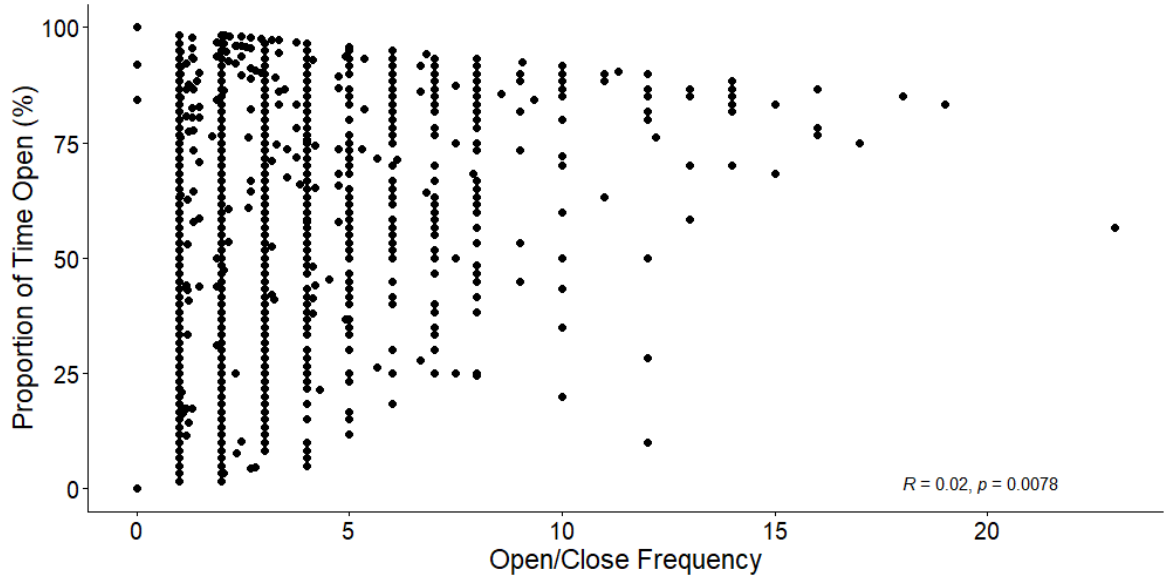

Figure S 4-3: Scatterplot of the relation between the proportion of time open and the open/close frequency conducted by a Spearman correlation test, the correlation coefficient (R) and the significance level (p) are noted.

S4.4 Identifying and excluding outliers

The data from the phytoplankton removal capacity experiment were negatively skewed (medcouple = -0.234). Hence an outlier was considered any value falling outside the range described by Hubert and Van Der Veeken (2008) on univariate data. Identified outliers are labelled in Figure S4-4 and were excluded from any analysis.

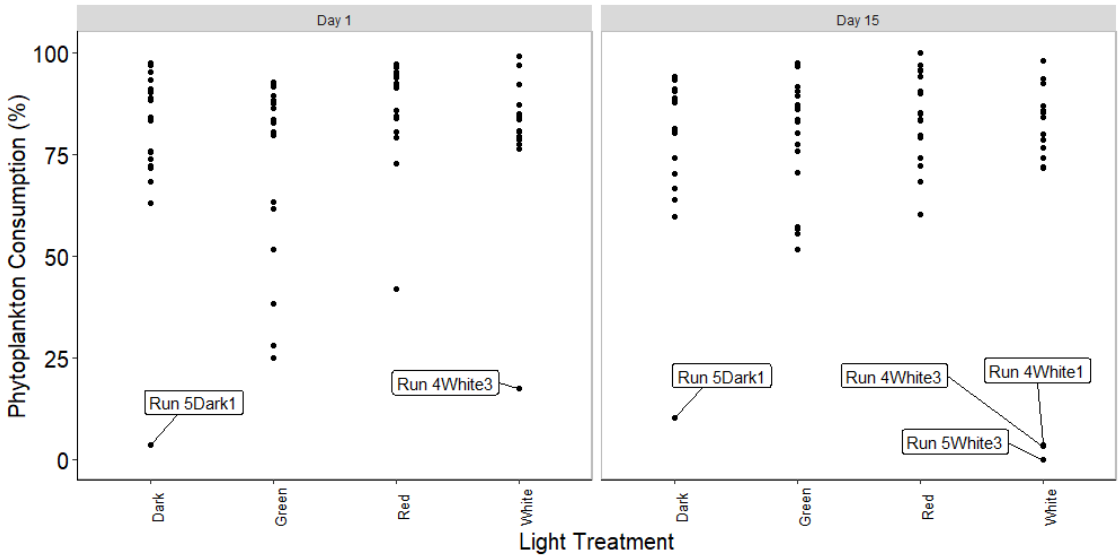

Figure S 4-4: The percentage of phytoplankton consumed by mussels, per experimental day, where the identified outliers are annotated with their mussel ID code.

