University of Southampton

Faculty of Natural and Environmental Sciences

School of Ocean and Earth Sciences

Understanding the direct and indirect effects of algal toxins on marine copepods

by

Ali Hassan Abdulhussain

Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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Copepods form an important link between phytoplankton and higher trophic levels. Several species of phytoplankton, including dinoflagellates of the genus *Alexandrium*, produce neurotoxins commonly known as paralytic shellfish toxins (PSTs). The toxins from harmful algae (HA) may impact copepod survival, feeding, and fitness by acting as a feeding deterrent and/or by causing physical incapacitation. However, copepods may be able to overcome these toxic effects and/or become tolerant to toxicity by partial metabolism. Published information on how HA affect survival, feeding and other physiological processes in copepods are difficult to compare due to the different concentrations of HA used as food, the level of toxins in the food, and the various responses measured on different copepod species from different locations. Very few experiments have examined how HA toxins influence the survival, feeding and fecundity of copepods within UK waters. This thesis aims to address this knowledge gap whilst also choosing organisms of wider geographical relevance. This study examined the effects of a toxin-producing dinoflagellate, *Alexandrium catenella*, on two physiologically different copepods: *Acartia tonsa*, a pelagic coastal copepod that is found in the UK and other coastal waters including Northern & Southern America and Australia, and *Calanus helgolandicus*, which is spread across the North East Atlantic with high numbers on the European shelf and in oceanic waters. In Chapter 3, short-term (24 h) survival and feeding experiments revealed that adult female *A. tonsa* can survive exposure to field-recorded bloom concentrations of toxic *A. catenella*. Survival only decreased when exposure levels exceed reported environmental concentrations by two orders of magnitude. The lethal median concentration (LC_{50}) was 12.45 ng STX eq L^{-1}. Ingestion rates were higher when offered *A. catenella* in the absence of alternative prey, potentially suggesting compensatory feeding. *A. tonsa* actively selected non-toxic *Rhodomonas* sp. over toxic *A. catenella* when offered a mixed diet. Chapter 4 demonstrated that the survival of female *A. tonsa* is not affected by prolonged (10 days) exposure to toxic *A. catenella*. However, additional feeding and egg production experiments suggested that whilst *A. tonsa* can obtain enough energy from ingesting toxic *A. catenella* to survive, it suffers reproductive impairment when feeding on this prey alone. In Chapter 5, *C. helgolandicus* showed a decrease in feeding rate when feeding on toxic *A. catenella* compared to when feeding on the non-toxic congener, *Alexandrium tamarense*. On the other hand, the egg production and hatching success rates were not affected by the relative abundance of toxic *A. catenella* and non-toxic *A. tamarense* in diet, suggesting they may have used biomass reserves to sustain egg production. Body toxin analysis of *C. helgolandicus* showed they may bioaccumulate toxins in their bodies; however, the retention efficiency was very low. Full toxin profiles for *A. catenella*, including 8 to 12 PSTs, are presented in all experiments. This study furthers our understanding of PST-producing HA-copepod interactions, and how they may be affected by the increased frequency and magnitude of HA blooms.

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Research Thesis: Declaration of Authorship

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I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

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Definitions and Abbreviations

Abbreviations	Definitions/ Description of the term
HA	Harmful Algae
PSP	Paralytic Shellfish Poisoning
PST	Paralytic Shellfish Toxins
ASP	Amnesic Shellfish Poisoning
DA	Domoic Acid
NSP	Neurotoxic shellfish poisoning
PbTxs	Brevetoxins
DSP	Diarrhetic Shellfish Poisoning
OA	Okadaic Acid
Acat	*Alexandrium catenella* (toxic)
Atam	*Alexandrium tamarense* (non-toxic)
TAC	Toxic *Alexandrium catenella*
NAC	Non-toxic *Alexandrium catenella* (This name was only used in Chapter 4 published paper: taxonomic revision of *A. catenella* designated the non-toxic 'strain' as a different species (*A. tamarense*).
LC_{50}	Lethal Concentration 50 %: the concentration that causes 50 % mortality within a population
ASW	Artificial Seawater
SFSW	0.2 µm sterile filtered seawater

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Chapter 1

Introduction

1.1 Harmful algal blooms

Phytoplankton are single-celled algae that form the base of the marine food web. There are around 5000 different species of marine phytoplankton (Hallegraeff, 1993; Hallegraeff et al., 2021), and approximately 300 of these can occur in high numbers and sometimes discolor the sea (Anderson et al., 2012). The proliferation of phytoplankton (algal bloom) can be beneficial for wild fisheries and aquaculture, where they act as important food for zooplankton and other filter feeding species (Hallegraeff, 1993; Anderson et al., 2012; Hallegraeff et al., 2021). However, algal blooms can also have a deleterious effect on marine animals, including fish and mammals, through direct contact with anoxia or hypoxia (oxygen depletion in a water body that may follow an algal bloom due to the decomposition of algae), and toxins produced by certain species of algae (Hallegraeff, 1993; Cook et al., 2010; Turner, 2010; Anderson et al., 2012; Dam, 2013; Turner, 2014; Jensen et al., 2015; Fire et al., 2021; Hallegraeff et al., 2021; Kershaw et al., 2021).

There are approximately 40 species of phytoplankton that have the capacity to produce potent neurotoxins, such as saxitoxins, brevetoxins, okadaic acid, and domoic acid (Hallegraeff, 1993; Cook et al., 2010; Anderson et al., 2012; Turner, 2014; Bresnan et al., 2021). These toxins are secondary metabolites which are produced naturally but are not required for physiological processes, such as feeding, growth, and reproduction (Landsberg 2002). There are two main hypotheses for why harmful algae (HA; hereafter) produce toxins: to avoid grazing of predators by causing physiological incapacitation of grazers or by acting as feeding deterrents, and to reduce competition for resources with other algae by affecting their growth (Legrand et al., 2003; Ianora et al., 2006; Pohnert et al., 2007).

The frequency of HA have changed in the past few decades both spatially and temporally (Hallegraeff, 1993; Anderon et al., 2012; Edwards et al., 2006; Nohe et al., 2020; Bresnan et al., 2021; Hallegraeff et al., 2021; Marampouti et al., 2021; Zingone et al., 2021). In the 1970s, the problem was sporadic and scattered, but nowadays, almost every coastal country is impacted by HA. The number of toxic blooms, the number of toxins and toxic species reported, the economic losses, and affected resources have all increased over the past 50 years (Anderson et al., 2012; Bresnan et al., 2021; Hallegraeff et al., 2021; Karlson et al., 2021).

There has been much disagreement in the literature over the causes of the expansion in HA blooms. Studies have suggested that humans have induced the expansion of HA by

transporting toxic species in ship ballast tanks or by shellfish seeding (Hallegraeff 1993; Anderson et al., 2012; Marampouti et al., 2021), increased aquaculture activity (Bresnan et al., 2021; Hallegraeff et al., 2021), and also global climate change (Edwards et al., 2006; Hallegraeff et al., 2021; Nohe et al., 2020; Marampouti et al., 2021). However, these events might also easily be attributed to natural dispersal via currents, rather than human impacts (Anderson et al., 2012). HA may always have been there, but we are only now seeing them because of new procedures that look for them routinely (Anderson et al., 2012; Bresnan et al., 2021; Hallegraeff et al., 2021). Another possibility is that watersheds used for agriculture, housing, recreation, and industry have increased nutrient loadings and, therefore, increased the frequency of HA (Anderson et al., 2012). For example, in the Inland Sea of Japan, red tides (a form of HA bloom) have increased from 44 per year in 1965 to > 300 per year a decade later. This increase correlates with the increase in nutrient enrichment from pollution (Okaichi, 2002; Imai et al., 2006). The control of pollution in Japan in the mid-1970's resulted in a 70 % reduction in the number of red tides up to the mid-1990's (Okaichi, 2002; Imai et al., 2006); this is evidence to suggest that nutrient loading is in someway responsible.

HA blooms are routinely recorded along the Atlantic margin of Europe, and impacts from toxin-producing HA have been recorded in this region over the last 50 years (Bresnan et al., 2021). A long-term increase in the occurrences of the HA blooms including both diatoms and dinoflagellates has been found in the North Sea (Nohe et al., 2020). Similar to the North Atlantic, it has been observed that at least 14 toxic dinoflagellate species are widely distributed in the Arctic, and this is likely to increase due to climate change (Okolodkov et al., 2005; Arrigo et al., 2011; Søgaard et al., 2021). HA bloom events (i.e. non-toxic) have also increased in the Mediterranean with no clear trend in toxic HA events (Garcés et al., 2000; Zingone et al., 2021), although 20 invasive non-native HA species are linked to toxic HA blooms (Marampouti et al., 2021). It is now widely acknowledged that there was a period of hydroclimatic change during the twentieth century, manifested in significantly higher sea surface temperatures, which dramatically altered planktonic ecosystems in the Northeast Atlantic (Beaugrand, 2004; Edwards et al., 2006; Philippart et al., 2011; Nohe et al., 2020). This warming of the oceans has continued, and in only 40 years, there has been a significant shift in plankton and fish communities (Beaugrand et al., 2002, 2009; Choquet et al., 2017; Nohe et al., 2020). By the end of the twenty-first century, the Intergovernmental Panel on Climate Change (IPCC) climate projections indicate a mean increase in the sea surface temperature of between 0.73 °C to 2.58 °C (IPCC, 2019). Global warming is thought to have expanded the niche of HA blooms and warming is predicted to

further increase the impact of HA blooms in the future (Berdalet et al., 2016; Dees et al., 2017; Hallegraeff et al., 2021).

In summary, HA blooms are a natural phenomenon; however, the frequency of these events may be increased by anthropogenic pollution, and aquaculture activities which may pose a significant threat to marine organisms (Hallegraeff 1993; Bresnan et al., 2021; Hallegraeff et al., 2021; Karlson et al., 2021; Marampouti et al., 2021). Global warming may also stimulate the frequency with which HA events occur in space and time (Edwards et al., 2006; Dees et al., 2017; Nohe et al., 2020; Hallegraeff et al., 2021).

1.2 Harmful algal toxins

Toxins produced by phytoplankton are variable in their solubility, mode of action and toxicity (Landsberg, 2002; Cembella, 2003). Multiple factors determine the ability of species to produce toxins, including genetic strain, nutritional status, age, environmental parameters such as temperature and salinity, (Legrand et al., 2003) as well as the species of grazer they encounter (Bergkvist et al., 2008; Selander, 2008; Wolhrab et al., 2010; Griffin et al., 2019; Selander et al., 2019). For example, copepodamides (also known as taurine-containing lipids; Mayor et al., 2015), which are exuded by calanoid copepods, have been reported to increase toxin production in dinoflagellates and diatoms (Selander et al., 2019).

Toxic diatoms (*Pseudo-nitzschia* spp.) that produce the water-soluble amino acid domoic acid (DA) can cause Amnesic Shellfish Poisoning (ASP). DA acts as a substitute for the excitatory neurotransmitter L-glutamic acid (Turner, 2014). When an organism is unable to regulate this substitute transmitter, it results in large neuronal depolarization, especially in species that contains abundant glutamate receptors, such as the vertebrate hippocampus. DA can, therefore, result in brain damage in humans, seabirds, and marine mammals due to the degeneration of the hippocampus (Turner, 2014, Luckas et al., 2015). *Pseudo-nitzschia* blooms can be found in most coastlines around the world including Europe, North America, Southern America, Africa and Australia (Trainer et al., 2012). *Pseudo-nitzschia* is known to form dense blooms with between 1,000,000 - 100,000,000 cells L^{-1}, accounting for 99 % of the phytoplankton assemblages, and last up to two months (Bates et al., 1989; Martin et al., 1990; Fryxell et al., 1997; Gallacher et al., 2001). The impact of DA can be detected in the open ocean as well as in upwelling systems (Trainer et al., 2012). DA has been detected in many European shellfish and poses a threat in many European countries including France, UK and Ireland (EFSA, 2009). Bresnan (2003) reported high densities of *Pseudo-nitzschia*

spp. in 2002 at Loch Striven and Loch Rog of 191,904 cells L^{-1} and 280,685 cells L^{-1}, respectively. Gallacher et al. (2001) found that *Pseudo- nitzschia* cell counts in the waters of the west coast of Scotland rarely exceeded 500,000 cells L^{-1}, with highest levels at most sites reaching around 100,000 cells L^{-1}. However, concentrations of these diatoms in the west coast of Scotland did not correlate with DA toxicity in mussels. Blooms of *Pseudo-nitzschia* spp. have also been recorded in the Great Bay in the northwestern Sea of Japan, with abundances exceeding 1,000,000 cells L^{-1} and accounting for 75–98 % of the total density of the phytoplankton assemblage (Stonik et al., 2011). According to García-Mendoza et al. (2009) a *Pseudo-nitzschia australis* bloom detected in Todos Santos Bay of the Pacific coast during April 2007 reached a concentration of 300,000 cells L^{-1} and was associated with a DA concentration of 0.86 ug DA L^{-1} in particulate matter.

Dinoflagellates are capable of producing paralytic shellfish toxins (PSTs) (e.g. *Alexandrium catenella, Alexandrium minutum, Alexandrium fundyense, Pyrodinium bahamense,* and *Gymnodinium catenatum*), brevetoxins (e.g. *Karenia brevis*), and okadaic acid (e.g. *Dinophysis acuminata* and *Prorocentrum minimum*). Dinoflagellates are responsible for the majority of toxic HA blooms (Sopanen et al., 2011) and are often associated with major environmental and economic issues (Hallegraeff, 1993; Anderson et al., 2012; Hallegraeff et al., 2021) and causing disease and death in a variety of marine animals, including fish, seabirds, and mammals (Wang, 2008; Jensen et al., 2015; Kershaw et al., 2021).

Brevetoxins (PbTxs) are cyclic polyether neurotoxins. They activate lipid-soluble sodium channels and eventually deplete acetylcholine at synapses (Yasumoto & Murata 1993). This causes repetitive firing of nervous impulses and can kill fish by paralyzing the gill muscles (Kirkpatrick et al., 2004). Shellfish appear unaffected by brevetoxins, but can accumulate these toxins, and ingestion of these or contaminated fish can result in neurotoxic shellfish poisoning (NSP) in humans. *K. brevis* occur naturally in the Gulf of Mexico and reach a concentration of ~1000 cells L^{-1} and can form blooms with a concentration of >100,000 cells L^{-1} that result in NSP which impacts fish, marine mammals and marine life (Tester & Steidinger 1997; Landsberg, 2002; Flewelling et al., 2005). Brand and Compton (2007) studied the long-term abundance of *K. brevis* along Southwest Florida coast between 1954 and 2002, and showed that the concentration categories above 1000 cells L^{-1} have increased over time. These incidents may allow for the accumulation of the brevetoxin in the tissues of organisms (Fire et al., 2021).

Okadaic acid and other lipid-soluble toxins act as inhibitors to the enzyme phosphatase and impact regulatory processes, including feeding, membrane transport, secretion, and cell division (Yasumoto & Murata 1993). Accumulation in shellfish and their subsequent consumption by humans results in Diarrhetic Shellfish Poisoning (DSP). *Dinophysis* spp. reported in August 2002 at Stonehaven in Scottish waters reached maximum concentration of 1740 cells L^{-1} and 3020 cells L^{-1} at offshore sites (Bresnan, 2003). *Dinophysis* spp. blooms found in Penobscot and Frenchman Bay off the central coast of the Gulf of Maine, USA have been reported at concentrations of 2000 cells L^{-1}, with a maximum concentration observed of 54,300 cells L^{-1} in July 2016 (Deeds et al., 2020).

The derivatives of PSTs are categorized into three main groups: carbamate, N-sulphocarbamoyl, and decarbamoyl toxins. Saxitoxin (STX) and neosaxitoxin (NEO) (carbamate toxins) are considered to be the most potent compared to other HA toxins. Other highly potent carbamate toxins include gonyautoxins 1 to 4 (GTX1, GTX2, GTX3, GTX4). N-sulphocarbomoyl, (C) toxins, and decarbomoyl (dc) toxins are considered less potent. Saxitoxins inhibit nerve transmission by blocking water-soluble sodium channels (Luckas et al., 2015). This affects muscle functioning and causes paralysis and respiratory failure in humans, seabirds, fish, and marine mammals, and are collectively termed PSTs.

Different blooms of the same HA species can be different with respect to the strains present, their toxin potency, and cell/ toxin concentration (Turner, 1998; Bresnan et al., 2005; Collins et al., 2009; Brown et al., 2010). According to Bresnan et al., (2005) low cell densities of *A. catenella* cells (1000 - 2000 cells L^{-1}) can result in levels of PSTs in shellfish flesh exceeding the EU regulatory limit of 800 ug STX eq./KG a situation that may persist for several weeks. High *Alexandrium* spp. cell densities were recorded in the Western Isles, UK during June 2003 (5000 cells L^{-1}), and Shetland, UK in July 2003 (18,000 cells L^{-1}) (Bresnan et al., 2008). Joyce et al., (2004) studied *Alexandrium tamarense* in Orkney, UK, between May 1998 and May 1999, and reported that *A. tamarense* is always present in water with a typical value of 200 - 400 cells L^{-1} and a maximum of up to 1600 cell L^{-1}. Samples collected by CEFAS (Centre for Environment, Fisheries and Aquaculture Science) during May 2010, showed that the maximum density of *Alexandrium* sp. found in Loch Creran was as high as ~3000 cells L^{-1}, with the second maximum in Loch Beag at 800 cells L^{-1} (Swan and Davidson, 2012). *Alexandrium* sp. blooms are not only found in the northern UK, but also in other seas. Strong interannual variation in *Alexandrium* sp. densities in Golgo Nuevo Patagonia (Argentina) between September 1995 and December 1998 were accompanied by a bloom in September 1995 that reached 15,000 cells L^{-1}, with a second peak that reached

5000 cells L^{-1} in October-November 1998 (Gayoso, 2002). Fauchot et al., (2005) reported that during a bloom in St. Lawrence, Canada, *A. tamarense* reached a maximum density of 2,300,000 cells L^{-1} in brackish surface waters during July 1998.

The dinoflagellate, *A. catenella*, (formerly *A. tamarense*, North American strain (Scholin et al., 1994) Group I, (Lilly et al., 2007) reassigned taxonomically by John et al., 2014 & Fraga et al., 2015, acknowledged in Prud'homme van Reine, 2017) is widely distributed in Northern Europe (Bresnan et al., 2021, Karlson et al., 2021). In Northern Europe: Scotland, Iceland, Faroe Islands, and Norway, *A. catenella* is the species responsible for causing paralytic shellfish toxin (PST) events while in Ireland, SW England, France, Spain and Portugal the species of *Alexandrium* responsible for PST events is *A. minutum* (Bresnan et al., 2021, Karlson et al., 2021). Some species historically thought to be the same species e.g. *A. tamarense* Western European Strain (Scholin et al.,2014), Grp III (Lilly et al., 2007) and now named *A. tamarense* (John et al., 2014; Fraga et al., 2015) are non-toxic (e.g. 1119/19 & 1119/33), whereas others (e.g. *A. catenella* 1119/27 &1119/28) produce PSTs and have been linked to closures of shellfish harvesting areas in Northern Europe (Bresnan et al., 2005, 2008; Brown et al., 2010). *A. catenella* has been found to vary in toxicity between 3103 and 10469 fg STX eq $cell^{-1}$ (Collins et al., 2009; Brown et al., 2010). The STX equivalent is toxin equivalent factor (TEF) provided from European Food Safety Authority (EFSA) recommendations (EFSA, 2009). The variability in toxin concentration can occur between different strains of the same HA under similar growth conditions (Loret et al., 2002; Etheridge et al., 2005). Some of the factors that can change the toxin levels within a HA bloom include: turbulence (Juhl et al., 2001), salinity (Grzebyk et al., 2003), and nutrient conditions (Turner, 1998; John & Flynn 2002; Leong et al., 2004).

HA toxins will not necessarily have a significant impact on invertebrates, including crustaceans and bivalves, but they may accumulate within their tissues and transmit toxins to vertebrates higher up the food chain. However, HA can strongly impact vertebrates, such as fish, birds, marine mammals and humans (Robineau et al., 1991; Durbin et al., 2002; Doucette et al., 2006; Wang et al., 2008; Fire et al., 2021; Kershaw et al., 2021; Marampouti et al., 2021). The decentralization of the invertebrate nervous system compared to vertebrates is one of the possible reasons that result in this difference (Turner, 2014). In summary, it is apparent from the existing literature that toxin-producing HA have varying levels of toxins that can occur in a wide range of different waters and with varying levels of toxicity.