S4.5 Actogram of individual mussels

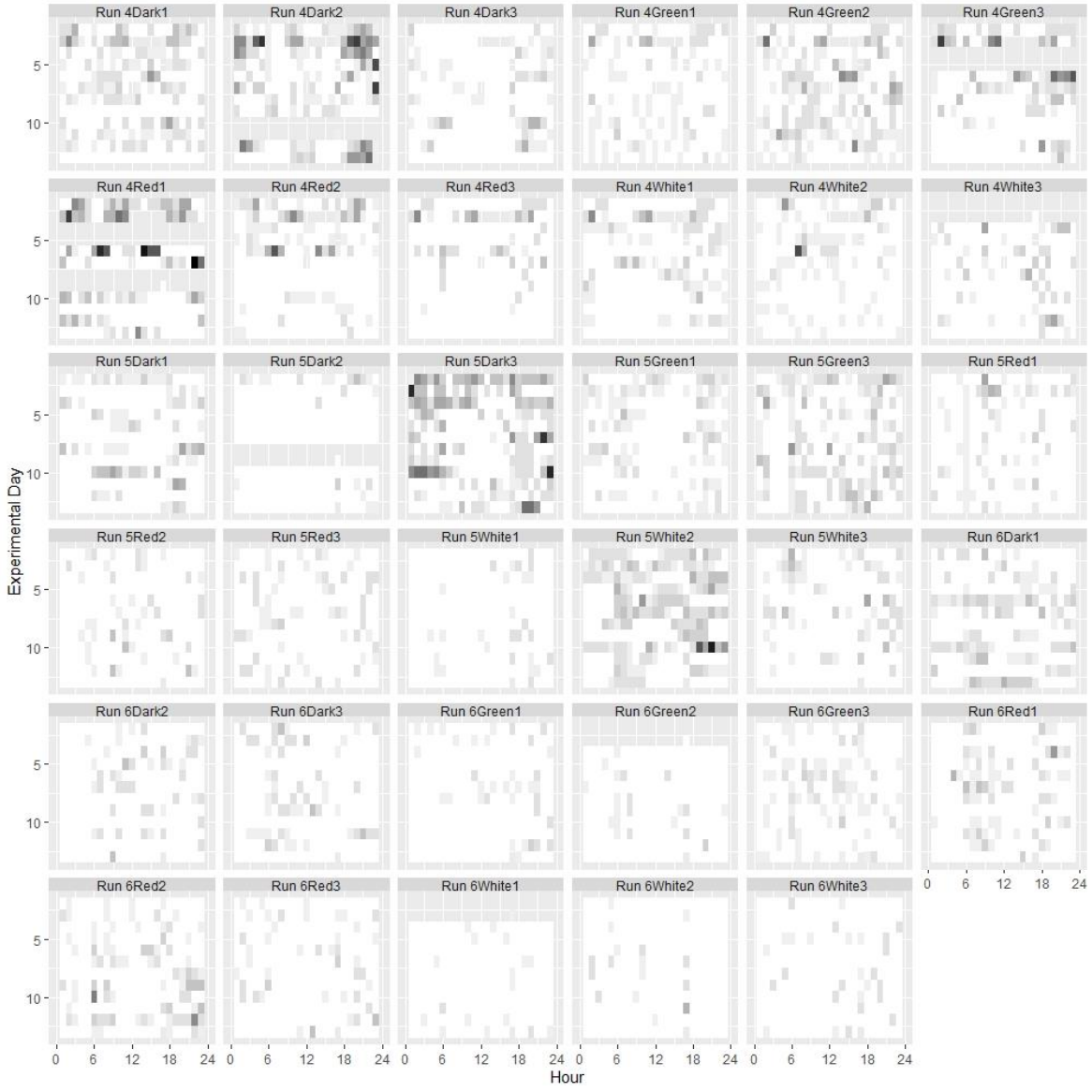

Figure S 4-5: Actogram of the mussels in each run of the experiment. The name of each plot represents the individual mussel ID, i.e., the run number, the light treatment and the replicate number. Within each plot, rows indicate the number of days since the start of the experiment, and columns the hours of day. Between the hours of 6:00 and 18:00 the lights were on (day-time) while 18:00 to 6:00 the ALAN light were on or the light were off at the control treatment. The colour intensity represents the amount of activity within each hour bin.

S4.6 GAM summary outputs

Table S 4-1: Detailed summary of the best supported model (GAM) of the response variables (A) proportion of time open and (B) Open/close frequency.

(A)

Explanatory Variable	**Estimate**	**Standard Error**	**Z-Value**	**P-Value**
Intercept	0.327	0.062	5.284	1.27×10^{-7}
Experimental day	-0.052	0.003	-17.5	$<2 \times 10^{-16}$
Water Change (Yes)	0.292	0.020	14.872	$<2 \times 10^{-16}$
	edf	**Reference df**	**Chi-square**	**P-Value**
s(Hour):Dark	6.369	8	92.78	$<2 \times 10^{-16}$
s(Hour):Green	5.979	8	106.17	$<2 \times 10^{-16}$
s(Hour):Red	6.947	8	226.40	$<2 \times 10^{-16}$
s(Hour):White	6.188	8	140.79	$<2 \times 10^{-16}$
Random effect: s(musselID, Run)	67.888	70	2396.41	$<2 \times 10^{-16}$
Deviance explained	27.5%			
Adjusted R^2	0.198			

(B)

Explanatory Variable	**Estimate**	**Standard Error**	**Z-Value**	**P-Value**
Intercept	-0.17	0.20	-0.89	0.37
Experimental day	-0.4	0.003	-14.11	$<2 \times 10^{-16}$
Water Change (Yes)	0.13	0.019	6.81	9.7×10^{-12}
Day-time/Night-time: Green	0.34	0.14	2.50	0.013
Day-time/Night-time: Red	-0.31	0.16	-2.00	0.046
Day-time/Night-time: White	-0.26	0.16	-1.57	0.12
	edf	**Reference df**	**Chi-square**	**P-Value**
s(Hour):Dark	6.98	8	108.6	$<2 \times 10^{-16}$
s(Hour):Green	4.961	8	106.3	$<2 \times 10^{-16}$
s(Hour):Red	6.382	8	110.6	$<2 \times 10^{-16}$
s(Hour):White	6.944	8	147.9	$<2 \times 10^{-16}$
Random effect: s(musselID, Run)	65.881	67	5796.5	$<2 \times 10^{-16}$
Deviance explained	22%			
Adjusted R^2	0.162			

S4.7 Relation between the Phytoplankton consumption capacity and the open/close frequency

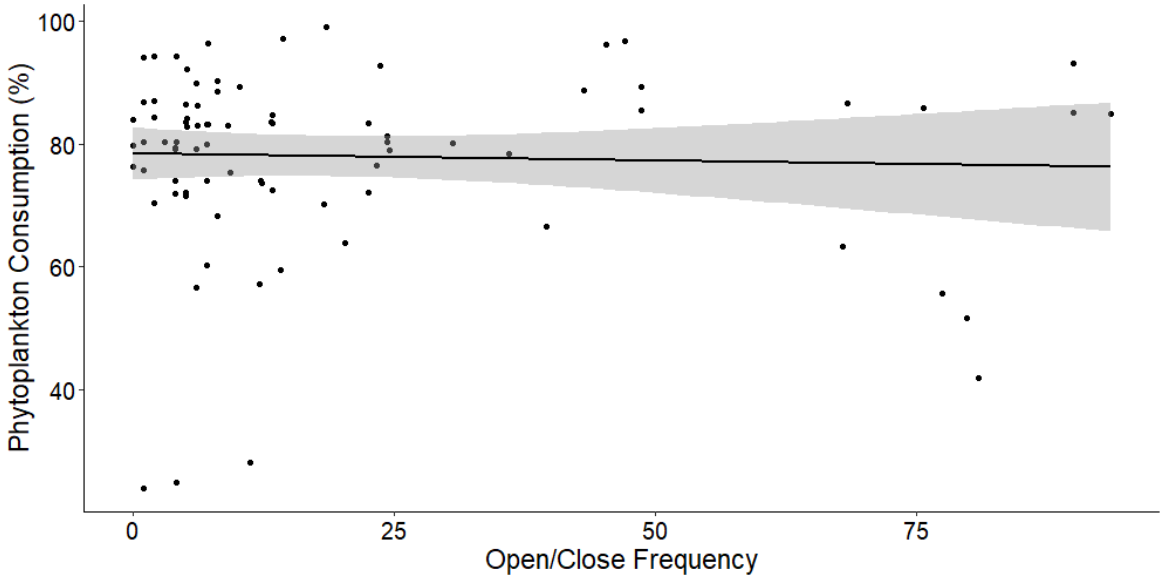

Figure S 4-6: The relation between the percentage of phytoplankton consumption and the open/close frequency of mussels represented by a regression line and the 95% confidence interval (grey band).

S4.8 Mussel gaping activity compared with the tidal pattern

Tidal data were provided by the British Oceanographic Data Centre (BODC). As data for Loch Eil were not available, the data used were from Tobermory, Scotland, the closest location to the mussel collection site. The tidal height at the dates the experiments were running were compared with the mussel activity, both averaged in a 24hour period (Figure S4-7).