1.3 Marine zooplankton (copepods)

Marine zooplankton play a pivotal role in marine ecosystems. The dominant component of zooplankton are copepods which provide an important link between phytoplankton and upper trophic levels (Runge, 1988; Mann, 1993). Copepods are the most diversified group of metazoans on earth (Humes, 1994). They also play a vital role in regulating phytoplankton blooms, including HA blooms, through grazing or the absence of grazing (Irigoien, 2005). Copepods are important in the biogeochemical cycles of the oceans where they represent a high proportion of ocean metazoan biomass (Steinberg & Landry, 2017). They play a vital role in carbon export where copepods contribute to the transfer of carbon from the atmosphere to the bottom of seas/oceans. They contribute to the biological carbon pump through different mechanisms such as mucous feeding webs, fecal pellets, molts (crustacean exoskeletons), carcasses and vertical migration. Diel vertical migration of planktonic copepods between surface waters where they feed at night, and the deeper waters (e.g. mesopelagic zone) where they reside and excrete during the day (Steinberg & Landry, 2017), actively transports carbon to the deep ocean. Seasonal vertical migration of copepods to deep waters also transports carbon into deeper waters: carbon is released at depth through respiration, defecation, and mortality (Dam et al., 1995; Steinberg et al., 2002; Jónasdóttir et al., 2015). Also, they can passively transfer carbon by producing sinking fecal pellets when feeding near the sea surface (Dam et al., 1995; Steinberg et al., 2002; Giering et al., 2014; Jónasdóttir et al., 2015). The contribution of these mechanisms towards carbon export are highly variable and dependent on the plankton community structure of each region. Due to the short generation times of marine copepods, they are used widely as model organisms to understand response to global change (Dam, 2013). The huge diversity of forms, short life span and high sensitivity as well as their link to upper trophic levels makes them a good model organism which can be used to understand the impact of toxic algae on marine pelagic food webs.

1.3.1 General characteristics of copepods

The name copepod generated from the Greek words, kope and podos, which means the oar foot, referring to the pairs of swimming legs that are moved together (Mauchline, 1998). Copepods are a subclass of small crustaceans that contain ten orders: Calanoida, Cyclopoida, Gelyelloida, Harpacticoida, Misophrioida, Monstrilloida, Mormonilloida, Platycopioida, Canuelloida, Siphonostomatoida.

The life cycle and morphological structure of copepods varies greatly, but there are some universal characteristics. Each copepod starts as an egg which, after fertilization, hatches as a nauplius larva (stage N1, Fig. 1). The nauplius moults five times (from stage N1 to N6) and after the sixth moult changes its morphology completely. The nauplius is now called a copepodite juvenile (stage C1). The copepodite also moults five times and transforms into an adult copepod (stages C1 to C6) (Boehler & Krieger, 2012). For some copepod species, such as *Calanus finmarchicus*, stage 5 copepodites may enter a diapause state in which they remain for ≥ 6 months before awakening and developing into adults (Mauchline, 1998). Other species can diapause as resting eggs (Dahms, 1995).

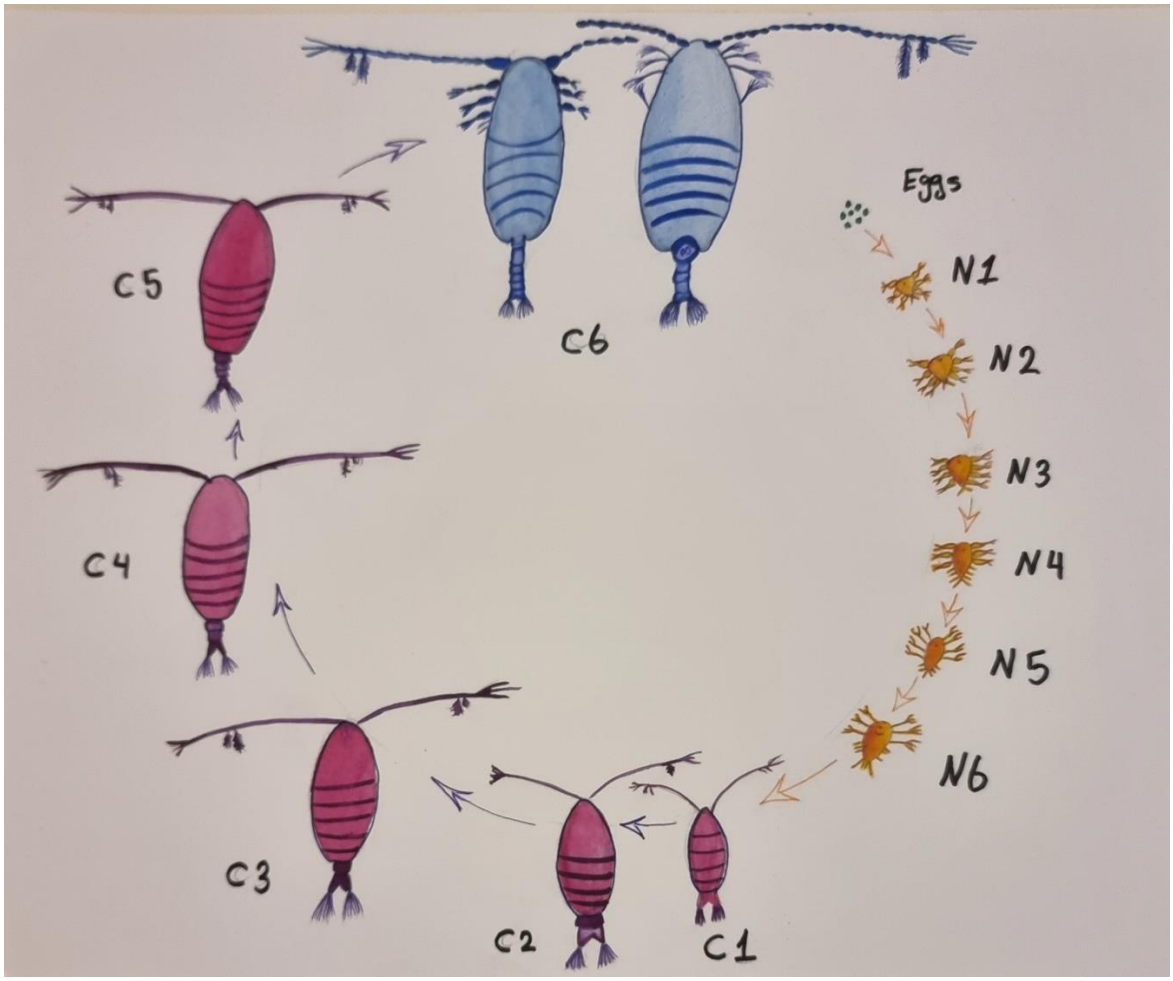

Figure 1. Schematic representation of the copepod life cycle. The eggs hatch and develop through 6 naupliar stages (N1-N6) and then 5 copepodite stages (C1-C5) before becoming adults (C6). (Credit: Ali H. Abdulhussain)

1.3.2 Model copepods for experiments

In this study, I have conducted experiments on two widely distributed calanoid copepods: *Acartia tonsa* (Chapters 2 & 3) and *Calanus helgolandicus* (Chapter 4) (Fig. 2 & 3). *A. tonsa* is a pelagic calanoid copepod that has been widely used as a test organism (Cook et al., 2010; Turner, 2014; Appendix A). It can live in various environmental conditions,

and has been shown to be susceptible to different harmful algae (Appendix A). They are euryhaline: 1–72 salinity (Cervetto et al., 1999), eurythermal: 5 – 34 °C (Holste and Peck, 2006), and inhabit a wide range of environments (Fig. 3A). The starvation tolerance for *A. tonsa* is 6-10 days at 15 °C (Dagg, 1977). Adults can reach a length of 1.5 mm, and N1 nauplii can reach a length of 70 μm. The eggs are spherical and 70-80 μm in diameter, and can be subitaneous (i.e. hatch immediately) or resting eggs (Drillet et al., 2011). Because the eggs have a slightly higher density than seawater, they sink when released. Females and males are distinguished by the composition of their antennae, urosome, and swimming legs.

C. helgolandicus is widely spread across the North East Atlantic with high numbers on the European shelf and in oceanic waters (Barnard et al., 2004; Bonnett et al., 2005: Fig. 3B) and can contribute 6-93 % of the mesozooplankton biomass in those regions (Bonnet et al., 2005). They are a warm, temperate species that prefer temperatures ranging from 9 to 20 °C. (Bonnet et al., 2005). They can live in various environmental conditions where they accumulate lipids, particularly wax esters, that enable them to survive periods of reduced food or starvation (Gatten et al., 1979). Biomass reserves are also used as a source of energy when diapausing in cold, deep water (Hirche, 1983, 1984; Williams & Conway, 1988; Stöhr et al., 1996). Although there is limited information on *C. helgolandicus* resting copepodites at deep waters during winter diapause, the diapause-stage of *C. finmarchicus* could be applied to *C. helgolandicus* (Bonnett et al., 2005). Temperature has been linked to the abundance and geographical distribution of *Calanus* spp in the North Atlantic (Barnard et al., 2004; Bonnett et al., 2005; Choquet et al., 2017). Thus, as regions warm or cool, we can expect dramatic changes in the abundance and dominance of different copepod species (Beaugrand et al., 2002, 2009; Richardson & Schoeman 2004; Choquet et al., 2017; Grieve et al., 2017).

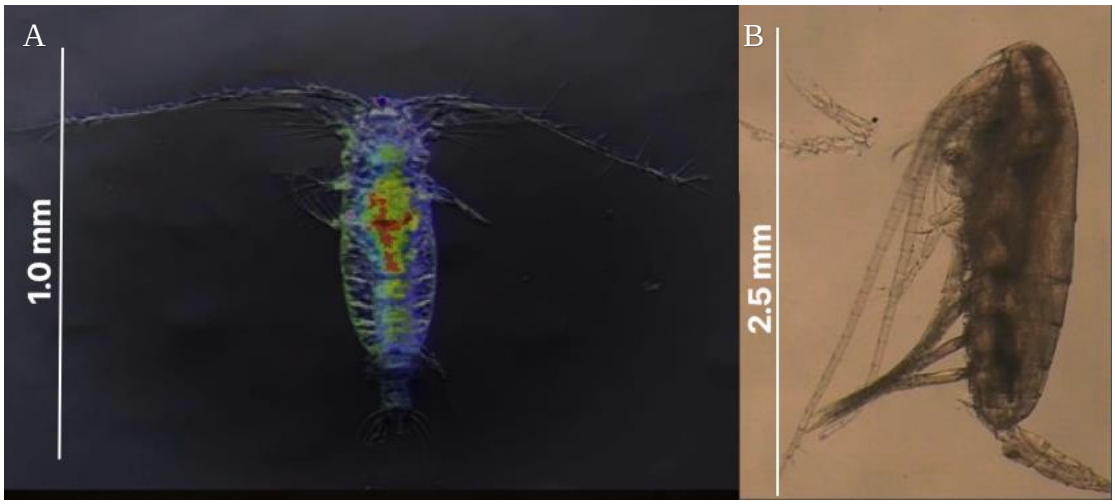

Figure 2. Marine Zooplankton Copepod. A: *Acartia tonsa* female - scale bar is ~ 1 mm (Credit: Ali H. Abdulhussain). B: *Calanus helgolandicus* female - scale bar is ~ 2.5 mm (Credit: Kathryn B. Cook).

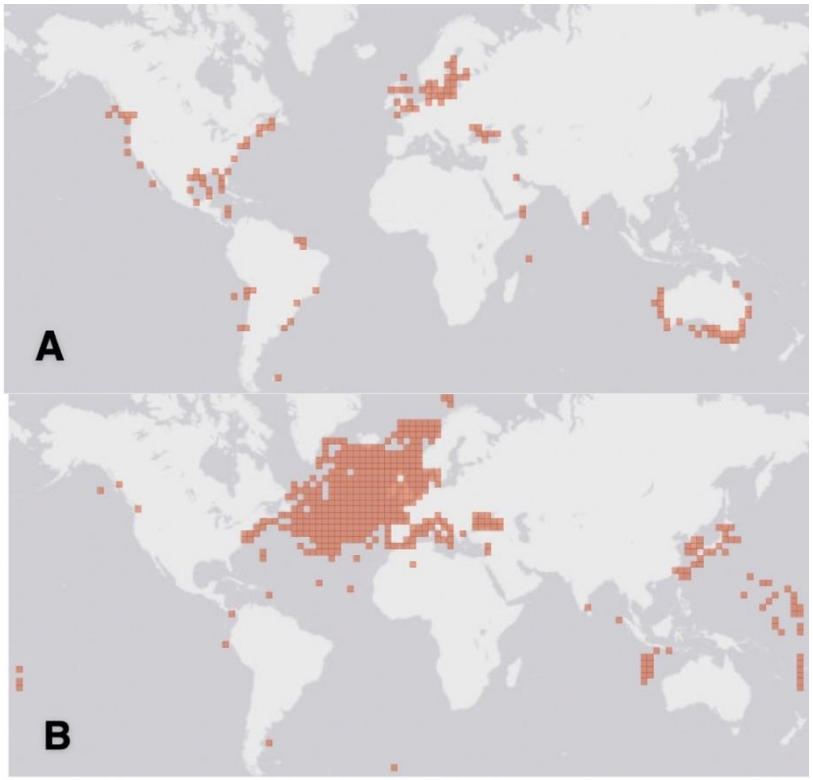

Figure 3. Distribution map of *Acartia tonsa* (A), and *Calanus helgolandicus* (B) (Ocean biodiversity information system. Intergovernmental oceanographic commission of UNESCO, 2021); open access agreement.

1.4 Impact of harmful algae on copepods

HA can be highly toxic to copepods (Anderson, 2012; Dam, 2013; Turner, 2014). Studies have shown that HA may impact their survival, grazing rates, swimming behaviour, and reproduction (Appendix A). Toxins mainly affect neurotransmission or inhibit enzyme functions in copepods (Baden et al., 1993; Turner & Tester 1997; Turner et al., 1998). Toxins may accumulate and be transported to higher trophic levels through feeding interactions (Maneiro et al., 2005; Tammilehto et al., 2012) which, through the process of bioaccumulation, will potentially impact the plankton community structure and ecosystem productivity by killing fish and marine mammals (Doucette et al., 2006; Turner, 2014; Fire et al., 2021; Kershaw et al., 2021).

1.4.1 Impact of HA on copepods survival

Due to the difference in HA mode of action and toxicity, survival experiments typically use monospecific cultures of toxin-producing species at different densities to understand the effects of HA on marine copepods. Short-term exposure to toxic algae might not result in significant mortality compared to non-toxic algae. However, extended periods of exposure to toxic algae may cause substantial mortality within a grazers' population (Table 1).

Table 1. Summary of survival experiments.

Copepod	Toxic Algae	Concentration	Mortality rate	Reference
Acartia tonsa	*Cochlodinium polykrikoides*	3300 and 4700 µgC L^{-1} = 1800 and 2600 cells mL^{-1}	100 % in 1.5 days	Jiang et al., 2009
Acartia tonsa	*Cochlodinium polykrikoides*	1500 and 2200 µg C L^{-1} = ~800 and 1200 cells mL^{-1}	100 % in 3.5 days	Jiang et al., 2009
Acartia tonsa	*Karenia brevis*	1.8´ 10^4 - 2.1´ 10^4 cells mL^{-1}	80 % in 5 days	Prince et al., 2006
Acartia clausi	*Alexandrium minutum*	650 cells mL^{-1}	50 % in 7 days	Barreiro et al., 2007
Calanus finmarchicus	*Alexandrium fundyense*	358 µgC L^{-1} = 200 cells mL^{-1}	17 %, and 0 % in 7-day, for June and July Experiments, respectively.	Roncalli et al., 2016
Calanus helgolandicus	*A. catenella* (Reported: *Gonyaulax tamarensis)*	234 µgC L^{-1} = 93 cells mL^{-1}	~ 50 % in 20 days	Gill & Harris 1987

There are several survivorship studies that have been conducted previously, yet they are difficult to directly compare to each other due to the large variation in methodological approaches (Table 1). For example, different species or even populations of copepods from different geographical regions have had different historical exposure to different concentrations of HA cells, which could contain different levels of toxins. Furthermore, many studies fail to quantify the toxin content of the algae. There is a distinct lack of studies to form a comprehensive understanding of how the survivorship of copepods changes when fed HA across a wide range of concentrations encountered under field conditions.

1.4.2 Impact of HA on copepods feeding

Grazing is the process through which copepods obtain both energy and materials for growth. HA can change copepod feeding behaviour by several mechanisms, including acting as a feeding deterrent resulting in reduced grazing rates (Teegarden, 1999; Costa & Fernández, 2002; Costa et al., 2005; Costa, 2012), physical incapacitation due to toxicity, or nutritional insufficiency (Brier & Buskey 2007; Cohen et al., 2007; Waggett et al., 2012; Lasley-Rasher et al., 2016). Feeding deterrents cause the copepod to behaviorally cease feeding or select against the toxic algae. For example, ingestion rates of *A. tonsa* fed on the toxic dinoflagellate *Cochlodinium polykrikoides* were 25 to 60 % lower than when feeding on non-toxic *Rhodomon*as sp. (Jiang et al., 2009). Another study measured the feeding rates of the copepods *Euterpina acutifrons* and *Acartia grani* every 6 h over a 24 h period to determine the effects of different concentrations of the toxic dinoflagellate *Gymnodinium catenatum* (Costa et al., 2012). They found that ingestion rates of toxic algae in both copepods decreased with increasing exposure time to the dinoflagellate and these results were attributed to the anti-grazing effects of the PSTs. Thus, toxic algae may act as a deterrent to feeding, with a time-dependent negative effect on the ingestion rates of copepods.

HA have also been reported to cause acute physiological incapacitation, such as suppressed swimming activity and erratic photosensitivity. For example, a behavioral study conducted on *A. tonsa*, *Temora turbinata* and *Centropages typicus* showed that swimming and their behavioral responses to light were affected in *T. turbinata* and *C. typicus* when fed *K. brevis* cells or exposed to dissolved brevetoxin with minimal sublethal behavioral effects on *A. tonsa* (Cohen et al., 2007). A toxic effect occurs after ingestion if the toxin interferes with a physiological function in the copepod that cannot be overcome by supplemental feeding on alternate prey (Walker et al., 2001).

Copepods have the ability to actively select or reject food to maximise their nutritional benefit (Turner & Tester, 1997). According to Colin and Dam (2002), the presence of *Tetraselmis* sp. in a mixed diet with HA, even at a low percentage, stimulated ingestion by *A. tonsa*. Feeding deterrents can reduce ingestion rates when fed monoalgal culture, but not necessarily when offered in a mixed diet (Koski 1999, Teegarden 1999; Colin & Dam 2002). For example, *A. tonsa* fed a diet of 100 % *K. brevis* had significantly lower ingestion rates than *A. tonsa* fed mixed diets with *K. brevis* (Brier & Buskey, 2007). Therefore, the mixed diet approach may change the pattern of copepod feeding, and help discern whether the suspected prey is beneficial, nutritionally inadequate, or toxic to copepods (Jónasdóttir et al., 1998; Colin & Dam, 2002).

Nutritional insufficiency occurs when the algae lacks an essential element for growth and reproduction in the copepod, which might result in increased feeding to compensate for the essential element. For example, *K. brevis* is unable to produce sterols, which are essential for copepod growth and reproduction (Ederington et al., 1995); thus, it is considered to be a nutritionally inadequate food source for some copepod species (Brier & Buskey, 2007). Studies of *A. tonsa* fed on toxic *K. brevis* found an impact on egg production and hatching similar to starvation (Collumb & Buskey, 2004; Prince et al., 2006). The dominant sterol found in copepods' tissues is cholesterol, which makes up 89 – 99 % of the total sterol composition of *A. tonsa* populations in wild and lab-reared environments (Ederington et al., 1995). Thus, low egg production rates of *A. tonsa* may result from a lack of dietary sterols. *K. brevis* also lack some chemical components such as fatty acids that are responsible for stimulating grazing by *A. tonsa*; thus, negative effects of HA may not always be due to toxicity (Breier & Buskey, 2007). Taken together, HA could influence fitness of copepods through toxicity or nutritional deficiency. Also, dinoflagellates that are not known to produce neurotoxins may also adversely affect copepod fitness (Ianora et al., 2004). Therefore, more studies are needed to estimate how the amount of HA in food influences copepod grazing rates by controlling and measuring the concentration of algae and their toxin concentration in diet.