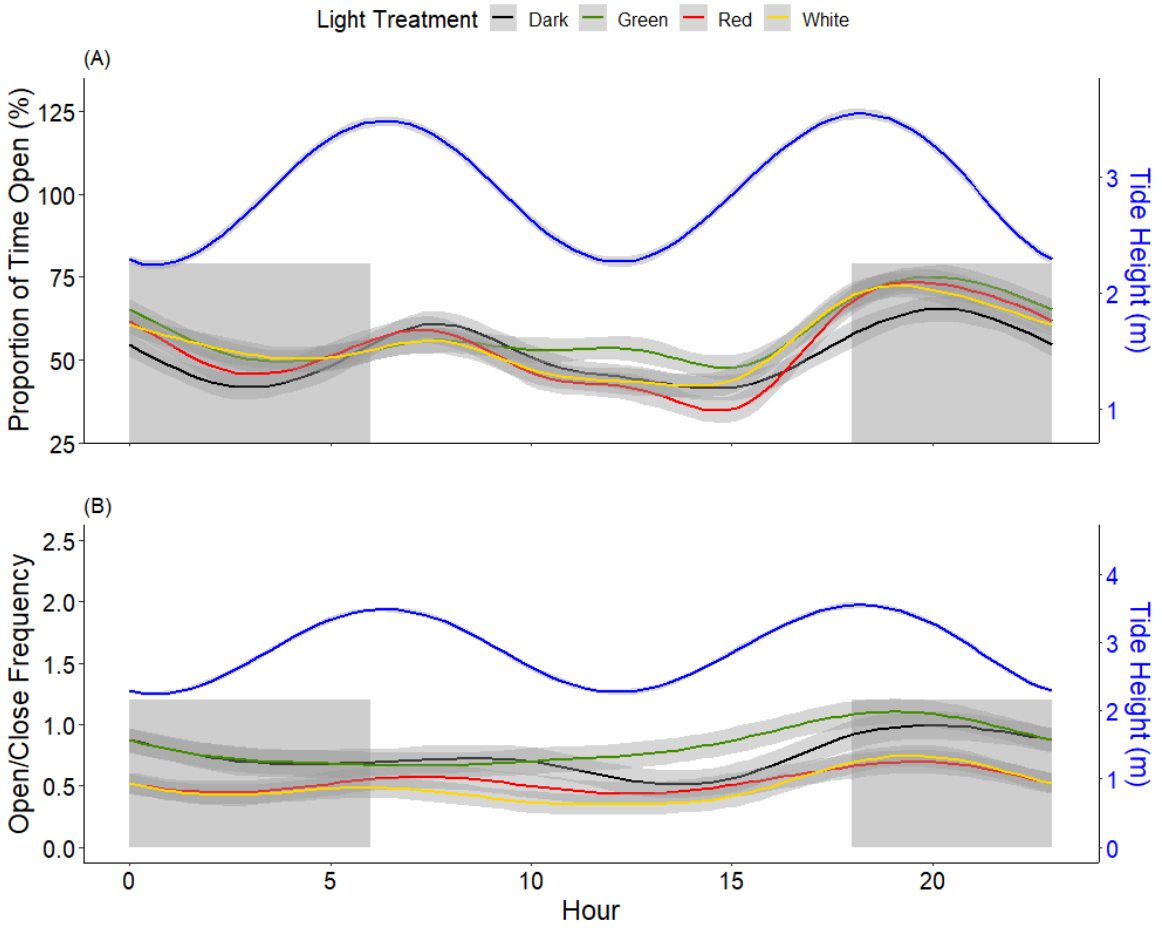

Figure S 4-7**:** The average (lines) and standard error (shading of lines) of the (A) proportion of open gape expressed as a percentage and (B) open/close frequency at each light treatment (dark, green, red, and white) through a 24hour period. The white and grey sections represent day-time and night-time respectively. The blue line (right y-axes) plots the hourly tidal height (m).

Appendix 2

Preliminary experimental work

To address the aims and objective of this PhD project, it was necessary to perform some preliminary experimental work. These pilot experiments were essential in developing methodological protocols and to identify the optimal experimental design and setup.

A1. Methodology - Phytoplankton quantification

To quantify the phytoplankton clearance capacity, which is used as a proxy of the ecosystem services provided by mussels throughout this thesis, I collected water samples from all the performed experiments: pilot and in-chapter experiment. These were stored in falcon tubes, preserved by the addition of 10 drops of Lugol iodine solution and kept in a dark location. Within a day of collections, each sample was filtered using filtration apparatus of 25mm diameter and the residue was collected on cellulose nitrate filter (SartoriusTM) with 0.45μm pore size.

The filters were then placed in glass petri dishes and were covered in aluminium foil. They were then inserted in a laboratory oven for one hour, which was the minimum time required to dry fully, at 40^{o}C. Each filter was then turned transparent on a glass slide by the addition of 2 drops of immersion oil: bellow and above the filter. After the addition of a cover slip, the filter was examined under a light microscope (40x/.065). During the drying process it was important that the filters were covered loosely with aluminium foil to enabling the evaporation of any residual humidity. This thorough drying is necessary for turning the filters transparent with the addition of immersion oil.

A microscope camera and the ToupView software were used to capture 15 randomly selected snapshots while moving along the filter in a systematic zigzag-motions. This number of snapshots was selected as the coefficient of variation of cell counts was kept below 0.7. The photos were then transferred to the ImageJ software and the cells were counted with the multi-point tool.

Through trials, I established that 25ml of water was the minimum volume required for phytoplankton quantification purposes, however in some experiments a greater quantity was collected as a reserve. Higher volumes, especially at the 0hour samples, would have a very dense cell distribution complicating their enumeration. The only occasion where a sample volume lower than 25ml would be filtered would be in the case of a mussel spawning, as gametes would clog the filters. On all occasions, the sample volume filtered was noted and accounted for in the data analysis.

Although more time consuming, this manual cell counting method was preferred to an automatic particle counter or software image analyser because these technologies are not able to distinguish, without errors, between microalgae, mussel faeces, pseudofaeces, gametes, airborne microfibres or a cluster of cells.

A2. Pilot experiments

A2.1. Pilot 1: Dose curve and sampling intervals

Aim

The aims of this experiment were to identify the microalgal concertation for optimum clearance capacity and to determine the most appropriate sampling intervals.

Approach

To achieve that, 32 mussels were acclimated in aerated glass vessels, used in the experimental setup, for two days and fed with $3x10^6$cells/L *Tetraselmis* sp.. Mussels were then starved for 24 hours prior to the initiation of the experiment. The treatments consisted of eight microalgal concentrations of *Tetraselmis* sp., ranging from oligotrophic to eutrophic (A-$3.0x10^3$, B-$1.5x10^4$, C-$7.5x10^4$, D-$3.75x10^5$, E-$1.87x10^6$, F-$9.37x10^6$, G-$4.68x10^7$ and H-$2.34x10^8$ cells/L) (Figure A 1), following a dose response curve (Figure A 2). Concentrations C ($7.5x10^4$cells/L) and F ($9.37x10^6$cells/L), in the absence of mussels, were used as controls to account for potential microalgae growth or mortality (Figure A 3). During the

experiment 25ml water samples were collected, with the use of a pipette, after mixing, at seven time points (0, 0.5, 1, 2, 4, 8, 24 hours). This experiment was replicated on three consecutive days with new groups of mussels each day for a total sample size of 96 mussels (12 per treatment). Throughout the experiment, ammonium, nitrate, and pH levels were tested daily to ensure optimal water chemistry conditions.

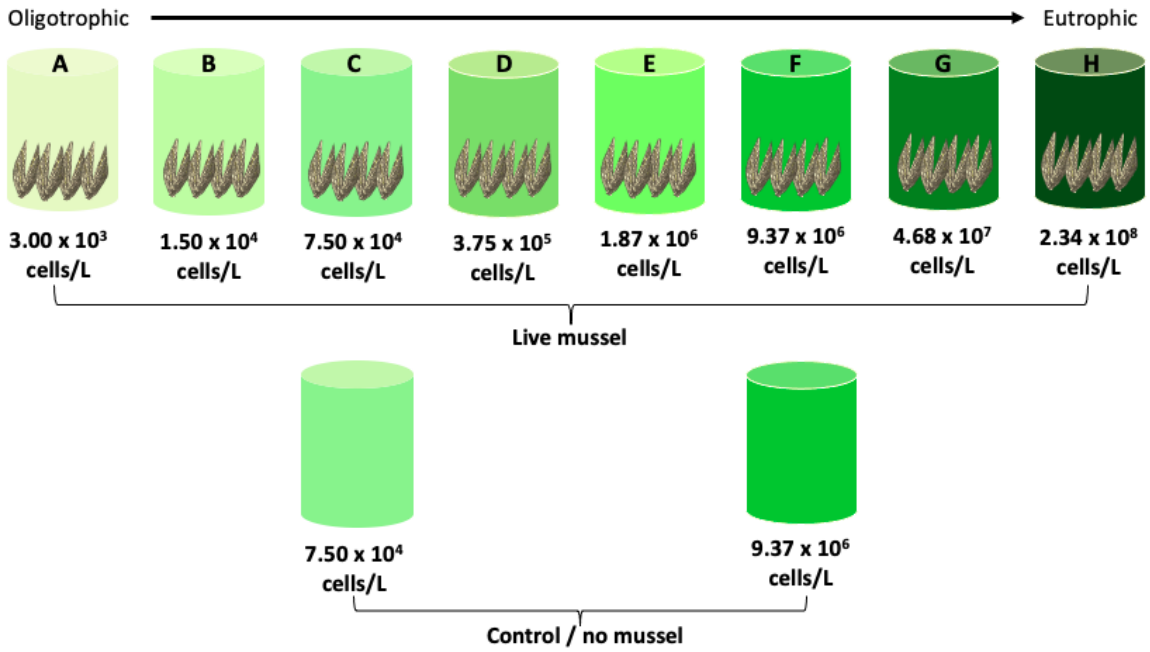

Figure A 1:Illustration of the experimental design of pilot 1. The treatments A-3.0x103 to H-2.34x108 cells/L range from oligotrophic to eutrophic *Tetraselmis* sp. monoculture containing 4 mussels per treatment while the treatments CC and CF were used as controls in the absence of mussel.

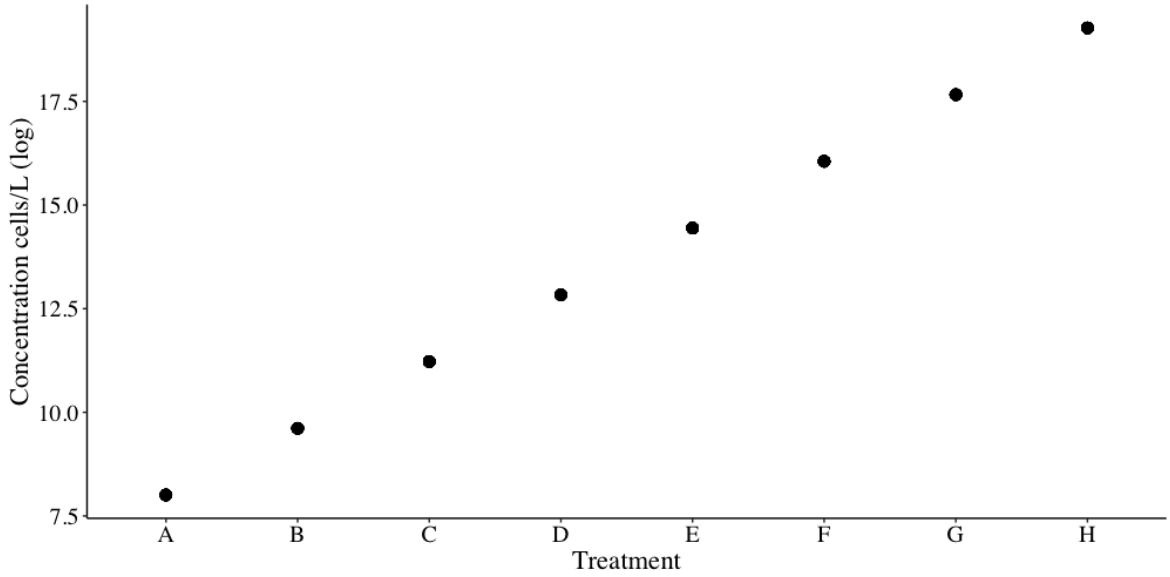

Figure A 2: Initial dose response curve of the concentrations used in pilot 1 treatments A to H following a logarithmic scale. (A-$3.0x10^3$, B-$1.5x10^4$, C-$7.5x10^4$, D-$3.75x10^5$, E-$1.87x10^6$, F-$9.37x10^6$, G-$4.68x10^7$ and H-$2.34x10^8$ cells/L).