1.4.3 Copepods tolerance to HA

Although some copepod species/populations have shown a decrease in survival/feeding, other species/populations have been shown to be tolerant to HA. In fact, several studies have shown that copepods with a history of co-occurrence with HA have higher survival and ingestion rates when exposed to a HA bloom compared to species with

no history of co-occurrence (Turner et al., 2000; Turner et al., 2005; Maneiro et al., 2005; Doucette et al., 2006; Deeds et al., 2007; Appendix A). They may have evolved tolerance to the HA species that co-occur in the same region. The adaptation of *Acartia* spp. (Colin & Dam 2002, 2005, 2007; Jiang et al. 2011; Zheng et al. 2011) and *Calanus* spp. (Turner & Borkman, 2005) to HA have been previously reported. For example, feeding experiments of five copepod species (*A. tonsa*, *Paracalanus quasimodo*, *Labidocera aestiva*, *Oncaea venusta*, and *C. typicus*), during the expatriate *K. brevis* bloom that was carried to North Carolina from the Gulf of Mexico in 1987, showed that the four species that grazed upon the toxic algae co-exist with *K. brevis* (Turner & Tester, 1989). On the other hand, *C. typicus*, which does not routinely co-occur in the same region as the HA, did not graze upon *K. brevis*. This may be due to a lack of co-evolutionary experience between the HA and the copepod. Moreover, *Acartia hudsonica* populations sampled from two regions, one of which co-occurs with HA, were fed the toxic dinoflagellate *A. fundyense* which showed that animals that were historically exposed to toxic *A. fundyense* ingested significantly more toxic dinoflagellates and had higher egg production rates than those without previous exposure to *A. catenella* (Colin & Dam, 2002).

Copepods that co-exist with HA may have developed a series of cellular mechanisms to avoid the effects of toxins. Toxins may be reduced by increasing the activity of detoxification enzymes in copepods (Dutz, 1998; Costa et al., 2008; Kozlowsky-Suzuki et al., 2009). However, a recent gene expression investigation in the marine copepod, *C. finmarchicus*, found that detoxification is not the main response to the toxic dinoflagellate *A. fundyense* (Roncalli et al., 2016). Rather, the genes that responded were those associated with digestion, implying that toxins might reduce food absorption and thus reproductive production without affecting survival. In addition, some copepods have a distinct toxin-resistant phenotype, implying that a simple genetic mechanism has evolved to adapt and withstand toxins during HA blooms (Avery & Dam, 2007; Lauritano et al. 2012, 2013; Wang et al., 2021). For example, after feeding on *A. fundyense* for four generations, the fitness of *A. hudsonica* increased, which indicates that adaptation occurred (Finiguerra et al., 2014). Although sodium channel mutations have been proposed as a possible mechanism (Avery & Dam, 2007; Chen et al., 2015), *A. hudsonica* exposure to *A. fundyense* did not result in an increased proportion of mutant isoforms (Finiguerra et al., 2014), suggesting that sodium channel mutations are unlikely to be responsible for *A. hudsonica* adaptations to toxigenic HA. It is widely acknowledged that there is no clear link between phytoplankton neurotoxicity and impacts on plankton grazers, and that trials with sympatric grazers and

prey species are required to understand fully trophic interactions in the plankton (Smayda, 1997; Turner & Tester, 1997).

1.4.4 Impact of HA on copepods fitness

Increased tolerance, survival and grazing rate of copepods exposed to HA might not be the only factors that are indicative of a copepod fitness. It might be expected that there would be no effect on copepod survival rate as well as ingestion rate if copepods have the ability to detoxify. However, detoxification mechanisms may cost energy by causing other sub-lethal effects. Thus, even if copepod survival remains high, responses in some parameters, particularly egg production and hatching success, typically decline over time if female copepods continue to consume teratogenic compounds that interfere with reproduction, such as dinoflagellate toxins (Turner, 2014). The calanoid copepod, *Temora longicornis*, was exposed to the HA *A. fundyense* to examine their survival, ingestion, egg production, and swimming behaviour (Lasley-Rasher et. al 2016). *T. longicornis* fed 320 cells mL^{-1} of *A. fundyense* + 1200 cells mL^{-1} of *Rhodomonas lens* in the same bottle ingested the *A. fundyense* with no significant change in their survival, ingestion, and egg production compared to the control. However, an alteration in copepod swimming behaviour was reported in that copepods swimming speed increased, and they no longer swam in a straight path after they were removed from the *A. fundyense* treatment. In addition, despite the fact that *A. clausi* females could consume toxic *Alexandrium lusitanicum* at high rates without impact on feeding, egg production was limited to intermediate levels even at high food concentrations (Dutz, 1998).

It is important to note that the majority of HA-copepod studies have been conducted over approximately one day (24 h). This is sufficient time to quantify grazing and discern patterns of selectivity. However, it may be insufficient time to determine any deleterious effects of HA on grazers such as egg production and hatching success since there is a considerable delay in the effect of grazers' response to the same concentrations of food suspensions, as well as acclimatisation of grazers to HA (Ianora & Miralto, 2010; Turner et al., 2012).

1.5 Harmful algae and fisheries in UK waters

There are four types of toxic *Alexandrium* species found in Northern UK waters, and the most abundant toxic species is *A. catenella* (reported as *A. tamarense*) (Bresnan et al., 2005). *A. catenella*, which is responsible for causing PSTs is found in Northern Europe:

Scotland, Iceland, Faroe Islands, Norway (Bresnan et al., 2021, Karlson et al., 2021). There is a long history of PST occurrence in Scotland, which is occasionally of high intensity (The Scottish Government, 2017). The first recorded PST outbreak occurred in 1827 and was associated with toxins in mussels. In 1968, an *A. catenella* bloom was associated with 78 clinical cases for people that consumed mussels from Budle Bay (Ayres & Collum, 1978; Smayda, 2006). After the 1968 incident, a weekly monitoring program (from March to August) began to measure PSTs in mussels collected at approximately 20 different sites along the east coast of Scotland. From 1968-1990, the quarantine level was exceeded 17 times (Smayda, 2006). The distribution of HA toxins can be widespread; they have been detected in the tissues of mussels, scallops, crabs, lobster, prawns, fish, seabirds, and marine mammals (Mortensen, 1985; Jensen et al., 2015; Kershaw et al., 2021) and there was an incident with 77 % mortality of the farmed Salmon in the Faroe Islands in 1985 (Mortensen, 1985). Since the 1990s, the presence of PSTs in mussels and the presence of toxic *Alexandrium* species in Scottish waters are reported on an annual basis (Bresnan et al., 2008). Recent testing of 40 different species of fish for toxin accumulation in Scottish waters showed that there was DA in 92.3 % of the samples, and PST in 96.2 % of the samples. Since there were no HA recorded during the months when the fish were sampled, the concentrations reported in the study are unlikely to represent the acutely high doses ingested by fish during HA events (Kershaw et al., 2021). Jensen et al., (2015) reported a decrease in the abundance of Scottish harbour seal (*Phoca vitulina*) populations that is linked to consuming contaminated fish with both DA and PST.

HA may impact marine ecosystems by impacting copepod survival, which will result in less food for higher trophic levels, and by toxin accumulation, so that copepods serve as a vector for toxin transfer to higher trophic levels. Therefore, understanding HA-copepod interactions is key to understanding the link between HA and fisheries. The role of copepods in passing the energy up the food chain is an important factor in determining the size of fisheries. Scotland is one of the largest sea fishing nations in Europe; in 2017 Scottish registered vessels landed 464,000 tonnes of fish, worth £559 million (The Scottish Government. 2017). Therefore, HA impacts on copepods and the wider ecosystem may have economic consequences. However, relatively few studies have examined the response of common North East Atlantic copepods to toxic strains of algae (Arendt et al., 2005; Jansen et al. 2006; Peters et al., 2007; Koski et al., 2008). The concern is that the present global expansion of toxic blooms may indirectly threaten the survival and recruitment of larval fish by reducing the survival and abundance of copepods in UK waters. The role of environmental managers to regulate any increase in the HA densities and their impact on

fisheries requires information on the lethal and sub-lethal effects of naturally occurring HA and the population-scale implications.

1.6 Aims of my thesis

Observations of how HA affect copepod grazers in UK waters remain scarce, and it is difficult to predict their effects on the basis of information reported in studies conducted elsewhere due to differences in the concentrations of HA used as food, the level of toxins in the food, the length of exposure and the various responses by different species of copepod from various locations. The aim of this study was to investigate the effects of PST-producing HA on the survival, feeding and fitness of important species of marine copepods found in UK waters. In order to address some of the knowledge gaps in previous work, a range of important advances were made:

1. The level of toxins in the experimental diets were quantified.
2. Experiments were conducted using conditions that are representative of UK HA bloom conditions, including realistic concentrations of toxin-producing cells.
3. Lethal and sub-lethal effects were quantified.
4. Short- (24 h) and long-term (10 days) exposures were examined.
5. Different species of copepods were examined to compare and constrast their responses.

Two physiologically different copepods (*A. tonsa* and *C. helgolandicus*) were cultured and also grew and maintained live cultures of toxic *A. catenella*, non-toxic *Rhodomonas* sp. and *A. tamarense* for several months. I also used a combination of analytical approaches, including liquid chromatography mass spectrometry (LCMS/MS), coulter counter, carbon and nitrogen analyses, microscopic counting, and statistical modelling.

Chapter 2 provides an overview of the methods that I used throughout the thesis.

Chapter 3 quantifies how the presence of toxic *A. catenella* and non-toxic *Rhodomonas* sp. affect survival (LC_{50}) and feeding in *A. tonsa*. This Chapter presents a detailed toxin profile of *A. catenella* and tests the hypothesis that exposure to toxic *A. catenella* negatively impacts the survival and feeding of *A. tonsa*. My results show that *A. tonsa* survival and feeding was not affected by toxic *A. catenella*.

Chapter 4 quantifies how the presence of toxic *A. catenella* and non-toxic *A. tamarense* affect long-term survival (10 days) and egg production and hatching success in *A. tonsa*. This Chapter also describes a detailed toxin profile of *A. catenella* and specifically tested the hypothesis that *A. tonsa* survival was influenced by the type of food available, and egg production and hatching success was influenced by the type of food available and its concentration. My results show that *A. tonsa* egg production and hatching success are influenced by the type of food and its concentration. However, survival was not affected by either toxic or non-toxic *Alexandrium*.

Chapter 5 examined how mono-specific and mixed-diets of toxic *A. catenella* and non-toxic *A. tamarense* affect ingestion, egg production and egg viability in *C. helgolandicus*. It also presents a detailed toxin profile of *A. catenella* and body toxin analysis of *C. helgolandicus*. The Chapter tested the hypothesis that exposure to toxic *A. catenella* negatively impacts the feeding, and egg production and viability of *C. helgolandicus. C. helgolandicus* ingestion was negatively affected when fed toxic *A. catenella* comparing to the control non-toxic *A. catenella*. However, egg production and hatching success were not affected by either toxic or non-toxic *A. catenella*. Thus, results were the exact opposite to the results found for *A. tonsa*.

Chapter 6 discusses the results of Chapters 3, 4 and 5 in the context of how they update current knowledge of HA-copepods interactions and how they improve understanding of the impacts of PST-producing HA on coastal and oceanic marine copepods. I also identify futher knowledge gaps and discuss future research priorities.

Chapter 2

Experimental and analytical methods

2.1 Phytoplankton culture

The original stock cultures of *Rhodomonas* sp. were obtained from the National Oceanography Centre (NOC) Aquarium culture collection. The toxic and non-toxic *Alexandrium* spp. cultures (toxic: 1119/27 and 1119/28; non-toxic: 1119/19 and 1119/33) were obtained from the Culture Collection of Algae and Protozoa (CCAP; https://www.ccap.ac.uk), Oban, UK. Phytoplankton were cultured at the NOC in seawater collected from the Western Channel Observatory monitoring site L4 off Plymouth, UK (https://www.westernchannelobservatory.org.uk/) for species in Chapters 3 and 4, and were cultured in Marine Scotland and the seawater collected from Stonehaven, UK for species in Chapter 5. The subculturing and counting (using the Coulter Counter) steps were similar for both toxic and non-toxic algae for species in Chapters 3 and 4, and cultures were counted under an inverted microscope (1 mL using Sedgewick-rafter cell) for species in Chapter 5.

Subculturing took place when the algal growth was exponential, based on the growth curve shown in Fig. 4. The working area and pipette were wiped with 70 % ethanol, and pipette tips were autoclaved. 100 µL of the old culture and 150 µL of F/2 medium were added to flasks containing 100 mL ASW (artificial sea water) for *Rhodomonas*, and 350 µL of L1 medium in sterile filtered L4 seawater (0.2 µm; SFSW hereafter) for *A. catenella*. The new sub-culture was grown in an incubator at 15 °C with a 16:8 h (Light: Dark) photoperiod under constant cool white fluorescent light with a light intensity of 15 µmol photons m^{-2} $s^{-1.}$

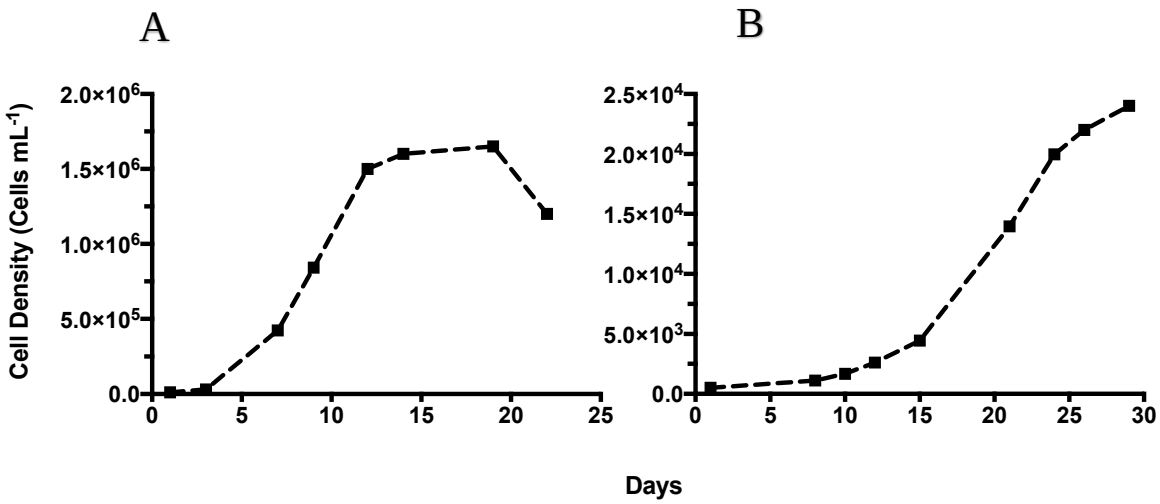

Figure 4. Example growth curves of *Rhodomonas* sp. (A), and toxic *Alexandrium catenella* (1119/27) (B) cultured in the laboratory and measured using Beckman Multisizer 3 Coulter Counter.

All phytoplankton samples were counted using a Beckman Multisizer 3 Coulter Counter equipped with a 70 μm aperture. 1 mL of the algal culture was added to 9 mL diluent solution (3 % NaCl) and mixed well. Each sample was counted 3 times to obtain the mean value of cell counts. The specific cell size range of the algae was determined when calculating the cell counts (the size range for *Rhodomonas* was 6 - 8 μm, and the size range for *A. catenella* was 15 - 42 μm). The cell counts were multiplied by 10 to correct for the dilution factor and obtain the number of cells in 1 mL of sample.

Prior to experimentation, phytoplankton samples were subcultured in 4 - 6 different 100 mL flasks in case contamination occurred in any flask especially during regular counting of the growth phase. For feeding copepod cultures, *Rhodomonas* sp. was grown in 20 L carboys (Fig. 5). Subculturing took place every 7 - 10 days when the carboy was nearly empty or when the color of the *Rhodomonas* sp. culture was very dark. The carboy was washed with pre-filtered seawater from the University of Southampton aquarium and filled with 18 L of pre-filtered seawater. 25 mL of the algal nutrient stock solution (F/2 medium) and 50 mL of sodium hypochlorite solution (bleach) were added to sterilize the water and get rid of all microbial agents. After 5 h, a 25 mL pipette was sterilised with 100 % ethanol and 25 mL of sodium thiosulphate solution was added to the carboy and mixed well to neutralise (dechlorinate) the effect of sodium hypochlorite. About 2 L of the old culture was added to the carboys containing 18 L sterilized seawater.

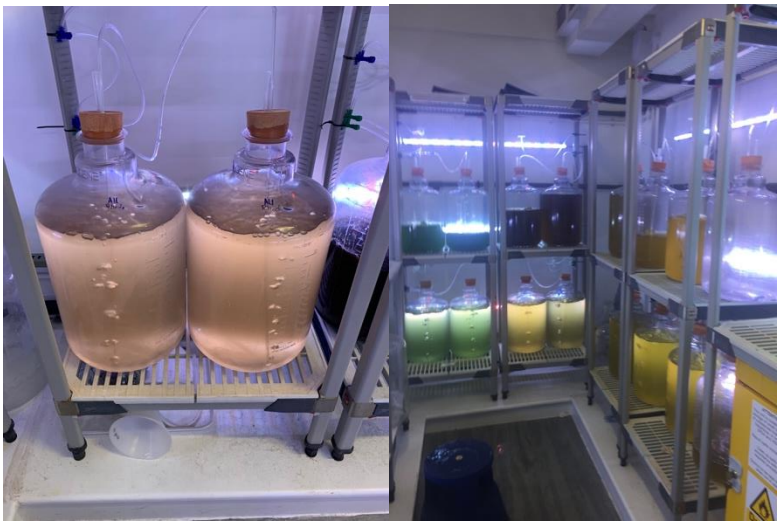

Figure 5. Carboys in a controlled temperature room with aeration to culture *Rhodomonas* sp.

2.2 Copepod culture

A. tonsa was obtained from a culture held at Reefshotz plankton production in Cardiff, UK where it has been reared in the NOC for several generations. *A. tonsa* were cultured in 4 tanks: 3 L for nauplii, 7 L for adults, and two large tanks (50 L) for a mixture of nauplii and adults. All cultures were maintained at room temperature, with natural sun light, and ASW with a salinity 31 made using Tropic Marin Seasalt. They were fed *Rhodomonas* sp. three times a week at a final concentration of 10,000 cell mL^{-1} for the nauplii tanks (3 L), and 60,000 cells mL^{-1} for the other tanks. Copepods were cultured in 4 tanks in case any individual tank became contaminated. The water was changed weekly for the 7 L tank where a 120 µm mesh was used to separate adults from nauplii, and a 53 µm mesh was used to collect eggs and nauplii. The eggs and nauplii were transferred into a 3 L tank, and then transferred to the 50 L tank after one week when most of the nauplii had developed into copepodite stages. It took 2-3 weeks for the nauplii to become a mature adult. 10 L of the water in the 50 L tanks was changed 2-3 times a week, and the whole tank was replaced every 4-6 weeks, depending on the amount of detritus that was found when checked under microscope.

C. helgolandicus were collected from the Scottish Coastal Observatory site at Stonehaven in the North Western North Sea (56° 57.8N 02° 06.2W) using a 1 m ring net fitted with a 350 µm mesh net and a non-filtering cod-end. Upon collection, copepods were diluted with fresh seawater and transported to the laboratory in Marine Scotland within 4 h. The copepods were maintained in 10 L tanks at 13 °C with a 12 h photoperiod, and fed Phyto Feast® Live (a mix of *Tetraselmis*, *Isochrysis*, *Pavlova*, *Nannochloropsis*, *Thalassiosira*, *Amphora* and *Synechoccus*) and Roti-Feast® (*Brachionus plicatilis* animals and eggs) produced by Reed Mariculture.

2.3 Elemental analysis

The carbon and nitrogen content of phytoplankton and *A. tonsa* copepods were measured using a Carlo Ebra EA-1108 elemental analyser. The samples (phytoplankton or copepods) were filtered onto 13 mm filters and wrapped in a tin capsule to ensure complete combustion. To achieve this, 25 mm GFF filters were cut to 13 mm using a steel hole punch, wrapped in aluminium foil, and combusted overnight in an oven (500 °C). The smaller size filters resulted in less ash accumulating in the C/N analyser after each combustion, which helped to prevent blocking of the furnace. After adding the samples, the filters were dried

overnight at 40 °C before analysis. The dried filter was folded using clean tweezers and placed into standard weight tin capsules (8 5 mm) (http://www.microanalysis.co.uk). The capsules were closed from the top using tweezers and placed into a caspule press to crush them into appropriate sizes. The prepared samples were then stored in a desiccator until analysis.

2.3.1 Calibration and running

In order to use the elemental analyser, a simple calibration was required to measure the carbon and nitrogen contents. First, it was necessary to run two bypasses (runs without filters) to check that the combustion tube was clear from the previous analysis and to allow the correct flow of gases. Second, a tin capsule containing 5 mg of standard with known carbon and nitrogen was run as a 'bypass'. The carbon and nitrogen in this sample was detected and the retention times were calculated. The new values of column and retention times were then entered. Third, two blank samples (pre-combusted 13 mm GF/F filter wrapped in tin capsule) were analyzed to determine the amount of carbon and nitrogen found in the filters and tin discs, and then reduces detected values in filters (theoretically zero) when running samples. Fourth, blank samples containing 5 mg of standard (C = 6.1 % and N = 0.46 %) were combusted, allowing the machine to calculate the K-factor, which is the constant area that was calculated as:

$$K_{factor} = \frac{S_{\%} S_w}{S_C B_C} \quad \text{Equation 1}$$

where $S_{\%}$ is the % of carbon (or nitrogen) in the standard (C = 6.1 % and N = 0.46 %), S_w is the weight of the added standard (5 mg), and S_c and B_c are the carbon (or nitrogen) areas of the standard and blank samples, respectively.