Experimental outcome

From this pilot experiment, I realised that the phytoplankton quantification methodology was not sensitive enough for the lower concentrations tested (A-$3.0x10^3$, B-$1.5x10^4$) therefore they were not used in any following experiments. Because of the use of four mussels per experimental vessel, with the initial thought of capturing the population's ecosystem service, it was impossible to determine the individual contribution which might be quite variable. Furthermore, towards the end of the 24 hour period some mussels were spawning suggesting that the conditions were not optimal, probably due to the limitation in space. Hence, I decided to maintain one mussel per experimental vessel in the following experiments.

In terms of the sampling intervals, about 65% of the phytoplankton was filtered within the first 30 minutes and remained at similar concentrations throughout the sampling points. Hence, I decided that I would use a 24hour experimentation period as that involves both daylight and nigh-time filtration activity, therefore capturing any nocturnal activity.

A2.2.Pilot 2: Sample collection and Chlorophyll-α as a secondary response variable

Aim

This small experiment was running in parallel to pilot 1 (A1A2.1) and was aiming to identify the importance of mixing the water in the experimental vessels at the time of sample collection and to establish whether chlorophyll-α could act as another response variable expressing algal filtration by mussels.

Approach

Three groups of four mussels were acclimated in glass vessels for two days (800ml saltwater) and fed with $3x10^6$cells/L *Tetraselmis* sp.. Mussels were then starved for 24 hours before the initiation of the experiment. Then each group was exposed to the three high concentrations of *Tetraselmis* sp. according to the initial concentrations used in Pilot 1(A2.1) (F – $1x10^6$, G –$1x10^7$, H –$1x10^8$ cells/L). Each treatment was replicated three times with a respective control without mussels. During the experiment 50ml water samples were taken at four time points (0, 4, 8, and 24 hours) with the use of a pipette. The 8 hour sample was collected in the dark with the use of a red-light headlamp and a tarp preventing light reaching the mussels and causing a stress response. At all time points, the water was mixed before sampled but the samples at eight and 24 hours were also taken before mixing. Chlorophyll-α was established spectrophotometrically using ethanol solution (Strickland & Parsons, 1977).

Experimental outcome

The results revealed the chlorophyll-α concentration was not different in the presence or the absence of mussels, in contrast to Pilot 1 (A2.1) manual cell counts. An explanation to this unexpected outcome could be that chlorophyll-α can still be found in phytoplankton cells that have been filtrated by mussels but not digested and ejected as pseudofaeces. *Tetraselmis* cells in pseudofaeces usually have a deformed shape and therefore can be distinguished from complete cells (Figure A 3). With the assumption that in nature, cells encapsulated in

pseudofaeces would not be available in the water column, to filter feeder, but to bottom feeders these cells were not counted by the manual cell counting method. Therefore, based on the results, chlorophyll-α was not used in subsequent experiments as a proxy for the ecosystem services provided by mussels.

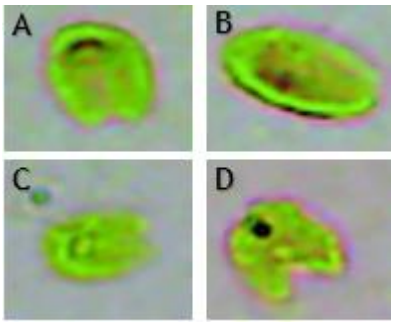

Figure A 3: Photos taken by a microscope camera of (A)(B) complete Tetraselmis sp. cells and (C)(D) cells that were in pseudofaeces (images not to scale but attention is drawn on the oval shape of the complete cells and chloroplast seen by the darker shade within the cells).

In addition, a large variation in the chlorophyll-a concentration of the non-mixed samples, in comparison to the mixed samples suggested that a standardised mixing technique should be used before the collection of each sample. After this experiment it was also established that it might be best for the mussels to be in continuous suspension to allow better water aeration, water mixing and separation from the pseudofaeces and faeces that accumulated at the bottom of the experimental vessels. To do that, plastic mesh was used to create a 4cm heigh base for the mussels to rest on.

A2.3.Pilot 3: Phytoplankton settlement and control determination

Aim

This small experiment aimed at identifying the potential interference of the bivalve's shell structure on phytoplankton benthic settlement. That would help in establishing the control that should be used in the following experiments (i.e., empty shells or absence of shell) as a variation in the phytoplankton settlement between the two surfaces would obscure any differences identified between the control and the live mussel treatments.

Approach

All the organic matter was removed from eight mussel shells using a scalpel. The two valves of each mussel were then re-attached using aquarium safe glue (JBL PRO HARY®) to represent the shape of a closed mussel. Two empty pairs of bivalve shells were placed in each of four vessel, and additional four vessels were used as controls where no shells were added. All vessels contained 800ml artificial salt water (32ppm) with $3x10^8$cells/L *Tetraselmis* sp.. Such a high concentration would allow for the visualisation of any microalgal settlement withing a short period of time and difference in the colouration of the water column. During the experiment, photos were taken at zero (mixed) and eight hours (mixed and unmixed) to allow visual comparison between any settlement observed at each treatment and the possible difference in the re-suspension of phytoplankton after mixing.

Experimental outcome

It was visually evident that phytoplankton cells had accumulated on the shells and their byssus threads after eight hours of exposure (Figure A4C). This was also causing a difference in the colouration between the unmixed shells and no-shells treatments where, in the latter less phytoplankton had settled at the bottom of the experimental vessel, and it seemed to have remained suspended in the water column. These results lead to the decision of using empty shells in the control treatments of future experiments. Moreover, the difference in colouration between the mixed and non-mixed treatments after eight hours (Figure A 4: A) A comparison of the pictures taken at the beginning (0 hour) and the end (8 hour) of Pilot 3 comparing the differences in colouration between the shells and no-shells treatments as well as the differences between mixed and unmixed water in both treatments. (B) Detailed comparison of the colour shade of the mixed and unmixed shell treatments. (C) Enlarged image of the phytoplankton settled on the shell and its byssus threads. This highlights the importance of thorough mixing before the collection of any samples, even though the filtration mechanism of mussels and the aeration also facilitate the continuous suspension of microalgal cells.

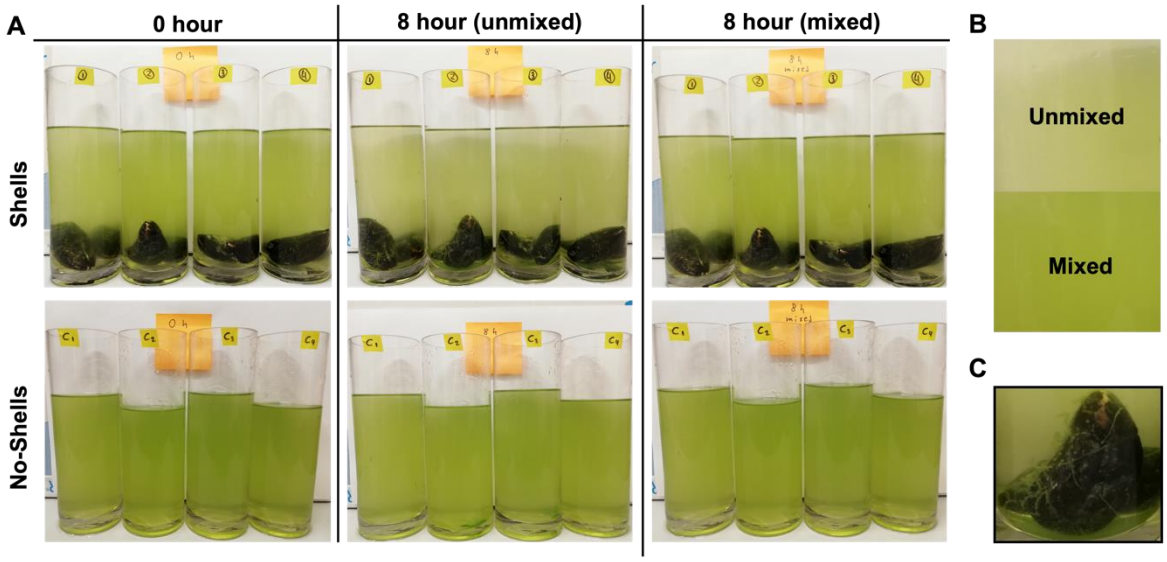

Figure A 4: A) A comparison of the pictures taken at the beginning (0 hour) and the end (8 hour) of Pilot 3 comparing the differences in colouration between the shells and no-shells treatments as well as the differences between mixed and unmixed water in both treatments. (B) Detailed comparison of the colour shade of the mixed and unmixed shell treatments. (C) Enlarged image of the phytoplankton settled on the shell and its byssus threads.

A2.4. Pilot 4: Verification of the ecosystem services provided

Aim

The aim of this pilot experiment is to verify the ecosystem services provided by mussels, at different phytoplankton bloom conditions, while implementing the experimental design that had been established after pilot experiments one, two and three.

Approach

The experimental design consisted of six treatments where a live mussel or a mussel shell acting as a control was exposed to one of the three phytoplankton concentrations: 7.5×10^4, 1.87×10^6 and 4.68×10^7 cells/L of *Tetraselmis* sp. monoculture (Figure A 5). The experiment was repeated ten times for a total of 60 mussels (2 mussel/shell x 3 phytoplankton concentrations x 10 replicates).

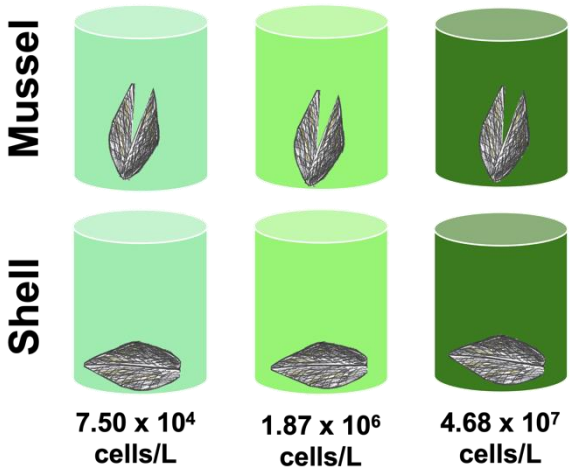

Figure A 5: Illustration of the treatments used in pilot 4. The three shades of green represent the phytoplankton concentrations ($7.50x10^4$, $1.87x10^6$ and $4.68x10^7$cells/L). The open and close mussel signify the presence of a live mussel and a shell only, respectively.