Once the K-factor was calculated, the machine was ready to run samples. To test the accuracy of the calibration, a blank sample containing a known amount of standard was run in the machine, and analysis was continued if the detection limits were <0.5 % of theoretical maximum, which is C = 6.1 % and N = 0.46 %.

The amount of carbon and nitrogen in the sample were expressed as the areas under the curve. The values were calculated using the following formula:

$$A = \frac{ScFc}{X}$$ Equation 2

Where A is the adjusted sample area, Sc is the carbon (or Nitrogen as Sn and Fn) area of the standard closest to the theoretical maximum, Fc is the carbon area of the sample, and X is the average carbon (or nitrogen) area of the standard analysed before and after sample F. Using the adjusted area (A), absolute values of carbon and nitrogen can be calculated using the following formula:

$$M = \frac{WA}{Sc}$$ Equation 3

Where M is mg of carbon (or nitrogen), W is mg of C (or nitrogen) in the standard, A, and SC is similar to equation above.

2.4 Detection of toxins and toxicity levels using LC-MS/MS

For Chapters 3 and 4, toxin samples were analysed at CEFAS (Centre or Environment Fisheries & Aquaculture Science), Weymouth, UK. PSTs were determined using Multiple Reaction Monitoring (MRM) transitions and the instrument settings used with the 3200 Qtrap mass spectrometer MS/MS triple quadrupole. Toxin quantitation was measured against calibrations made with approved reference standards received from the Institute of Biotoxin Metrology of the National Research Council of Canada (NRC). Certified reference calibration solutions for PSTs contained gonyautoxins (GTX1, GTX2, GTX3, GTX4, GTX5, GTX6), decarbamoyl-gonyautoxins (dcGTX 1, dcGTX2, dcGTX3, dcGTX4), saxitoxin (STX), neosaxitoxin (NEO), decarbamoyl- STX (dcSTX), decarbamoyl-NEO (dcNEO), (doSTX) deoxydecarbamoyl-saxitoxin, (N-sulfocarbamoyl toxins (C1, C2, C3, C4).

On the day of the experiments, triplicate 50 mL samples of HA were centrifuged at 3000 rpm for 20 min. The supernatant was removed and the pellets were transferred to 2 mL tubes and stored in the -80 $^{\circ}$C freezer until analysis. On the day of analysis, the sample was taken to the CEFAS laboratory. The pellet was mixed with 5 mL of 1% Acetic acid before being mixed using a vortex-mixer for 90 s at 2500 rpm. A Gilson (Middleton, WI) Aspec XL-4 SPE (solid phase extraction) liquid handler was used for automated SPE clean-up processes during all HILIC-MS/MS validation. The sample injection volume was 2 µL. Mobile phase A1 consisted of water + 0.015 % formic acid + 0.06 % of 25 % ammonia. Mobile phase B1 comprised 70 % MeCN + 0.01 % formic acid. The column used in the

MS/MS was ZIC-HILIC analytical column. The results quantified by the quadrupole mass spectrometer (MS/MS) are presented in Chapters 3 and 4. The STX equivalent was calculated by multiplying the toxins by toxin equivalent factor (TEF) provided from European Food Safety Authority (EFSA) recommendations (EFSA, 2009).

For Chapter 5, the samples of both *Alexandrium* spp. and *C. helgolandicus* were analysed in Marine Scotland for PSTs using the PCOX method (Van de Riet et al., 2011). The survived animals (or 250,000 cells of *Alexandrium*) were centrifuged at 3,000 rpm for 20 minutes (multiple stages) in 2 mL Eppendorf tubes. The supernatants were removed using a pipette and the pellets were then stored at -20 ºC until extraction. Glass beads (180 µm, 100 ± 20 mg) were acid-washed and added to the Eppendorf tubes. The extraction solvent (0.5 M acetic acid, 100 µL) was then added with a calibrated pipette. The cells were extracted for two minutes at 25 Hz using a TissueLyser 2. Following microscopic confirmation that the cells had ruptured, they were centrifuged at 14,000 rpm for 5 minutes. The supernatants were transferred to 0.2 µm Ultrafree-MC centrifugal filters using a pipette fitted with a long tip and this was followed by centrifugation for five minutes at 10,000 rpm. All extracted filtrates were transferred to pre-insert amber vials and were immediately analysed by HPLC following the PCOX method (Van de Riet et al., 2011).

Chapter 3

The influence of the toxin producing dinoflagellate, *Alexandrium catenella* (1119/27), on the feeding and survival of the marine copepod, *Acartia tonsa*

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AA designed and conducted the experiments. ME assisted with culturing. Toxin extraction and analyses were conducted by AA at CEFAS with assistance from AT and AL. AA analysed the data, created the figures and wrote the manuscript. Comments were provided by AL, AT, KC, ME and DM.

Chapter 3 The influence of the toxin producing dinoflagellate, *Alexandrium catenella* (1119/27), on the feeding and survival of the marine copepod, *Acartia tonsa*

Ali H. Abdulhussain [1,2], Kathryn B. Cook[3], Andrew D. Turner[4], Adam M. Lewis[4], Mohamed A. Elsafi[5], Daniel J. Mayor[3]

[1] *Ocean and Earth Science, University of Southampton, National Oceanography Centre, Southampton, United Kingdom.*

[2] *Department of Marine Sciences, Kuwait University, Fintas, Kuwait.*

[3] *National Oceanography Centre, Southampton, SO14 3ZH, United Kingdom.*

[4] *Centre for Environment, Fisheries, and Aquaculture Science (Cefas), Barrack Rd, Weymouth, Dorset, DT4 8UB, United Kingdom.*

[5] *Oceanography Department, Faculty of Science, Alexandria University, Egypt.*

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3.1 Abstract

Blooms of harmful algae are increasing globally, yet their impacts on copepods, an important link between primary producers and higher trophic levels, remain largely unknown. Algal toxins may have direct, negative effects on the survival of copepods. They may also indirectly affect copepod survival by deterring feeding and thus decreasing the availability of energy and nutritional resources. Here we present a series of short-term (24 h) experiments in which the cosmopolitan marine copepod, *Acartia tonsa*, was exposed to a range of concentrations of the toxic dinoflagellate, *Alexandrium catenella* (strain 1119/27, formerly *Alexandrium tamarense*), with and without the presence of alternative, non-toxic prey (*Rhodomonas* sp). We also present the toxin profile concentrations for *A. catenella*. The survival and feeding of *A. tonsa* was not affected across the range of concentrations recorded for *A. catenella* in the field; increased mortality of *A. tonsa* was only discernible when *A. catenella* was present at concentrations that exceed their reported environmental concentrations by two orders of magnitude. The observed lethal median concentration (LC_{50}) for *A. tonsa* exposed to *A. catenella* was 12.45 ng STX eq L^{-1}. We demonstrate that *A. tonsa* is capable of simultaneously ingesting both toxic and non-toxic algae, but increases clearance rates towards non-toxic prey as the proportional abundance of toxic *A. catenella* increases. The ability to actively select non-toxic algae whilst also ingesting toxic algae suggests that consumption of the latter does not cause physical incapacitation and thus does

not affect ingestion in *A. tonsa*. This work shows that short-term exposure to toxic *A. catenella* is unlikely to elicit major effects on the grazing or survival of *A. tonsa*. However, more work is needed to understand the longer-term and sub-lethal effects of toxic algae on marine copepods.

3.2 Introduction

Blooms of harmful algae (HA) refer to the high biomass accumulation of toxic or noxious species (Anderson, Cembella, and Hallegraeff, 2012) and can be caused by microalgae such as phytoplankton and cyanobacteria (blue-green algae) or macroalgae (seaweeds). HA blooms can be toxigenic and produce blooms that cause illness and death in a variety of marine organisms, including fish, seabirds, and mammals. The frequency of HA blooms has increased over the past five decades (Anderson et al., 2012; Hallegraeff, 1993) as has the number of toxic blooms, the number of toxins and toxic species reported, and the associated economic losses (Anderson et al., 2012).

Zooplankton grazing plays a vital role in regulating phytoplankton blooms, including those of toxic species (Irigoien, 2005). Toxin-induced mortality may directly affect zooplankton populations (Jiang, Tang, Lonsdale, and Gobler, 2009; Prince, Lettieri, McCurdy, and Kubanek, 2006) and hence their ability to control blooms of HA. The unpalatability of HA (Costa and Fernandez, 2002; Costa, Franco, Cacho, and Fernandez, 2005; Teegarden, 1999; Costa 2012) and physical incapacitation induced by toxicity (Sopanen et al., 2011) can also affect the ability of grazers to control HA blooms. Blooms lacking specific elements or nutrient compounds may further affect zooplankton populations (Breier and Buskey, 2007; Cohen, Tester, and Forward, 2007; Waggett, Hardison, and Tester, 2012). All of these mechanisms ultimately result in reduced rates of zooplankton growth and reproduction (Dam, 2013; Turner, 2014).

The effects of HA on copepods appear to be species- and/or population- specific. Some copepod species/populations have shown a decrease in feeding and/or survival when exposed to HA (Barreiro et al., 2007; Jiang et al., 2009), whereas others appear to be tolerant to HA (Colin and Dam, 2002, 2005, 2007). Several studies have shown that copepods with a history of co-occurrence with HA have higher survival and ingestion rates when exposed to a HA bloom compared to species with no history of co-occurrence (Doucette et al., 2006; Maneiro et al., 2005; Turner et al., 2000; Turner, Doucette, Keafer, and Anderson, 2005;

Deeds et al., 2014), potentially suggesting an evolved tolerance to HA species that co-occur in the same region (Colin and Dam, 2002, 2005, 2007). Some copepods have a discrete toxin-resistant phenotype which suggests evolution of a simple genetic mechanism to adapt and tolerate PSP toxins during bloom conditions (Avery and Dam, 2007). For example, the fitness of *Acartia hudsonica* increased after feeding on *Alexandrium fundyense* for over four generations, which shows that adaptation occurred (Finiguerra, Avery, and Dam, 2014). Sodium channel mutations have been suggested as a possible mechanism (Avery and Dam, 2007; Chen et al., 2015); however, *A. hudsonica* exposure to HA did not induce an increased proportion of mutant isoforms (Finiguerra et al., 2014) which suggests that the mutant isoforms are unlikely to be responsible for adaptations of *A. hudsonica* to toxigenic HA.

Differences between studies may also arise from experimental differences, e.g. concentrations of HA used as food, the level of toxins within the food, and different species of copepod examined (Turner, 2014). It is therefore currently difficult to make generic predictions about how copepods will respond to future blooms of HA. In UK waters, the dinoflagellate, *A. catenella* (previously recorded in this region as *A. tamarense* Group I, reassigned taxonomically by John et al., 2014 & Fraga, Sampedro, Larsen, Moestrup, and Calado, 2015, accepted in Prud'homme van Reine, 2017), is capable of producing Paralytic Shellfish Poisoning (PSP) toxins and is frequently associated with shellfish toxicity (Bresnan, Fryer, Hart, and Percy, 2005; 2008). Densities of *A. catenella* between 1000 and 2000 cells L^{-1} typically result in shellfish flesh exceeding the regulatory limit for PSP toxins, and blooms usually last for a few days (Bresnan et al., 2005; Turner et al., 2014). Maximum reported densities of this species around the UK are 5000 and 18,000 cells L^{-1} in the waters around the Western Isles and Shetland, respectively (Bresnan, Turrell, and Fraser, 2008). These ‘blooms’ do not necessarily result in *A. catenella* becoming dominant within the phytoplankton community but can nevertheless result in an environmental problem, depending on the specific levels of toxins present.

The derivatives of PSP toxins are categorized into three main groups: carbamate-, N-sulphocarbamoyl-, and decarbamoyl- toxins. The carbamate toxins saxitoxin (STX) and neosaxitoxin (NEO) are considered to be the most potent compared to other PSP toxins. Other highly potent analogues include gonyautoxins 1 to 4 (GTX1, GTX2, GTX3, GTX4). N-sulphocarbomoyl (C) toxins and some of the decarbomoyl (dc) congeners are considered less potent (Cook, Bresnan, and Turrell, 2010). Saxitoxins inhibit nerve transmission by blocking watersoluble sodium channels (Luckas, Erler, and Krock, 2015). The consumption

of shellfish contaminated with PSP toxins can cause difficulty in breathing, gastrointestinal problems, and a sense of dissociation followed by complete paralysis in humans and other vertebrates (Wang, 2008); however, how PSP toxin exposure/consumption affects invertebrate fitness remains unclear.

The composition and concentrations of toxins within any species of HA, including *A. catenella*, can be variable, even when grown under similar conditions (Loret et al., 2002; Turner et al., 1998; Etheridge et al., 2005; Brown et al., 2010; Waggett 2012). Some of the factors that can change the toxin levels within a HA bloom include turbulence (Juhl, Trainer, and Latz, 2001), salinity (Grzebyk et al., 2003), and nutrient conditions (John and Flynn, 2002; Leong, Murata, Nagashima, and Taguchi, 2004; Turner et al., 1998). HA can also significantly increase the production of PSP toxins in response to nutrient stress and chemical cues from copepods (Griffin, Park, and Dam, 2019; Wohlrab, Iversen, and John, 2010). A group of predator cues named copepodamides, which are exuded by three calanoid copepod species (*Centropages typicus*, *Pseudocalanus* sp, and *Calanus* sp), were found to trigger increased PSP toxin production in dinoflagellates, and also amnesic shellfish poisoning (ASP) toxins in diatoms (Selander et al., 2015). However, toxin profile concentrations are rarely reported, particularly in the context of copepod grazing studies. This makes it difficult to robustly explain the observed results and hinders comparisons between different studies.

We studied the survivorship and grazing of a globally distributed marine copepod, *Acartia tonsa*, feeding on a toxic strain of *A. catenella* across the range of concentrations above and below those that are typically encountered in UK waters. Additional experiments examined how ingestion rates and prey selection in *A. tonsa* were influenced by the relative abundance of *A. catenella* in the prey field. We present our results alongside a full toxin profile for the *A. catenella* used in our experiments, which allows us to calculate the lethal median concentration (LC_{50}).

3.3 Methods

3.3.1 Culture conditions

A toxic strain of *Alexandrium catenella* (Strain 1119/27; CCAP, Loch Ainort, Scotland) and the non-toxic cryptomonad, *Rhodomonas* sp, were both grown in a culture

cabinet at 15 °C with a 16:8 h (Light:Dark) photoperiod. *A. catenella* was cultured in seawater collected from the Western Channel Observatory monitoring site L4 off Plymouth, UK (https://www.westernchannelobservatory.org.uk/) amended with L1 medium (3.5 mL of L1 medium for every 1 Litre). *Rhodomonas* sp was cultured using artificial seawater (Tropic Marin seasalt, 31) and F/2 medium (1.5 mL of F/2 medium for every 1 Litre). *Acartia tonsa* (Reefshotz, Cardiff, UK, where they have been cultured for > 10 years) were cultured in artificial seawater (Tropic Marin Seasalt; 31) for 5 months prior to experimentation and fed *Rhodomonas* sp three times a week at a concentration of 60,000 cells mL^{-1}. All cell counts were undertaken using a Beckman Multisizer 3 Coulter Counter equipped with a 70 μm aperture. The carbon and nitrogen content of phytoplankton and copepods were determined for each experiment using a Carlo Erba EA-1108 elemental analyser.

3.3.2 Influence of *A. catenella* concentration on the feeding and survival of *A. tonsa*

Experiment 1 simultaneously examined how the ingestion rate and survival of *A. tonsa* changed across a plausible environmental range of *A. catenella* concentrations. A total of 150 adult female *A. tonsa* were carefully transferred via pipette into 500 mL of 0.2 μm sterile filtered L4 seawater (SFSW hereafter) and incubated for 24 h to clear their guts. The carbon and nitrogen content of *A. catenella* and toxin concentration profiles were determined from the stock culture of *A. catenella* prior to their exposure to copepod grazing. Triplicate 5 mL samples from the *A. catenella* stock culture were collected onto glass fibre filters (Whatmann GF/F) and dried at 40 °C for subsequent elemental analysis (Carlo Ebra EA-1108 elemental analyser). Triplicate 50 mL samples of the *A. catenella* stock culture were also centrifuged at 3000 rpm for 20 mins and the resulting pellets were transferred into 2 mL Eppendorf tubes and stored frozen at −80 °C until toxin analysis. Survival and grazing experiments were conducted in 215 mL clear bottles containing *A. catenella* at nominal concentrations of 20, 100, 200 and 1000 μg C L^{-1}. These concentrations were achieved by determining the concentration of the *A. catenella* stock culture and subsequently diluting this with an appropriate volume of SFSW. Nine bottles at each of the required concentrations were prepared. Three initial bottles at each concentration were immediately preserved with acidified Lugol's iodine to enumerate the number of cells at the start of the experiment. Ten female *A. tonsa* were added to each of three grazing bottles at each concentration and incubated alongside triplicate control bottles at each concentration on a plankton wheel (2 rpm) for 24 h at 19 °C with a 12:12 h (L:D) photoperiod. At the end of the experiment, the grazing and control bottles were preserved with acidified Lugol's iodine after removing

copepods from the grazing bottles using a 200 μm mesh and transferred into SFSW. The number of motile copepods observed after mechanical stimulus was recorded. Surviving animals were transferred onto glass fibre filters (Whatmann GF/F) and dried at 40 °C for subsequent elemental analysis (Carlo Erba EA-1108 elemental analyser).

Experiment 2 was conceptually similar to experiment 1, but examined a broader concentration range of *A. catenella* and made no attempt to quantify ingestion rates. A total of 210 adult female *A. tonsa* were incubated in SFSW for 24 h prior to experimentation. Triplicate groups of 10 animals were subsequently incubated for 24 h with *A. catenella* at nominal concentrations of 100, 1000, 3000, 5000, 7500, and 10,000 μg C L^{-1}, where *A. catenella* from the stock culture was diluted with SFSW. Samples to determine the elemental content of *A. catenella* and *A. tonsa* were collected at the beginning and end of the experiment, respectively. The number of animals responding to mechanical stimulus was recorded at the end of the experiment.

3.3.3 Ingestion and selection for/against *A. catenella* in the presence of nontoxic cells

Experiment 3 examined how, for a given total quantity of food, ingestion rates and food selection in *A. tonsa* changed in response to proportional changes in the availability of toxic and non-toxic food. Experimental females were incubated in SFSW for 24 h prior to experimentation. Experiments were conducted in 215 mL culture bottles, each containing a total of 1250 μg C L^{-1} of algae. The four treatment levels contained 0 %, 25 % 50 %, and 75 % of *A. catenella*-derived carbon, with the remainder being provided via *Rhodomonas* sp. Nine bottles at each level were initially set up, with three being sampled to determine cell numbers and their elemental content at the outset of the experiment. Ten female *A. tonsa* were added to each of three grazing bottles and incubated alongside triplicate control bottles on a plankton wheel for 24 h. At the end of the experiment, samples were collected to determine the elemental content of the copepods and the amount of food ingested (as above).

3.3.4 Toxin analysis

The frozen pellets of *A. catenella* from each experiment were mixed with 1.5 mL of 1 % acetic acid before being vortex-mixed for 90 s at 2500 rpm. Solutions were subjected to probe sonication (Sonic Dismembrator, Fisher Scientific) set to 30 % power for 1.5 mins per

sample. After sonication was complete, vials were re-mixed, and 400 μL taken for desalting clean-up using carbon solid phase extraction (SPE) as described by Turner, McNabb, Harwood, Selwood, and Boundy, 2015. A Gilson (Middleton, WI) Aspec XL-4 SPE liquid handler was used for automated SPE clean-up processing prior to a 4:1 dilution in acetonitrile and HILIC-MS/MS analysis. Quantification of individual PSP toxin concentrations was conducted, enabling the assessment of toxin profiles of *A. catenella* for each experiment. An Agilent (Manchester, UK) Infinity II 1290 Ultra-high Performance Liquid Chromatograph (UHPLC) coupled to a 6495B tandem mass spectrometer (MS/MS) was used for acquiring two Multiple Reaction Monitoring (MRM) transitions for each toxin analogue. Certified reference calibration solutions for PSP toxins to allow identification and quantification were sourced from the Institute of Biotoxin Metrology, National Research Council, Canada. These were used to prepare working calibration solutions, which were utilized as external standards for quantification of individual analogues. PSP toxins incorporated into the method included the gonyautoxins (GTX1, GTX2, GTX3, GTX4, GTX5, GTX6), decarbamoyl-gonyautoxins (dcGTX1, dcGTX2, dcGTX3, dcGTX4), saxitoxin (STX), neosaxitoxin (NEO), decarbamoyl- STX (dcSTX), decarbamoyl-NEO (dcNEO), (doSTX) deoxydecarbamoyl-saxitoxin and the N-sulfocarbamoyl C toxins (C1, C2, C3, C4). HILIC chromatography and MS/MS detector conditions were those described by Turner et al., 2015 and Turner et al., 2019 respectively. Mobile phase A1 consisted of water + 0.015 % formic acid + 0.06 % of 25 % ammonia. Mobile phase B1 comprised 70 % MeCN + 0.01 % formic acid. For chromatographic separation of PSP toxins, a Waters (Manchester, UK) BEH Amide HILIC column (2.1 × 150 mm; 1.7 μm) together with an equivalent 5 mm guard cartridge was used. The column was held at 60 °C and sample injection volumes were 2 μL.