Mussels were collected from Arrochar (56.199716, -4.747824), at low tide, on May 16th, 2018. After their transport to the laboratory, epibionts like barnacles and macroalgae were scrapped off and the mussels were placed in 5L aquariums containing artificial salt water (salinity: 32 ppm). Mussels were purified for 24 hours, after which the water was changed and $3x10^6$cells/L of *Tetraselmis* sp. monoculture was added daily. Three mussels were randomly selected from the aquarium and placed individually in glass experimental vessels where they acclimated for two days with again daily water changes and fed with *Tetraselmis* sp. On the third day, mussels were starved to ensure equal feeding during the experiment. The experiment was performed on the fourth day. Three empty mussel shells were prepared, as per pilot 3 (A2.3Pilot 3: Phytoplankton settlement and control determination), and individually placed in experimental vessels. This process was repeated daily with a new set of mussels and shells each day. The aquariums and experimental vessels were constantly aerated and located in a water bath maintaining a constant temperature of 13°C and were enclosed in wooden boxes with a 12:12 hour photoperiod.

For each experimental treatment, 825ml of artificial salt water were prepared and the allocated phytoplankton concentration was added, as required per treatment. A water sample of 25ml was collected from the prepared solutions and

the rest was added to the experimental vessels. The duration of the experiment was 24 hours after which another 25ml sample was collected and the phytoplankton in both initial and final samples was quantified as per section A1. The phytoplankton consumed by mussels was calculated by the following equation:

$$Phytoplankton\ Consumption = \frac{Concentration\ (0h) - Concentration\ (24h)}{Concentration\ (0h)} * 100.$$

Due to the nature of the proportionate data, beta distribution was used for the analysis and the following transformation was performed:

$$Ytransformed = \frac{[Y * (N-1) + 0.5]}{N},$$

where *Ytransformed* is the transformed value of the proportion of phytoplankton consumed, Y, and N is the sample size (Smithson and Verkuilen, 2006). The phytoplankton enumeration method proved inconsistent for the lowest concentration of $7.5x10^4$cells/L, hence data from that treatment was discarded and the final sample size was 40 mussel.

To confirm the ecosystem services provided by mussels at different phytoplankton concentrations we performed general linear models (GLMs). The explanatory variables used were the mussel/shell treatments, the phytoplankton concentrations ($1.87x10^6$ and $4.68x10^7$cells/L) and the interaction of these variable. Model selection was conducted by the Likelihood Ratio Test (LRT) and the Tukey method in the emmeans package (Lenth, 2018) was used to perform pairwise comparison of the significant variables. The analysis was performed in R v.4.0.4 (R Core Team, 2021).

Experimental outcome

The results verify the ecosystem service, provided by mussels, of phytoplankton clearance from the water column. In the presence of mussels, 64.52% more phytoplankton was cleared in comparison to the empty mussel shells (Tukey,

p<0.001, Figure A6). There was no significant difference between the percentage of phytoplankton consumed at the two microalgal concentration.

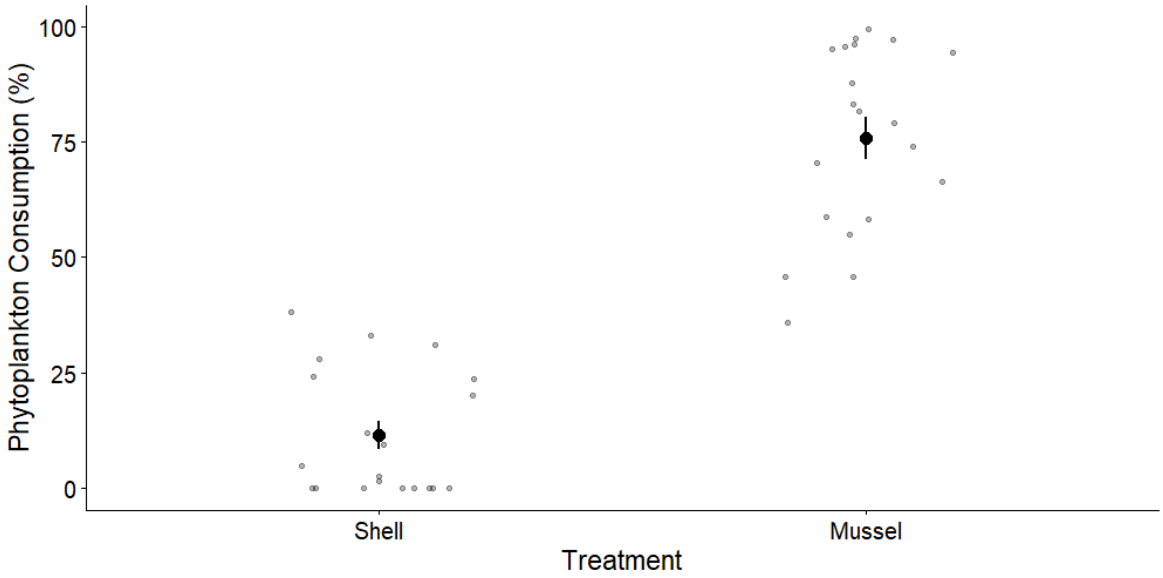

Figure A 6: The percentage of phytoplankton consumption (mean ± SE) in the presence of live mussels and empty shells.

Incidentally, 11.38% of the phytoplankton initially added to the shell treatment was not accounted for after 24 hours. This could be attributed to the settlement of the microalgal cell on the surface of the shells with the potential to form periphytic biofilm. Hence, this pilot experiment verified another ecosystem services provided by bivalves which is the increase of surface area where periphytic biofilm can develop (Ozersky *et al.*, 2013). An advantage of this biofilm formation, as suggested by Bremner et al. (2020), is the removal of dissolved heavy metals finding their way to the ocean through stormwater runoff.

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