3.3.5 Data analyses

Copepod daily ingestion rates were calculated using established equations (Frost, 1972) and expressed relative to the carbon content of the females, giving carbon-specific ingestion rates (CSI, % C day^{-1}). The number of copepods surviving each experiment was expressed as a percentage of the ten that were initially added to each grazing bottle. The influence of increasing concentrations of *A. catenella* on the survival and ingestion of *A. tonsa* were examined using separate one-way ANOVAs in which the concentration of *A. catenella* was treated as a categorical variable. Post-hoc comparisons were conducted using Tukey's test with alpha = 0.05. Feeding selectivity of *A. tonsa* for or against *A. catenella* and *Rhodomonas* sp. was examined by plotting the proportion of these cells in the available food

against their proportion in the ingested diet. Values above and below a 1:1 line indicate positive and negative selection, respectively.

The concentration of PSP toxins, expressed in STX equivalence, of *A. catenella* in each experiment was calculated using a six-point calibration curve drawn from the included toxin standards and then converting molar concentrations of each toxin to STX equivalence using the toxin equivalent factors (TEF) for each PSP toxin analogue detected and quantified (EFSA 2009). These same conversion factors are used to standardize data generated in the UK regulatory monitoring programmes (Turner et al., 2014). The lethal median concentration that killed 50 % of the population (LC_{50}), expressed in STX equivalents, was determined by applying a non-linear regression using a dose-response model. All statistical analyses were performed using Prism Graphpad software (v.7.0).

3.4 Results

The average carbon content of the *A. tonsa* used in the experiments was 2.6 μg C Copepod^{-1} (± 0.53, SD). Table 2 presents the nominal and actual concentrations of *A. catenella* offered in the different experiments, along with the cellular carbon quotas. The cell toxicity analysis detected 12 different toxins out of the 19 included in the calibration standard mix, these different congeners showed variability in their concentrations and STX equivalence leading to an average total cellular toxin quota of 2.7 pg STX eq cell^{-1}. (Table 3).

Table 2. The nominal and actual concentrations of *Alexandrium catenella* offered in the different experiments, along with the cellular carbon & nitrogen quotas

Experiment	***Rhodomonas* sp Carbon content (Nitrogen Content) ng cell^{-1}**	***Alexandrium catenella* Carbon content (Nitrogen Content) ng cell^{-1}**	**Nominal Concentration (Actual concentraton) µg C L^{-1}**
1	-	1.62 – 1.75 (0.48-0.52)	20 (22) 100 (112), 200 (224), 1000 (1120)
2	-	2 (0.4)	100 (150) 1000 (1150), 3000 (3450), 5000 (5750), 7500 (8625), 10000 (11500)
3	0.053-0.054 (0.010)	1.96-2.11 (0.05)	*A. catenella*: 25% (30%) 50% (55%) 75% (79%)

Table 3. *Alexandrium catenella* (1119/27-Ainort) toxin profile concentrations (fg Cell^{-1} & fg STX eq. cell$^{-1)}$ cultured in L1 media and analysed using HILIC-MS/MS (Liquid Chromatography Mass Spectrometry) at CEFAS.

Toxin Concentration fg cell^{-1} (fg STX eq cell^{-1})									
C1	**C2**	**C3**	**C4**	**dcGTX 2**	**dcGTX 3**	**dcGTX 1**	**dcGTX 4**	**GTX6**	**dcNEO**
18.8 (0.2)	1130.8 (113.1)	nd	44.3 (4.43)	nd	nd	nd	nd	nd	nd
GTX2	**GTX3**	**GTX1**	**GTX4**	**GTX5**	**doSTX**	**dcSTX**	**STX**	**NEO**	**STX eq cell^{-1}**
88.9 (35.6)	307.3 (184.4)	88.8 (88.8)	450.5 (315.4)	42.9 (42.9)	40.2 (2.0)	6.5 (6.5)	1089.7 (1089.7)	888.3 (888.3)	2732.5

3.4.1 Influence of *A. catenella* concentrations on the feeding and survival of *A. tonsa*

Survival, and daily rates of clearance, total ingestion and carbon specific ingestion (CSI) for *A. tonsa* offered *A. catenella* at nominal concentrations between 20 and 1000 µg C L^{-1} (experiment 1) and between 100 and 10,000 µg C L^{-1} (experiment 2) are presented in Figs. 6 and 7, respectively. The survival of *A. tonsa* was not affected by the increase in concentration of *A. catenella* between 20 and 1000 µg C L^{-1} (ANOVA, df = 14, p = 0.310; Fig. 6A). Survival was only significantly reduced when offered *A. catenella* at the highest concentration treatment (Experiment 2: Fig. 7A) (ANOVA, df =20, p = 0.001).

The calculated LC_{50} was 12.45 ng STX eq L^{-1} (Fig 7B), with a 95 % confidence interval between 10.31–14.0 ng STX eq (Fig. 7B). This corresponds to an *A. catenella* concentration of 7707 μg C L^{-1}, or ~4561,000 cell L^{-1}.

Clearance rates were variable, ranging between 5.1 and 13.5 mL $copepod^{-1}$ day^{-1} and were not significantly affected by prey availability (ANOVA, df = 11, p = 0.52; Fig. 6B). Total ingestion rates ranged between 0.87 and 6.17 μg C $copepod^{-1}$ day^{-1}, and, by contrast, changed significantly as a function of prey concentration (ANOVA, df = 11, p = 0.0009; Fig. 6C). Post-hoc comparisons revealed that the ingestion rate observed when *A. catenella* was offered at a concentration of 1000 μg C L^{-1} was significantly higher than the ingestion rates in all other treatments. Daily CSI rates ranged between 0.27 to 2.27 and also increased significantly as a function of the concentration of *A. catenella* (ANOVA, df = 11, p = 0.0003; Fig. 6D); the CSI rate in the treatment offering *A. catenella* at 1000 ug C L^{-1} was significantly higher than in all of the other treatments.

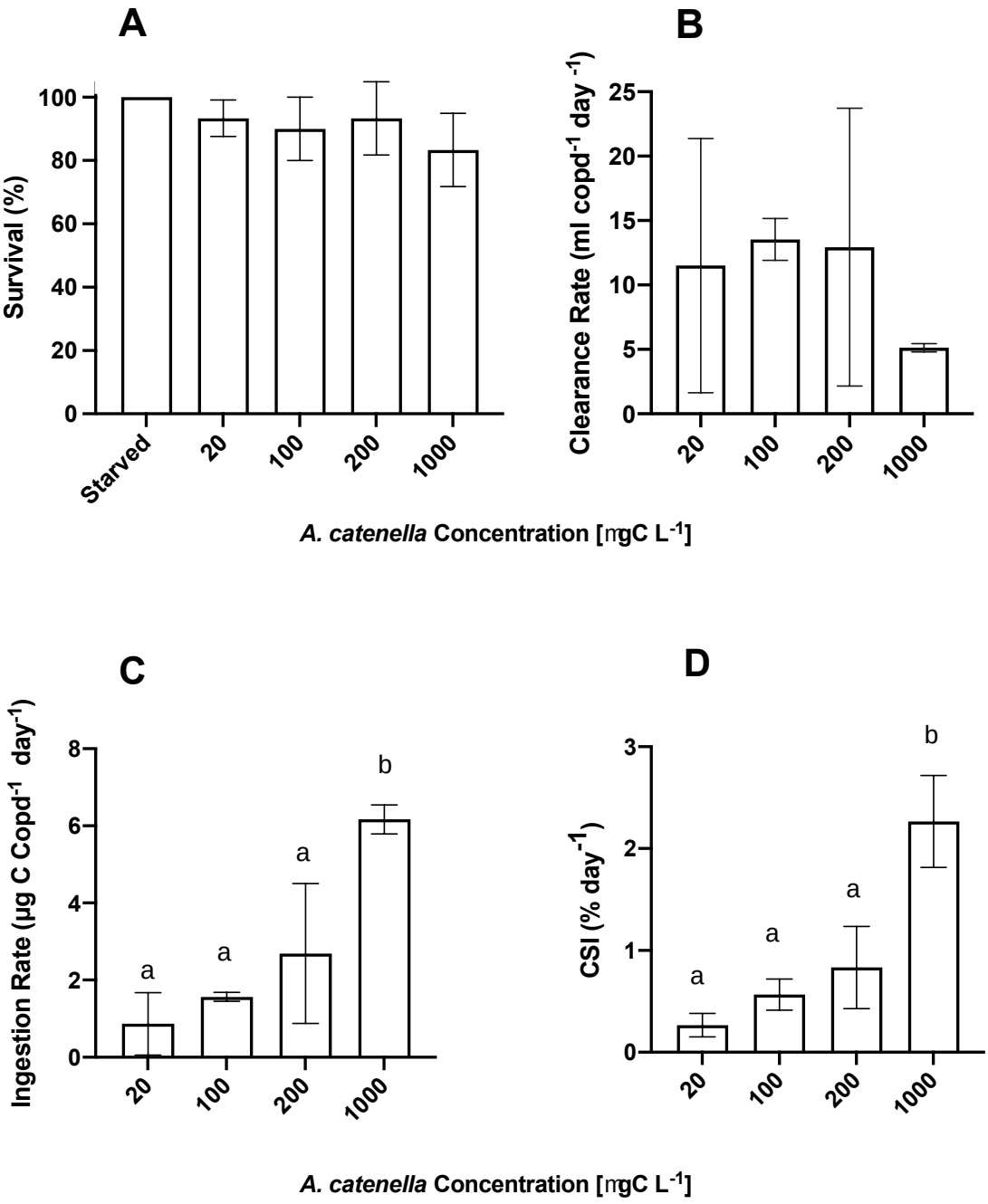
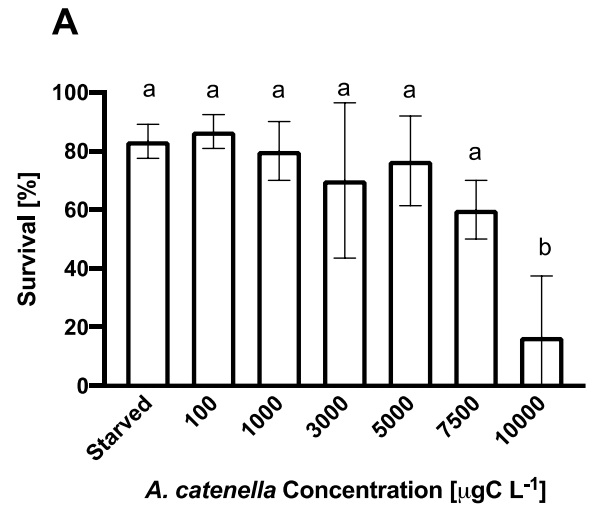


Figure 6. Survival and feeding of *Acartia tonsa* fed toxic algae (*Alexandrium catenella*) at 4 different concentrations. The bars show the mean value ± standard deviation (SD). A: survival (%). B: clearance rates (mL $Copd^{-1}$ day^{-1}). C: Ingestion rate (µg C $Copd^{-1}day^{-1}$). D: Carbon Specific Ingestion (CSI: % C day^{-1}). Different letters denote significant differences (Tukey's test, $p < 0.05$).



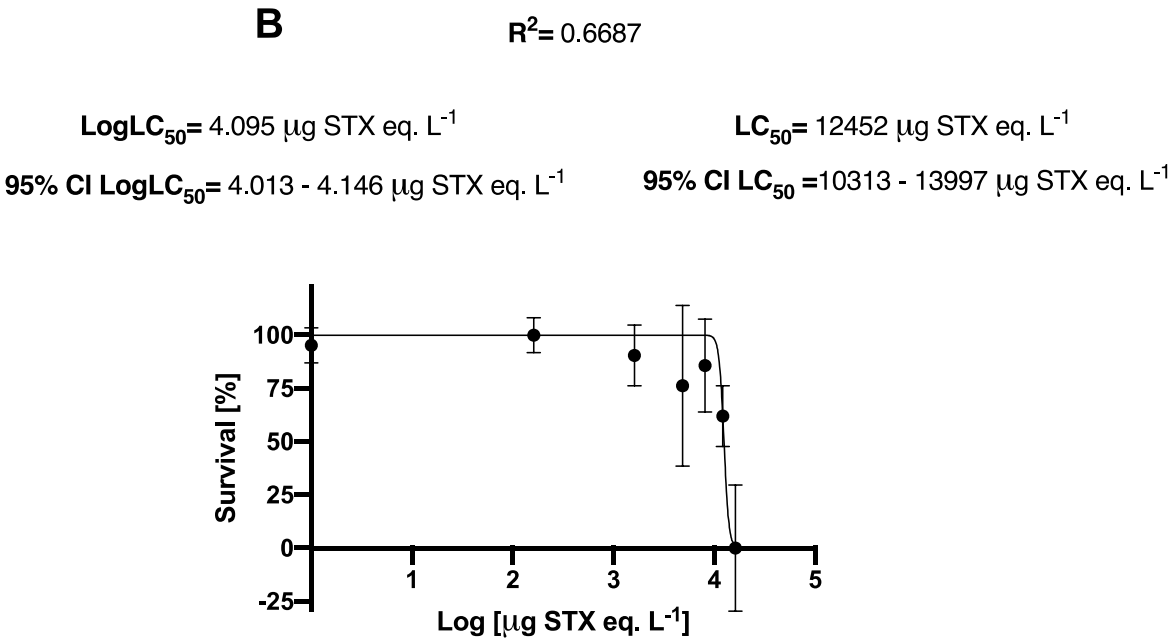


Figure 7. Survival of *Acartia tonsa* females across 7 different concentrations of *Alexandrium catenella*. The bars show the mean ± SD. A: survival. Different letters denote significant differences (Tukey's test, $p < 0.05$). B: The log10 curve of *A. catenella* concentration with normalized % of survival to determine the LC_{50} of the survival experiment. The LC_{50} was calculated as 12452 µg STX eq L^{-1} (7707 µg C L^{-1}; 4561 cell mL^{-1}), with a 95 % confidence interval between 10313-13997 µg STX eq L^{-1}.

3.4.2 Ingestion and selection for/against *A. catenella* in the presence of nontoxic cells

Clearance rates of *A. tonsa* were consistently lower (0.0–1.9 mL copepod^{-1} day^{-1}) when fed *A. catenella* than *Rhodomonas* sp. (2.0–11.2 mL copepod^{-1} day $^{-1}$) (Fig. 8A). The total CSI rate of *A. tonsa* (*Rhodomonas* sp. & *A. catenella)* was not affected by the proportion of *A. catenella* in the available prey field (ANOVA, df=10, p = 0.780). The majority of ingested carbon was always derived from *Rhodomonas* sp. (Fig. 8B & C), but the difference in the amounts ingested of *Rhodomonas* sp. and *A. catenella* was only statistically discernible at toxic algae (Tukey's test, df=11, p> 0.329 in both cases). Fig. 9 shows that the percentage of *Rhodomonas* sp. in the diet was consistently higher than that of the available food, whereas the percentage of *A. catenella* in the diet was consistently lower than available in the prey field.

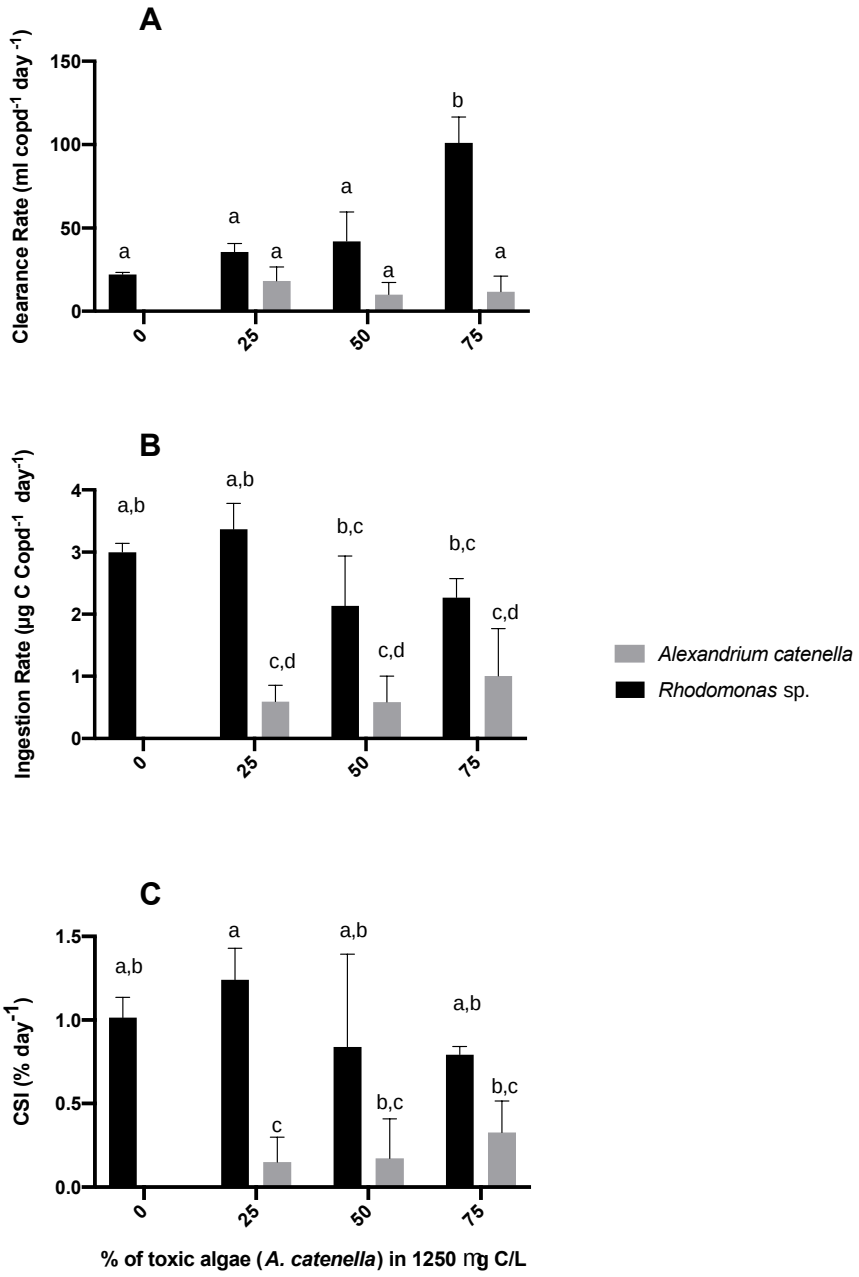


Figure 8. Mean (± SD) clearance and ingestion rates of *Acartia tonsa* fed a mixed diet of *Alexandrium catenella* (grey bars) and *Rhodomonas* sp. (black bars), where the total concentration of carbon in all treatments remained constant (1250 µg C L^{-1}). A: Clearance rates (mL $Copd^{-1}$ day^{-1}). B: Ingestion rate (µg C $Copd^{-1}$ day^{-1}). C: Carbon Specific Ingestion (CSI: % C day^{-1}). Survival was 100 % in all the of treatment levels. Different letters denote significant differences (Tukey's test, $p < 0.05$).

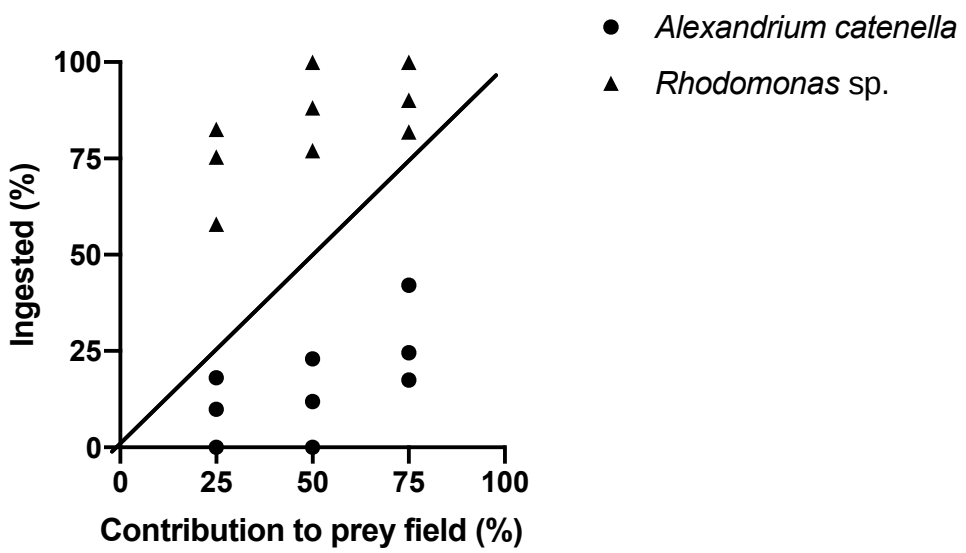


Figure 9. Prey selection of *Acartia tonsa* fed a mixed diet of *Alexandrium catenella* and *Rhodomonas* sp., where the total concentration of carbon in all treatments remained constant (1250 µg C L^{-1}). Percent (%) Carbon *Rhodomonas* sp./*Alexandrium catenella* ingested (based on CSI: % C day^{-1}) versus % carbon of *Rhodomonas* sp./*Alexandrium catenella* in the prey field.

3.5 Discussion

This study examined how the feeding and survival of *A. tonsa* were influenced by the concentration of the toxin-producing dinoflagellate, *A. catenella*. It also examined how the presence of alternative, non-toxic prey influenced food ingestion and patterns of prey selection.

3.5.1 Survival of *A. tonsa* feeding on *A. catenella*

Our study failed to discern any negative effects of a toxic strain of *A. catenella* on the survival of *A. tonsa* across a range of plausible environmental concentrations. Maximum reported densities of *A. catenella* in UK waters range between 5000 and 18,000 cells L^{-1} (Bresnan et al., 2008), which equate to between 10 and 36 µg C L^{-1} (assuming *A. catenella* contains 2.0 ng C $cell^{-1}$; Table 2). Significant mortality was only observed at a concentration of 10,000 µg C L^{-1}, equivalent to ~5919,000 cells L^{-1}. These data suggest that *A. tonsa* survival is unlikely to be affected during natural blooms of toxic strains of *A. catenella*. Our experimental animals had been maintained in culture for > 10 years without exposure to HA. We therefore suggest that it is unlikely that they possessed genetic resistance to the toxins of *A. catenella*. Indeed, the observed lethal median concentration (LC_{50}) of 12.45 ng STX eq L^{-1} is equivalent to 7707 µg C L^{-1} or ~4561,000 cells L^{-1}. Our lethal median concentration

(LC_{50}) was estimated using the toxin concentration profile of *A. catenella* that was determined prior to their exposure to copepod grazing. We cannot exclude the possibility that *A. catenella* increased toxin production during our grazing experiments (Griffin et al., 2019; Selander et al., 2015; Wohlrab et al., 2010) and therefore consider our LC_{50} estimate to be conservative. Nevertheless, this concentration is two orders of magnitude higher than any of the maximum densities of *A. catenella* recorded in natural systems (Bresnan et al., 2008; Fauchot, Levasseur, Roy, Gagnon, and Weise, 2005). Our experiments were not designed to determine which of the toxins present (Table 3) were responsible for the observed effects. We therefore present toxicity as a saxitoxin equivalent (EFSA 2009). Through use of an ultra-sensitive detection method, we have the first reported evidence for the presence of certain PST analogues. For example, *A. catenella* is well known to contain C1&2, STX, NEO and GTX1–4 – but here we also demonstrate the presence of C4, GTX5, dcSTX and doSTX (Table 3). Our findings support the understanding that *Acartia* spp. are able to ingest toxic *Alexandrium* spp. without negative impacts on their survival (Colin and Dam, 2002; Dutz, 1998; Teegarden and Cembella, 1996). Nevertheless, it is probable that the effects of *A. catenella* are strain-specific; the toxicity of six different strains of *A. catenella* found in Scottish waters ranged between 2.8 - 10.5 pg STX eq cell $^{-1}$ (Brown et al., 2010).

3.5.2 Ingestion and prey selection

The ingestion rates observed in this study (μg C Copd $^{-1}$ day $^{-1}$) agree well with those previously reported for the genus *Acartia* (Colin and Dam, 2002; Jiang et al., 2009; Zheng, Dam, and Avery, 2011), and demonstrate that *A. tonsa* can readily achieve CSI rates of > 1 % day^{-1} feeding on a toxin-producing species. Indeed, our results suggest that CSI rates observed when feeding on *A. catenella* alone may exceed those when feeding on non-toxic *Rhodomonas* at a similar concentration (compare Figs. 6C and 8C). This result is consistent with the observation that *Acartia grani* ingested more of the toxic dinoflagellate, *Alexandrium minutum*, than the non-toxic *Rhodomonas* controls (Costa, Pereira, and Fernandez, 2008). It has been suggested that copepods increase ingestion in order to compensate for the increased energy required for detoxification (Costa et al., 2008; Dutz, 1998). Compensatory feeding may also be required if the nutritional value of *A. catenella* is lower than the control diet of *Rhodomonas* sp. This has been shown to occur when *A. tonsa* was fed the nutritionally insufficient species of toxic algae, *Karenia brevis*, which lacks various fatty acids and other nutritional components, and affected egg production and

hatching success in a manner similar to starvation (Collumb and Buskey, 2004; Prince et al., 2006). Unfortunately it is not possible to robustly distinguish between these two non-mutually exclusive mechanisms from the data presented herein. This study demonstrated that, in the absence of alternative prey, *A. tonsa* achieves similar daily CSI rates when fed either *A. catenella* (Fig. 6) or *Rhodomonas* sp (Fig. 8). However, when offered both species together, *A. tonsa* consistently selected for *Rhodomonas* and against *A. catenella*, even as the proportional abundance of the latter was increased (Figs. 8 & 9). This agrees with the understanding that *A. tonsa* can discriminate between different species of *Alexandrium* with different toxin contents, showing preference for the least toxic species (Colin and Dam, 2003; Selander, Thor, Toth, and Pavia, 2006; Teegarden, 1999; Turner and Tester, 1989). Similar results have been reported for *A. tonsa* fed on toxic *K. brevis* (Turner and Tester, 1989). Exactly how copepods achieve this food selection remains unclear; they may be able to physically distinguish different prey items prior to ingestion, or alternatively, their selection mechanisms may be based on a cell's palatability/toxicity or nutritional content. Physical incapacitation caused by toxicity would have resulted in decreased ingestion rates of the non-toxic algae (Colin and Dam, 2003), but the total CSI of *A. tonsa* (*Rhodomonas* sp. & *A. catenella*) was not statistically different between copepods fed a mixed diet and the control (0 % toxic algae) (Fig. 8C). It is possible that the apparent selection was size-related, as the equivalent spherical diameters (ESD) of *A. catenella* (25 μm) and *Rhodomonas* sp. (7 μm) were different. However, we suggest that this is unlikely as previous work has shown that female *A. tonsa* have an optimal prey size of 14.8 μm ESD, but are able to achieve approximately equal clearance rates when fed *Rhodomonas baltica* (ESD = 7 μm) and the dinoflagellate *Scripsiella faroense* (ESD= 19 μm) (Berggreen, Hansen, and Kiørboe, 1988). Further work is required to determine if the apparent rejection of *A. catenella* is because of its palatability or its nutritional content.

3.5.3 Future-work

The presented 24 h experiments are an important first step towards understanding how marine copepods respond to toxin-producing algae. However, HA blooms in natural ecosystems last from a few days up to few months (Bresnan et al., 2008) and extended periods of exposure to toxic algae may cause substantial mortality within a population of copepods (Barreiro et al., 2007; Jiang et al., 2009; Prince et al., 2006; Turner et al., 2014). Previous work has shown a time-dependant decrease in the feeding rates of the copepods *Euterpina acutifrons* and *A. grani* when exposed to the toxic dinoflagellate *Gymnodinium catenatum* (Costa, Pereira, and Ferrn.ndez, 2012). The presence of toxin-producing algae

can also lead to reduced rates of growth, respiration and egg production in copepods (Colin and Dam, 2007; Dutz, 1998). However, it is not yet known if these effects arise because of direct toxicity, the costs of detoxification, unpalatability of toxin producing cells, or the absence of particular elemental and biochemical components in the HA that are essential for healthy functioning of copepods. Indeed, it is possible for all four of these mechanisms to operate simultaneously, and determining the relative importance of each remains a challenge. We suggest that additional, longer-term experiments are required to better appreciate the population-scale effects of interactions between HA and copepods. Furthermore, a more detailed understanding of the energetic and nutritional requirements of copepods such as *A. tonsa* is required for us to determine if HA are in some way nutritionally inferior to other, non-toxin producing algae.

3.6 Conclusion

Our short-term experiments demonstrate that the dinoflagellate *Alexandrium catenella* does not have a direct impact on *Acartia tonsa* at concentrations typically encountered in natural systems. Decreased survival of *A. tonsa* was only apparent when offered *A. catenella* at a concentration two orders of magnitude higher than previously reported maximum densities. Ingestion rates of *A. tonsa* were not reduced when offered *A. catenella*, suggesting that the concentrations of toxins present in our experiments did not incapacitate *A. tonsa*. However, active selection towards non-toxic cells when presented simultaneously with *A. catenella* suggests that the toxins do act as a feeding deterrent. Further studies are required to understand the longer-term, sub-lethal effects of *A. catenella* on *A. tonsa*.

Chapter 4

The influence of the toxin-producing dinoflagellate, *Alexandrium catenella* (1119/27), on the survival and reproduction of the marine copepod, *Acartia tonsa*, during prolonged exposure

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AA designed and conducted the experiments, performed the statistical analyses, and wrote the manuscript. Toxin extraction and analyses were conducted by AA at CEFAS with assistance from AT and AL. Comments were provided by AL, AT, KC, TB and DM.

Note: non-toxic *Alexandrium catenella* (NAC): this name was used in this published paper, and taxonomic revision of *A. catenella* designated the non-toxic 'strain' as a different species (*A. tamarense*).

Chapter 4 The influence of the toxin-producing dinoflagellate, *Alexandrium catenella* (1119/27), on the survival and reproduction of the marine copepod, *Acartia tonsa*, during prolonged exposure

Ali H. Abdulhussain[1,2,3]*, Kathryn B. Cook[1], Andrew D. Turner[4], Adam M. Lewis[4], Thomas S. Bibby[2] and Daniel J. Mayor[1]

[1] National Oceanography Centre, Southampton, United Kingdom,
[2] Ocean and Earth Science, National Oceanography Centre, University of Southampton, Southampton, United Kingdom
[3] Department of Marine Science, College of Science, Kuwait University, Kuwait City, Kuwait
[4] Centre for Environment, Fisheries and Aquaculture Science (Cefas), Weymouth,United Kingdom

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4.1 Abstract

Copepods can feed on, and may regulate, the blooms of harmful algae (HA), and may also facilitate dinoflagellate blooms by inducing toxin production and through selective grazing. However, exposure to HA may also cause mortality and reproductive impairment in copepods, with detrimental effects at the population-scale. Here we present the toxin profile of the dinoflagellate, *Alexandrium catenella* (formerly *Alexandrium tamarense*), and examine how it affects the survival and reproduction of the cosmopolitan marine copepod, *Acartia tonsa*. Healthy adult copepods were exposed to mono-specific diets of toxic and non-toxic strains of *A. catenella* (1119/27 and 1119/19, respectively) and non-toxic *Rhodomonas sp.* for 10 days alongside unfed controls to examine how their survival was influenced by likely HA bloom conditions. Additional 2-day experiments examined how their egg production rate and hatching success were affected by food deprivation, toxic *A. catenella*, a non-toxic alternative and a mixture of toxic and non-toxic prey, at high and low concentrations. Survival of *A. tonsa* declined over the 10-day experiment in all treatments but was not significantly lower in the toxic *A. catenella* treatment; mortality was only significantly enhanced in the unfed animals, which showed 100 % mortality after 9 days. Egg production rates and hatching success from females in the unfed and toxic *A. catenella* treatments were all significantly lower than values observed in females fed *Rhodomonas* sp. or nontoxic *A. catenella*. Animals offered 1,000 μg C L^{-1} of *Rhodomonas* sp. and a 50:50

mixture of toxic *A. catenella* and *Rhodomonas* sp. produced significantly more eggs than animals fed toxic *A. catenella* alone. These results were not apparent at prey concentrations of 100 μg C L^{-1}. The percentages of eggs to successfully hatch from females offered mono specific diets of toxic *A. catenella* were always close to zero. Collectively, our results indicate that adult female *A. tonsa* can acquire sufficient energy from toxic *A. catenella* to survive, but suffer reproductive impairment when feeding on this prey alone.

4.2 Introduction

In the past five decades, harmful algal (HA) blooms, which are caused by toxic or noxious species, have increased in frequency, duration, and distribution, resulting in major environmental and economic impacts (Hallegraeff, 1993; Anderson et al., 2012). Regardless of whether or not these increases are due to changing observational frequencies, HA blooms can be toxigenic, creating blooms that cause disease and death in a number of marine species including fish, seabirds, and mammals (Hallegraeff, 1993; Anderson et al., 2012). The impacts of HA on marine life and humans due to consuming intoxicated shellfish might result in illness or mortality (Wang, 2008). However, the interactions between toxic algae and potential grazer species are complex, inconsistent, and difficult to compare between studies due to different concentrations of HA used as food, the level of toxins in the food and the various responses of the species examined.

The dinoflagellate, *Alexandrium catenella* [formerly reported in this area as *Alexandrium tamarense* Group I, reassigned taxonomically by John et al. (2014) and Fraga et al. (2015), acknowledged in Prud'homme van Reine, 2017] can be found in the United Kingdom waters and some strains (such as 1119/27) are associated with shellfish toxicity and produce paralytic shellfish poisoning toxins (PSP), while others can be found naturally non-toxic (such as 1119/19). PSP toxin derivatives are divided into three main groups: carbamate-, N- sulfocarbamoyl-, and decarbamoyl- toxins. Compared to other PSP toxins, the carbamate toxins saxitoxin (STX) and neosaxitoxin (NEO) are considered as the most potent. Gonyautoxins 1 to 4 (GTX1, GTX2, GTX3, and GTX4) are other highly potent analogs, whereas N-sulfocarbamoyl (C) toxins and some of the decarbamoyl (dc) are considered less potent (Cook et al., 2010). Saxitoxins have mainly neurological effects through the blockage of sodium channels (Luckas et al., 2015). Consumption of shellfish contaminated with PSP toxins can cause breathing difficulties, gastrointestinal problems, and a sense of dissociation followed by complete paralysis in humans and other vertebrates

(Wang, 2008). However, it remains unclear how exposure to and/or consumption of PSP toxins affect invertebrate fitness.

Zooplankton, and particularly copepods, are major grazers of phytoplankton and can influence phytoplankton dynamics (Runge, 1988; Mann, 1993; Mitra and Flynn, 2006). Copepods dominate the zooplankton communities in most marine water bodies worldwide, and play an important role in the transfer of carbon from phytoplankton to higher trophic levels such as fish and marine mammals (Mann, 1993; Dam, 2013). Toxic algae may affect the survival, feeding and fitness of copepods, and lead to lower growth and reproduction rates in their populations (Barreiro et al., 2007; Jiang et al., 2009; Waggett et al., 2012). These impacts would ultimately result in less food available to higher trophic levels, and may therefore have negative ecological effects (Dam, 2013; Turner, 2014).

In some cases, copepods are reported to show negative effects when feeding on neurotoxic dinoflagellates, with symptoms consistent with toxicity (Dutz, 1998; Xu and Kiørboe, 2018; Xu et al., 2018; Prevett et al., 2019) and decreased fitness (Cohen et al., 2007; Jiang et al., 2009). In other cases, neurotoxic dinoflagellates were consumed by copepods without obvious adverse effects (Teegarden and Cembella, 1996; Abdulhussain et al., 2020). Dinoflagellates that are not known to produce neurotoxins may also adversely affect copepod fitness, e.g., by reducing egg production and hatching success (Ianora et al., 2004). Copepods may produce detoxification enzymes to reduce the impact of toxins (Dutz, 1998; Costa et al., 2008). However, a recent gene expression study that examined the impact of the toxic dinoflagellate, *A. catenella* (reported as *Alexandrium fundyense*), on the marine copepod, *Calanus finmarchicus*, showed that detoxification is not the main component of the response (Roncalli et al., 2016). Rather, the main genes that responded were related to digestion, suggesting that toxins may reduce the absorption of food and therefore the reproductive output without an impact on survival. It is generally recognized that there is no single obvious relationship between phytoplankton neurotoxicity and effects on plankton grazers, and that experiments involving sympatric grazers and prey species are necessary to understand trophic interactions in the plankton (Smayda, 1997; Turner and Tester, 1997).

The resistance of marine copepods to HA has been well documented (Colin and Dam, 2002, 2005, 2007; Jiang et al., 2011; Zheng et al., 2011). Copepods with a history of co-occurrence with HA were found to have higher survival and ingestion rates when exposed

to HA blooms (Turner et al., 2000, 2005; Maneiro et al., 2005; Doucette et al., 2006). They may have evolved tolerance to HA species that co-occur in the same region. Some copepods have a discrete toxin-resistant phenotype that suggests a simple genetic mechanism evolving to adapt and tolerate Paralytic Shellfish Poisoning (PSP) toxins during HA blooms (Avery and Dam, 2007). For example, the fitness of *Acartia hudsonica* increased for several generations after feeding on *A. catenella* (reported as *A. fundyense*), which indicates that adaptation occurred (Finiguerra et al., 2014). Mutations of the sodium channels have been suggested as a possible mechanism (Avery and Dam, 2007; Chen et al., 2015). However, exposure of *A. hudsonica* to HA did not induce an increased proportion of mutant isoforms (Finiguerra et al., 2014), which suggests that mutation in the sodium channel was not responsible.

The majority of studies examining how copepods respond to HA exposure have been conducted over approximately 1 day (Turner, 2014). This duration is sufficient to quantify grazing and discern patterns of feeding selectivity, but may be insufficient to determine any deleterious effects of the HA on grazers since there is a considerable delay in the effect of grazers' response to toxins, and acclimatization may also take time to occur (Ianora and Miralto, 2010; Turner et al., 2012), e.g., due to the time required to produce detoxification enzymes (Kozlowsky-Suzuki et al., 2009). Short-term exposure to toxic algae might not result in significantly increased mortality compared to non-toxic algae. However, extended periods of exposure to toxic algae may cause substantial mortality within a grazers' population (Prince et al., 2006; Barreiro et al., 2007; Jiang et al., 2009; Drillet et al., 2015). Results from short-term, e.g., 24 h, experiments may therefore hinder our ability to understand how copepods are affected by exposure to natural blooms of HA, which can last from a few days up to a few months (Bresnan et al., 2008).

Here we exposed adult female *A. tonsa* to mono-specific diets of toxic and non-toxic strains of *A. catenella* and nontoxic *Rhodomonas* sp. for 10 days alongside unfed controls to examine how their survival was impacted by probable conditions experienced during a HA bloom. In addition, 2-day experiments examined how their egg production rate and hatching success were influenced by the type of food available and its concentration. Our results are presented alongside a full toxin profile for *A. catenella* in each of the experiments conducted.

4.3 Materials and methods

4.3.1 Culture condition

All phytoplankton were grown in a culture cabinet at 15 °C with a 16:8 h (Light:Dark) photoperiod, using seawater collected from the Western Channel Observatory monitoring site L4 off Plymouth, United Kingdom (https://www.westernchannelobservatory.org.uk), amended with either F/2 (*Rhodomonas* sp.) or L1 (*A. catenella*) medium (Guillard and Ryther, 1962; Guillard and Hargraves, 1993). *Acartia tonsa* were purchased from Reefshotz, Cardiff, United Kingdom, where they have been cultured for >10 years, and were subsequently cultured in artificial seawater (Tropic Marin Sea salt, Classic Meersalz; salinity 31) at room temperature (21 ± 2 °C) for >15 months prior to experimentation. Details of the culturing conditions for all phytoplankton species and for *A. tonsa* can be found elsewhere (Abdulhussain et al., 2020). The toxic *A. catenella* cultured was strain 1119/27 (CCAP, Loch Ainort, Scotland), and the non-toxic *A. catenella* was strain 1119/19 (CCAP Port Ellen, Islay, Scotland). *A. tonsa* cultures were fed *Rhodomonas* sp. (59.0±7.8 pg C cell^{-1}) before experiments 1 and 2 and *Isochrysis galbana* (25.5 ± 0.3 pg C cell^{-1}) before experiment 3 at a concentration of 60,000 cells mL^{-1} (2,000 ± 1,000 μg C L^{-1}) three times a week.

4.3.2 Influence of toxic *A. catenella*, starvation, and non-toxic prey alternatives on the survival of *A. tonsa*

Experiment 1 examined how the survival of *A. tonsa* was affected by a 10-day exposure to either food deprivation (starved) or one of three different types of food: *Rhodomonas* sp. (RHO), toxic *A. catenella* (TAC) and non-toxic *A. catenella* (NAC). Prey cells were offered at a nominal concentration of 1,000 μg carbon (C) L^{-1} to ensure that feeding conditions were always saturating (Berggreen et al., 1988; Abdulhussain et al., 2020). Individual adult females were incubated in 15 mL glass dram vials with 10 mL of sterile filtered L4 seawater (0.2 mm; SFSW hereafter) for 10 days at 19 °C with a 12:12 h (L:D) photoperiod. Each of the four treatment levels was replicated fifteen times (total = 60 bottles). Nominal prey concentrations were achieved by determining the algal stock culture concentration using a Beckman Multisizer 3 Coulter Counter equipped with a 70 mm aperture and subsequently diluting it with an appropriate volume of SFSW. Each copepod was carefully transferred into a petri dish at the end of every 24 h incubation period and examined for survival, determined by movement after mechanical stimulus. Surviving copepods were subsequently pipetted into clean 15 mL vials of fresh SFSW containing the

same species and concentration of prey cells that they had previously been exposed to. Algal concentrations in the stock cultures were counted every other day using the Coulter Counter. Triplicate 5 mL samples from the stock cultures of each of the three prey types (RHO, TAC, and NAC) were collected onto pre-combusted 13 mm glass fiber filters (Whatman GF/F) on the start of days 1 and 9 of the experiment, dried overnight at 40 °C and stored in a desiccator prior to determining their carbon and nitrogen content (Carlo Erba EA-1108 elemental analyzer). Triplicate 30 mL samples of the TAC stock culture were collected at the outset of the experiment, centrifuged at 3,000 rpm for 20 min, and the resulting pellets were transferred into 2 mL Eppendorf tubes and stored frozen at −80 °C until toxin profile analysis (see section "Toxin Analysis").

4.3.3 Influence of toxic *A. catenella*, starvation, and non-toxic prey alternatives on the egg-production and hatching success of *A. tonsa*

Experiment 2 examined how the egg production rate of *A. tonsa* was affected by different types of food: RHO, TAC, and NAC. Prey cells were offered at a nominal concentration of 1,000 μg C L^{-1}. Four adults (three females and one male) were incubated in 150 mL LPDE plastic bottles with an inner mesh compartment of 120 mm (to separate the adult from the eggs produced during the experiment) filled with 100 mL of SFSW for 2 days at 19 °C with a 12:12 h (L:D) photoperiod. Each of the four treatment levels (starved, RHO, TAC, NAC: 12 bottles) was replicated three times. The experiment was run over 2 days, with the first day being considered as an acclimation period (Breier and Buskey, 2007; Cohen et al., 2007; Jiang et al., 2009; Waggett et al., 2012; Abdulhussain et al., 2020). Hatching success was therefore only calculated for eggs produced on day 2. After the first day, the mesh compartments containing copepods were transferred to another LPDE plastic bottle with fresh SFSW containing the same species and concentration of prey cells that they had previously been exposed to. The eggs left in the LPDE plastic bottles from day 1 were counted under a stereomicroscope (to observe the response of *A. tonsa* to TAC during the acclimation period: see Appendix B). At the end of day 2, copepods in each treatment were carefully transferred into a petri dish and examined for survival (see section "Influence of Toxic *A. catenella*, Starvation, and Non-toxic Prey Alternatives on the Survival of *A. tonsa*"). Eggs produced on day 2 were counted and then transferred into petri dishes for 48 h at 19 °C with a 12:12 h (L:D) photoperiod before counting the eggs hatched for each group, and calculating percentage hatching success (equation 1):

$$\%\ \text{hatching success} = \frac{total\ number\ of\ eggs\ hatched\ from\ day\ 2}{total\ number\ of\ eggs\ produced\ on\ day\ 2} \times 100 \quad \text{Equation 1}$$

Samples to determine the toxin- and elemental-content of the algal cultures were collected on the start of day 2 (see section "Influence of Toxic *A. catenella*, Starvation, and Non-toxic Prey Alternatives on the Survival of *A. tonsa*").

4.3.4 Influence of toxic *A. catenella*, starvation, and mixed diet prey alternatives on the egg-production and hatching success of *A. tonsa* at high and low concentrations

Experiment 3 was conceptually similar to experiment 2, but examined how the egg production rate and hatching success of A. tonsa was affected by low (100 mg C L^{-1}) and high (1,000 mg C L^{-1}) concentrations of: RHO, TAC, and a diet where the carbon content was derived from 50 % RHO to 50 % TAC (MIX hereafter). Each of the seven treatment levels (starved, low RHO, high RHO, low TAC, high TAC, low MIX, and high MIX) was replicated three times (two times for low MIX; total = 20 bottles). Samples to determine the toxin- and elemental-content of the algal cultures were collected at the start of the experiment (after the acclimation period) (see section "Influence of toxic *A. catenella*, starvation, and non-toxic prey alternatives on the survival of *A. tonsa*").

4.3.5 Toxin analysis

The frozen pellets of *A. catenella* from each experiment were mixed with 1.5 mL of 1 % acetic acid before being vortex-mixed for 90 s at 2500 rpm. Solutions were subjected to probe sonication (Sonic Dismembrator, Fisher Scientific) set to 30 % power for 1.5 min per sample. After sonication, vials were re-mixed, and 400 mL taken for desalting clean-up using carbon solid phase extraction (SPE). Details of the toxin extraction, column, mobile phases, chromatographic separation, and HILIC-MS/MS analysis conditions are described elsewhere (Turner et al., 2015, 2019; Abdulhussain et al., 2020).

4.3.6 Data analyses

The effects of 10 days of exposure to food deprivation, RHO, TAC, and NAC on the survival of *A. tonsa* were examined using Kaplan Meier survival analysis:

$$S_{t+1} = S_t \times \frac{N_{t+1} - D_{t+1}}{N_{t+1}} \quad \text{Equation 2}$$

Where S is the Survival probability; t is time in days; N is the number of *A. tonsa* that survived; and D is the number of *A. tonsa* that died. Curve comparisons were achieved using the Log-rank test. Standard errors are presented to show the deviation from the population mean. The influence of feeding conditions (starved, RHO, TAC, and NAC) on the egg production of *A. tonsa* in experiment 2 was examined using 1-way ANOVA. Egg hatching success was examined using a 1-way ANOVA. The influence of food type (starved, TAC, MIX) and food concentration (100 or 1,000 μg C L^{-1}) on the egg production of *A. tonsa* in experiment 3 were examined using 2-way ANOVA. Egg hatching success was examined using a 2-way ANOVA. All explanatory variables were treated as categories. Post hoc comparisons were conducted using Tukey's test with alpha = 0.05. Standard errors are presented for the egg production and hatching experiments to show the variation within each treatment. All statistical analyses were carried out using Prism Graphpad (v.8.4) software.

4.4 Results

The carbon and nitrogen contents of the prey cells offered in the different experiments are presented in Table 4. TAC toxin profile concentrations, and their saxitoxin (STX) equivalent, are presented in Table 5.

Table 4. The concentrations of carbon (C) and nitrogen (N) (pg $cell^{-1}$) ± Standard error (SE) in the different prey cells offered to *Acartia tonsa* in the three experiments (Exp.). NA = not applicable. ESD: mean equivalent spherical diameter (μm). See Methods text for further details.

Species	**Exp.**	**Day**	**C ± SE (pg $cell^{-1}$)**	**N ± SE (pg $cell^{-1}$)**	**C:N**	**ESD (μm)**
Rhodomonas sp.	1	1	57 ± 0.03	14 ± 0.43	4.1	6-8
	1	9	63 ± 0.21	8 ± 0.06	7.9	6-8
	2	NA	67 + 0.36	19 + 0.58	3.5	6-8
	3	NA	49 ± 0.97	9 ± 0.36	5.4	6-8
Non-toxic *Alexandrium catenella-1119/19*	1	1	3552 ± 57.3	732 ± 15.5	4.9	20-40
	1	9	2980 ± 524	609 ± 84	4.9	20-40
	2	NA	1488 ± 9.0	400 ± 31.5	3.7	20-40
Alexandrium catenella - 1119/27	1	1	4950 ± 30	1100 ± 53.3	4.5	20-40
	1	9	3336 ± 168	1042 ± 50.9	3.2	20-40
	2	NA	4640 ± 30	1070 ± 32.5	4.3	20-40
	3	NA	2103 ± 34.21	410 ± 19.53	5.1	20-40

Table 5. *Alexandrium catenella* (1119/27-Loch Ainort) toxin profile concentrations (fmol cell^{-1}). Cell toxicity of saxitoxin equivalent concentrations are calculated in (fg STX eq. cell^{-1}). A: toxin profile concentrations for experiment 1 and 2. B: toxin profile concentrations for experiment 3. Saxitoxin (STX), neosaxitoxin (NEO), Gonyautoxins 1 to 4 (GTX1, GTX2, GTX3, GTX4), N-sulfocarbomoyl (C1, C2, C3, C4) toxins, decarbomoyl (dc) toxins.

A. Toxin Concentration, fmol cell^{-1}									
C1	**C2**	**C3**	**C4**	**dcGTX2**	**dcGTX3**	**dcGTX1**	**dcGTX4**	**GTX6**	**dcNEO**
0.35	1.08	nd	nd	nd	nd	nd	nd	nd	nd
GTX2	**GTX3**	**GTX1**	**GTX4**	**GTX5**	**doSTX**	**dcSTX**	**STX**	**NEO**	**STX eq cell^{-1} (fg STX eq cell^{-1})**
0.76	0.55	nd	0.41	0.18	nd	nd	0.26	nd	501.1

B. Toxin Concentration, fmol cell^{-1}									
C1	**C2**	**C3**	**C4**	**dcGTX2**	**dcGTX3**	**dcGTX1**	**dcGTX4**	**GTX6**	**dcNEO**
0.27	3.06	nd	0.15	nd	nd	nd	nd	nd	nd
GTX2	**GTX3**	**GTX1**	**GTX4**	**GTX5**	**doSTX**	**dcSTX**	**STX**	**NEO**	**STX eq cell^{-1} (fg STX eq cell^{-1})**
0.43	1.49	nd	2.06	0.15	nd	nd	3.74	3.36	3575.5

4.4.1 Influence of Toxic *A. catenella*, starvation, and non-toxic prey alternatives on the survival of *A. tonsa*

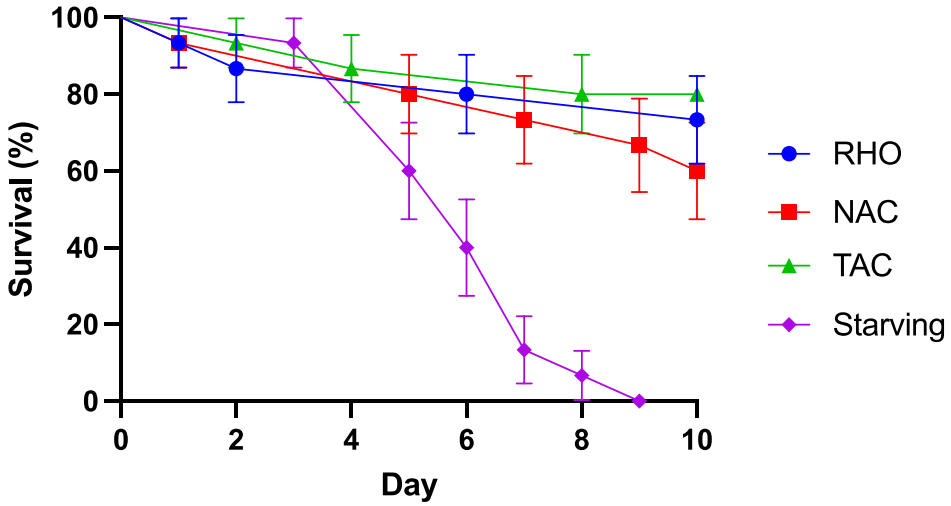

Figure 10. *Acartia tonsa* survival when exposed to RHO (*Rhodomonas* sp.; blue circles), TAC (toxic *Alexandrium catenella* 1119/27; green triangles), and NAC (non-toxic *A. catenella* 1119/19; red squares) at a concentration of 1,000 µg C L^{-1} or when starved (purple diamond). Dots (N=15) are mean value, and error bars show the standard error (SE).

The survival of female *A. tonsa* declined significantly across the 10-day duration of the experiment (Fig. 10; Log-rank test, Chi square = 29, df = 3, $p < 0.001$). However, survival was only significantly reduced in the starving group, where there was 100 % mortality by day 9 (Fig. 10; Log-rank test, Chi square = 29, df = 3, $p < 0.001$). Survival of the females offered RHO (73 %), TAC (80 %), and NAC (60 %) at nominal concentrations of 1,000 µg C L^{-1} were statistically indistinguishable, with an average survival of 71 ± 10 % across these treatments (Fig. 10; Log-rank test, Chi square = 1, df = 2, p = 0.525).

4.4.2 Influence of toxic *A. catenella*, Starvation, and non-toxic prey alternatives on the egg production and hatching success of *A. tonsa*

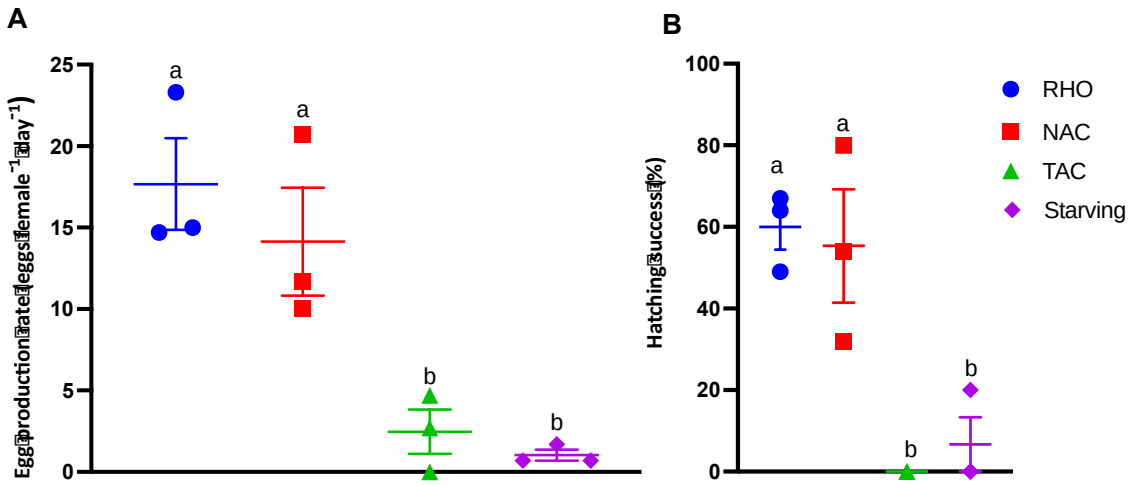

Figure 11. Egg production and hatching success of *Acartia tonsa* fed on RHO (*Rhodomonas* sp.; blue circles), TAC (toxic *Alexandrium catenella* 1119/27; green triangles), and NAC (non-toxic *A. catenella* 1119/19; red squares) at a concentration of 1,000 µg C L^{-1} or when starved (purple diamond). The dots are replicates and bars show the mean ± standard error (SE). **A**: Eggs produced (24-48 h) after acclimation period. **B**: Nauplii hatched (48-96 h). The a and b letters above bars show the post-hoc comparisons between the treatments with shared letters denoting no significant difference.

The egg production rate of *A. tonsa* was affected by the type of food they received (Figure 11A: 1-way ANOVA, F (3,8) = 13.24, $p \leq 0.0115$). The females offered RHO and NAC produced significantly more eggs (17.7 ± 4.9 and 14.1 ± 5.7 eggs $female^{-1}$ day^{-1}, respectively) than those offered TAC (2.4 ± 2.3 eggs $female^{-1}$ day^{-1}) and starved (1.0 ± 0.6 eggs $female^{-1}$ day^{-1}) (Figure 11A). The hatching success of eggs produced on day 2 was influenced by the maternal feeding conditions (1-way ANOVA, F (3,7) = 11.53, $p = 0.0042$). Hatching success of the eggs from females that had received RHO (60 ± 9.7 %) and NAC (56± 23.9 %) was higher than the eggs from females fed TAC (0 %) or starved (7 ±11.5 %) (Figure 11B).

4.4.3 Influence of toxic *A. catenella*, starvation, and a mixed diet on the egg production and hatching success of *A. tonsa* at high and low food concentrations

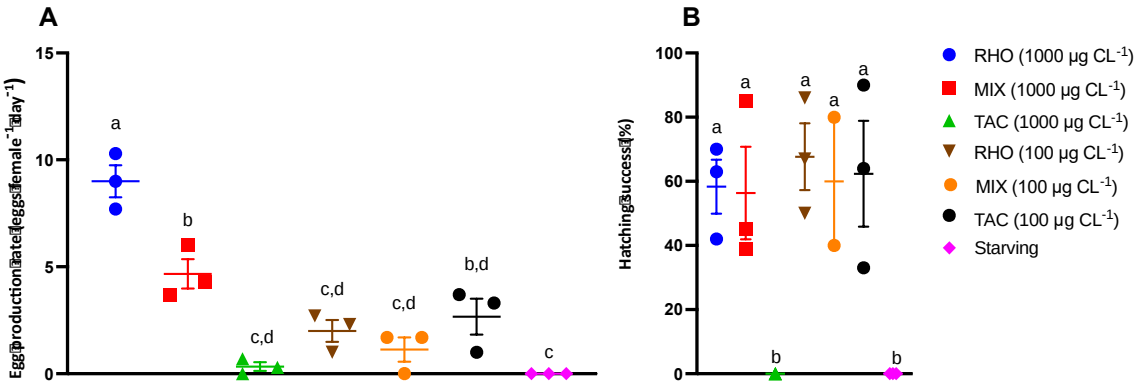

Figure 12. Egg production and hatching success of *Acartia tonsa* fed on RHO (*Rhodomonas* sp.; brown inverted triangles and blue circles, 100 & 1,000 µg C L^{-1}, respectively), TAC (toxic *Alexandrium catenella* 1119/27; black circles and green triangles, 100 & 1,000 µg C L^{-1}, respectively), and MIX (50:50 mixture of both cell types; orange circles and red squares, 100 & 1,000 µg C L^{-1}, respectively) at two concentrations: 100 & 1,000 µg C L^{-1} or when starved (purple diamond). The dots are replicates, and bars show the mean ± standard error (SE). **A**: Eggs produced (24-48 h) after acclimation period. **B**: Nauplii hatched (48-96 h). The a and b letters above bars show the post-hoc comparisons between the treatments with shared letters denoting no significant difference.

The egg production rate of *A. tonsa* was affected by food type and food quantity (Fig. 12 A, 2-way ANOVA, food type: F (3,15) = 40.0; food quantity F (1,15) = 25.0, $p \leq 0.002$ in both cases). Copepods that were fed on MIX at 1,000 µg C L^{-1} produced significantly fewer eggs (4.7 ± 1.2 eggs $female^{-1}$ day^{-1}) than in the RHO treatment at the same concentration (9.0 ± 1.3 eggs $female^{-1}$ day^{-1}) but significantly more than the females that received TAC (0.3 ± 0.3 eggs $female^{-1}$ day^{-1}) (Fig. 12A). Egg production rates in the 100 µg C L^{1} treatments were all similarly low (Fig. 12A). The hatching success of the eggs produced was influenced by the food type and food quantity (Fig. 12B, 2-way ANOVA, F (3,15) = 15.39 and 6.32, respectively, $p \leq 0.0238$ in both cases). The hatching success of eggs from females that were fed 1,000 µg C L^{-1} of RHO (58 ±14 %) and MIX (56 ± 25 %) were both higher than TAC (0 %) and starved (0 %) (Fig. 12B), whereas the hatching success of eggs from females fed at 100 µg C L^{-1} of RHO (67 ± 18 %), MIX (60 ± 28 %) and TAC (62 ± 28 %) were all similar.

4.5 Discussion

4.5.1 *A. catenella* toxin composition and concentrations

The elemental content, and composition and concentrations of toxins, of *A. catenella* (1119/27) were all variable (Tables 4, 5), despite being grown under near identical conditions. For example, the total toxin concentration was 501.1 STX eq $cell^{-1}$ for experiments 1 and 2, and 3575.5 STX eq $cell^{-1}$ for experiment 3. Some of the factors that can change the toxin levels within a HA bloom are turbulence (Juhl et al., 2001), salinity (Grzebyk et al., 2003), and nutrient conditions (Turner et al., 1998; John and Flynn, 2002; Leong et al., 2004). Toxin levels can also vary with growth phase in cultured algae (Hamasaki et al., 2001; Cook et al., 2010). We suggest that the differences in toxicity in our laboratory-based study were caused by differences in the nutrient concentrations of the seawater that was used for culturing TAC: Experiment 1 and 2 were conducted during January 2020, and experiment 3 during July 2019, when surface nitrate and phosphate concentrations were close to their highest and lowest, respectively (https://www.westernchannelobservatory.org.uk/l4_nutrients.php). Moreover, in response to nutrient stress and chemical signals from copepods, HA can dramatically increase the development of PSP toxins (Wohlrab et al., 2010; Selander et al., 2015; Griffin et al., 2019). A group of predator cues called copepodamides (also known as taurine-containing lipids; Mayor et al., 2015), which are exuded by three species of calanoid copepods (*Centropages typicus*, *Pseudocalanus* sp., and *Calanus* sp.) have been found to trigger increased PSP toxins in dinoflagellates, as well as amnesic shellfish poisoning (ASP) toxins in diatoms (Selander et al., 2019). It is therefore possible that the toxin content of the prey cells increased during our incubations and the concentrations presented in Table 5 should be considered minimal. These differences highlight the importance of reporting toxin profile concentrations, particularly in the context of copepod survival, grazing, and egg production studies. This makes it easier to robustly compare between different studies.

4.5.2 Survival of *A. tonsa* exposed to toxic *A. catenella*

Exposure of *A. tonsa* to a toxic strain of TAC for 10 days did not significantly affect their survival relative to the NAC treatment. Rather, increased mortality was only observed in the starved treatment (Fig. 10). These findings suggest that *A. tonsa* is able to feed and survive on TAC over the likely duration of a typical bloom of TAC (Bresnan et al., 2008), regardless of their toxicity. Previous 24 h experiments showed that *A. tonsa* can feed upon

TAC and survive blooms two orders of magnitude higher than the maximum densities of *A. catenella* recorded in natural systems (Abdulhussain et al., 2020), possibly by producing detoxification enzymes (Dutz, 1998; Costa et al., 2008) or by reducing food absorption efficiency (Asselman et al., 2012; Roncalli et al., 2016). Bloom concentrations of TAC are generally low and typically last for only a few days (Bresnan et al., 2005), although more persistent blooms are reported (Bresnan et al., 2008). Our findings therefore support the understanding that *Acartia* sp. tolerate exposure to TAC for the typical duration of naturally occurring blooms without negative impacts on the survival of adults (Liu and Wang, 2002; Estrada et al., 2008). However, exposure to high densities of HA for an extended period may cause substantial mortality within *Acartia* spp. populations (Prince et al., 2006; Barreiro et al., 2007; Jiang et al., 2009). Thus, the response of *Acartia* spp. appears to vary between different studies, possibly because of different toxin concentrations (section "*A. catenella* Toxin Composition and Concentrations"). Higher survival rates of *A. tonsa* in this study compared to other survival experiments could also be a result of reduced encounter rates between individuals as each experimental vial contained only one female per treatment replicate. However, in denser cultures, elevated encounter rates between individuals may increase levels of stress and cannibalism, potentially resulting in higher rates of mortality (Drillet et al., 2015).

4.5.3 Egg production and hatching success of *A. tonsa* exposed to toxic *A. catenella*

The average egg production rate of *A. tonsa* feeding on RHO in our 2-day exposure experiments, ~17 eggs female^{-1} day^{-1} (Fig. 11), agrees with values previously reported for the genus *Acartia* sp. (range = 9–76 eggs female^{-1} day^{-1}; Prince et al., 2006; Barreiro et al., 2007; Breier and Buskey, 2007; Jiang et al., 2009; Waggett et al., 2012). Our results reveal that egg production and hatching success were affected by the types of food available and the concentration at which it was offered (Figs. 11, 12), with the observed values being significantly lower in the TAC treatments relative to those in the NAC and RHO treatments (Figure 11). Egg production rates by females fed RHO, NAC and MIX (50 % RHO, 50 % TAC) at 1,000 μg C L^{-1} were also significantly higher than those fed TAC alone. By contrast, the rates of females fed TAC were not statistically different to those of the starved animals (Figs. 11, 12). Furthermore, the percentages of eggs to successfully hatch from females offered mono-specific diets of toxic *A. catenella* at concentration of 1,000 μg C L^{-1} were always close to zero. These results are consistent with the observation that reproduction in

Acartia clausi is negatively affected when consuming toxic *Alexandrium minutum*, and that these effects can be alleviated by offering mixed diets (Barreiro et al., 2006, 2007).

Acartia tonsa can discriminate between different species of *Alexandrium* with different toxin contents, showing preference for the least toxic species (Xu et al., 2018; Abdulhussain et al., 2020). Copepods are thought to be capable of rejecting toxic prey cells (Cook et al., 2010; Abdulhussain et al., 2020) or consume them at lower rates than a similarly sized non-toxic cells (Xu and Kiørboe, 2018). It is therefore possible that the reduced egg production rate in the TAC treatment could be caused by starvation. However, we know that *A. tonsa* is capable of surviving and feeding on TAC when alternative prey are not available (Abdulhussain et al., 2020). This understanding is further supported by the current study, where survival of *A. tonsa* in TAC treatment was significantly higher than in the starved treatment (section "Survival of *A. tonsa* exposed to toxic *A. catenella*"). The significantly lower egg production rates observed in the TAC treatments of both experiments is therefore unlikely attributable to a starvation effect. The negative effects of TAC on the observed egg production rates and hatching success may have occurred because (a) the females were directly impeded by the toxins, (b) the females needed to produce detoxification enzymes, and therefore had to channel energy and other resources away from producing viable eggs, (c) the presence of toxins reduced the efficiency with which food is absorbed, and therefore reduced the amount of energy and other resources available for producing viable eggs, or (d) TAC were in some way nutritionally inferior to the other diets offered.

Nutritionally deficient food and the presence of toxins have both been suggested to cause reduced egg production in *Acartia* sp. (Dutz, 1998; Collumb and Buskey, 2004; Prince et al., 2006; Costa et al., 2008). The survival of females exposed to TAC was not significantly lower than females exposed to the non-toxic prey treatments, apparently suggesting that TAC toxins do not necessitate additional metabolic costs associated with the production of detoxification enzymes to the extent that they have a discernible, negative effect on the overall physiological performance of *A. tonsa*. However, copepods may increase their ingestion rates when feeding on toxic dinoflagellates in order to compensate for the increased energy required for detoxification (Dutz, 1998; Costa et al., 2008) or reduce the efficiency with which they absorb food (Asselman et al., 2012; Roncalli et al., 2016). For example, ingestion rates of *A. tonsa* were higher when fed on toxic *A. catenella* relative to animals fed on non-toxic *Rhodomonas* sp. at a food saturating concentration

of 1,000 μg C L^{-1} (Abdulhussain et al., 2020). Compensatory feeding may also be necessary if the nutritional value of HA is lower than other non-toxic food (Collumb and Buskey, 2004; Prince et al., 2006). Our survival experiments were conducted at a food saturating concentration of 1000 μg C L^{-1} (Berggreen et al., 1988; Abdulhussain et al., 2020) and we cannot therefore exclude the possibility that the females increased their ingestion rates to offset any additional metabolic costs. Nevertheless, even if compensatory feeding did occur, it was clearly insufficient to mitigate the negative effects on reproduction associated with ingesting TAC. Egg production rates of females fed NAC, which we assume to have a highly similar nutritional make up to TAC, were indistinguishable from those fed on RHO, a food source that is widely assumed to support optimal reproductive output in *Acartia* spp. (Støttrup et al., 1986; Berggreen et al., 1988). We therefore suggest that the reduced rates observed in the TAC treatment (Fig. 11) were not attributable to the absence of nutritive compounds; they occurred because of the presence of toxins, although the specific mechanism cannot be elucidated here.

The negative effects of consuming TAC were less apparent when prey cells were offered at 100 μg C L^{-1}, where egg production rates were similarly low across all treatments (Fig. 12A). This agrees with the understanding that lower food concentrations limit growth, reproduction, and survival (Berggreen et al., 1988). Interestingly, the hatching success of the few eggs produced by females fed TAC at 100 μg C $L^{-1}$was similarly high when compared to the values observed in the other, non-toxic treatments. This may suggest that there is a toxin-threshold level, beyond which eggs do not hatch.

Prey cell size can influence the outcome of copepod feeding experiments. However, in our study, differences between Rhodomonas (6–8 mm ESD) and I. galbana (4–6 mm ESD) were trivial, suggesting that these are unlikely to cause differences in the feeding rates of our experimental animals. Indeed, previous work has shown that female *A. tonsa* have an optimal prey size of 14.8 mm ESD, but are able to achieve high clearance rates when feeding on both *Rhodomonas* and on the dinoflagellate *A. catenella* (ESD = 20–40 mm) (Berggreen et al., 1988; Abdulhussain et al., 2020). Moreover, despite differences in the feeding histories of the animals in experiments 2 and 3, the outcomes of these two experiments were qualitatively and quantitatively the same: EPR and hatching success in the TAC and starved treatments in both experiments were similarly low (not different to zero); EPR and hatching success in the RHO treatments in both experiments were always higher than in the TAC and starved treatments (Figs. 11, 12); ERP in the RHO treatments were not statistically different between

the two experiments (Paired t-tests: t = 3.965, df = 2, p = 0.058). These results would not have occurred if any feeding history effects had not been accounted for within the 24 h acclimation period.

Finally, in the real environment, there are usually more than one species of algae available for copepods to feed on. The availability of alternative prey suggests that the impact of HA on copepod egg production may be very low due to availability of other algae. Nevertheless, it is clear that further work is required to understand the energetic and nutritional requirements of *A. tonsa* and their developing embryos, and how these are directly and indirectly affected when ingesting toxin-producing prey species.

4.6 Summary

The survival of *A. tonsa* exposed to monospecific diets of either toxic and non-toxic *A. catenella* or *Rhodomonas* sp. declined over a 10-day incubation experiment. However, mortality only increased significantly in unfed copepods, which showed 100 % mortality after 9 days. Egg production and hatching success rates of *A. tonsa* exposed to 1,000 g C L^{-1} toxic *A. catenella* were not significantly different from starved animals, but were significantly lower than animals fed 1,000 µg C L^{-1} of non-toxic *A. catenella*, *Rhodomonas* sp., or a 50:50 mix of toxic *A. catenella* and *Rhodomonas* sp. Egg production and hatching success rates of eggs produced by females that were fed either *Rhodomonas* sp., toxic *A. catenella* or a 50:50 mix of toxic *A. catenella* and *Rhodomonas* sp. at 100 µg C L^{-1} were not significantly different. Further studies are required to understand the physiological basis of why female *A. tonsa* can feed and survive on toxic *A. catenella*, but display reproductive impairment as a result

Chapter 5

The influence of the toxin-producing dinoflagellate, *Alexandrium catenell*a (1119/28) on the feeding and reproduction of *Calanus helgolandicus*

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The raw data underpinning this Chapter were generated by KC, EB & JL and provided to me to mitigate the effects of laboratory closure throughout 2020 due to periods of national lockdown during the global pandemic.

AA analysed the data, created the figures and wrote the manuscript. KC and EB designed and conducted the experiments. Toxin extraction and analyses were conducted by JL. All authors contributed to manuscript revision, read, and approved the submitted version.

Chapter 5 The influence of the toxin-producing dinoflagellate, *Alexandrium catenella* (1119/28) on the feeding and reproduction of *Calanus helgolandicus*

Ali H. Abdulhussain [1,2,3], Kathryn B. Cook [1], Eileen Bresnan [4], Jean-Pierre Lacaze [4], Daniel J. Mayor [1]

[1] *National Oceanography Centre, Southampton, United Kingdom*
[2] *Ocean and Earth Science, University of Southampton, National Oceanography Centre, Southampton, United Kingdom.*
[3] *Department of Marine Science, College of Science, Kuwait University, Kuwait City, Kuwait.*
[4] *Marine Scotland Science, Marine Laboratory, Aberdeen, United Kingdom*

Keywords: Phytoplankton, Harmful algal bloom, Copepod feeding, Egg production, Paralytic shellfish poisoning, Saxitoxin.

5.1 Abstract

Copepods of the genus *Calanus* dominate the biomass of pelagic ecosystems from the Mediterranean Sea up into the Arctic Ocean and form an important link between phytoplankton and higher trophic levels. Impacts from toxin-producing harmful algae (HA) have been recorded throughout this region over the last 50 years, with potentially negative effects on *Calanus* spp. populations and the ecosystem functions and services they provide. Here we examine how ingestion, egg-production and egg-viability in *Calanus helgolandicus* are affected by the relative abundance of the toxin-producing dinoflagellate, *Alexandrium catenella*, in their diet. Our four-day experiments demonstrate that the ingestion rate of *C. helgolandicus* declined significantly as the percentage of toxin-producing *A. catenella* within their diet increased, whereas egg production and egg viability were unaffected. Toxin profile concentrations for *A. catenella* are presented alongside body toxin-loads in *C. helgolandicus* after 4 days of feeding on these cells. The body toxin concentrations of *C. helgolandicus* was 3.6-356.6 pg STX eq. copepod^{-1}, approximately 0.02–3.3 % of the toxins ingested. Our work suggests that the effects of exposure to *A. catenella* may be negligible in the short-term but could manifest if bloom conditions persist for longer than our experimental duration.

5.2 Introduction

Copepods of the genus *Calanus* are one of the most common calanoid copepods in the North Atlantic, with populations ranging from the mid-Atlantic Shelf off the east coast of the United States, the Mediterranean Sea, and up into to the Barents Sea north of Norway (Conover, 1988, Planque et

al., 1997; Bonnet et al., 2005). *Calanus* is an important food source for many commercially important fish and also seabirds in the North Atlantic (Gaard & Reinert, 2002; Gislason & Astthorsson, 2002; Ringuette et al., 2002; Beaugrand et al., 2003; Steen et al., 2007; Wold et al., 2011). The warmer-water species, *Calanus helgolandicus*, is widely distributed throughout the Eastern North Atlantic, with particularly high abundances in the Western European Shelf region (Barnard et al., 2004; Bonnet et al., 2005; Choquet et al., 2017). The abundance and geographical distribution of *C. helgolandicus* in the Atlantic are positively related to temperature (Bonnet et al., 2005). Indeed, *C. helgolandicus* and other species associated with warmer water have shifted northwards by ten degrees of latitude over the last 7 decades with *C. helgolandicus* replacing the boreal species, *Calanus finmarchicus*, in regions of warming (Beaugrand et al., 2002; Choquet et al., 2017). Changing the geographical distribution and abundance may result in a possible mismatch between copepods and their phytoplankton prey, with wider consequences for ecosystem functioning (Edwards & Richardson, 2004). However, although there is a strong link between *C. helgolandicus* distribution and temperature, other factors relating to environmental change may also affect their ability to survive (e.g. Cook et al., 2007; Mayor et al., 2012).

Of the ~5000 species of extant marine phytoplankton, ~40 are able to produce potent toxins that can reach humans through fish and shellfish (Hallegraeff, 1993; Hallegraeff et al., 2021). Blooms of these harmful algae (HA) are a globally recurring issue, the distribution and frequency of which appear to have changed in recent years (Hallegraeff, 1993; Edwards et al., 2006; Anderson et al., 2012). The apparent increase in the impacts from HA blooms can be attributed to increasing monitoring programs (Bresnan et al., 2021; Hallegraeff et al., 2021), but global climate change and anthropogenic pollution may also be contributing to the observed changes (Edwards et al., 2006; Hallegraeff et al., 2021; Nohe et al., 2020; Marampouti et al., 2021; Søgaard et al., 2021). Dinoflagellates are responsible for the majority of HA blooms (Sopanen et al., 2011) and are often associated with major environmental and economic issues (Hallegraeff, 1993; Anderson et al., 2012; Hallegraeff et al., 2021), causing disease and death in a variety of marine animals, including fish, seabirds, and mammals (Wang, 2008; Jensen et al., 2015; Kershaw et al., 2021).

Toxic algae can affect copepod survival, feeding, and reproduction by decreasing ingestion, growth and egg production rates in their populations (Colin & Dam 2003; Barreiro et al., 2007; Jiang et al., 2009; Abdulhussain et al., 2020, 2021). The presence of algal toxins may cause copepods to reject prey cells (Teegarden, 1999), or, once ingested, result in physical incapacitation that inhibits feeding (Sopanen et al., 2011). The paralytic shellfish toxin (PST)-producing dinoflagellate,

Alexandrium spp., has been observed to adversely affect the feeding (Turriff et al., 1995; Campbell et al., 2004) and fitness (Roncalli et al., 2016) of *C. finmarchicus*. However, other studies have shown that *C. finmarchicus* consumed neurotoxic dinoflagellates and diatoms without apparent negative effects (Turner & Borkman 2005; Leandro et al., 2010) and *C. helgolandicus* is also reported to feed on the okadaic acid-producing dinoflagellate, *Dinophysis* spp., with no apparent impact on their ingestion (Wexels Riser et al., 2003). Copepods may accumulate PSTs when feeding on *Alexandrium* spp. (Teegarden & Cembella, 1996; Teegarden et al., 2003; Campbell et al., 2004) and transfer these to higher trophic levels, including marine mammals (Durbin et al., 2002; Doucette et al., 2006). However, most observations show that the tissues of copepods retain only a small fraction of the ingested toxins after feeding. *C. finmarchicus* fed on *Alexandrium* spp. in the field and laboratory accumulated toxins at a rate of 0.41-0.89 ng STX eq. copepod day^{-1} with a retention efficiency of 1 – 2 % of the total toxin ingested (Campbell et al., 2004). *Acartia hudsonica* fed *Alexandrium fundyense* at ~ 3000 cells mL^{-1} accumulated toxins up to 54 μg STX eq g^{-1} of wet weight within only 6 h of exposure, equating to ~ 10 % of the total toxin ingested (White, 1981). In addition, the toxin retention efficiencies of two copepod species, *Acartia tonsa* and *Eurytemora herdmani*, were typically < 5 % of ingested *Alexandrium* spp. toxins (Teegarden and Cembella, 1996). These low retention efficiencies suggest that toxins are either transformed and excreted as other compounds and/or are directly eliminated in dissolved form, perhaps by regurgitation (Guisande et al., 2002; Teegarden et al., 2003) or excretion.

To date, the majority of studies examining how *Calanus* spp. respond to PST-producing HA have been conducted over ~ 24 h (Hassett, 2003, Teegarden et al., 2001, Turner, 2010, Turner & Borkman, 2005). This duration is sufficient to understand the immediate patterns of prey selectivity, but may not be sufficient to determine the effects of HA on reproduction as *Calanus* spp. may take > 24 h to convert ingested food into eggs (Hirche et al., 1997), and may also produce eggs from maternal biomass when feeding conditions are poor (e.g. Niehoff et al., 1999; Mayor et al., 2009). Here, we used 4-day incubation experiments to examine how feeding, egg production and egg viability in *C. helgolandicus* were affected by the relative abundances of the toxic dinoflagellate, *A. catenella* (1119/28), and the non-toxic species, *A. tamarense* (1119/33), in their diet. *A. catenella* [formerly *Alexandrium tamarense*, North American strain (Scholin et al.,1994) Group I (Lilly et al., (2007) reassigned taxonomically (John et al., 2014; Fraga et al., 2015), acknowledged in Prud'homme van Reine, (2017)] is widely distributed (Hallegraeff, 1993; Brown et al., 2010; Anderson et al., 2012) and reported as a nuisance species in Scotland, Iceland, the Faroe Islands and Norway. It can reach cell densities of between 1000 and 2000 cells L^{-1} and result in levels of PSTs in shellfish flesh that

exceed the EU regulatory limit of 800 µg STXeq/KG for several weeks resulting in closures of shellfish harvesting areas in Northern Europe (Bresnan et al., 2005; 2008; Brown et al., 2010). Our results are presented alongside a full toxin profile for *A. catenella*, as well as body toxin profiles for *C. helgolandicus* at the end of our experiments.

5.3 Materials and methods

5.3.1 Collection and culture conditions

Adult female *C. helgolandicus* were collected from the Scottish Coastal Observatory site at Stonehaven in the North West North Sea (56° 57.8N 02° 06.2W) using a 1m ring net fitted with a 350 µm mesh and a non-filtering cod-end. Upon collection, copepods were diluted with fresh seawater and transported to the laboratory within 4 h. The copepods were maintained in 10 L tanks with a 12 h photoperiod and fed Phyto Feast® Live (a mix of *Tetraselmis, Isochrysis*, *Pavlova*, *Nannochloropsis*, *Thalassiosira*, *Amphora* and *Synechoccus*) and Roti-Feast® (*Brachionus plicatilis* animals and eggs) produced by Reed Mariculture. All experimental work was conducted in a controlled temperature room at 13 °C.

5.3.2 Collection and culture of organisms

The dinoflagellate, *Alexandrium catenella*, was isolated from Scapa, Orkney, UK, between 2007-2008. The two species examined were toxic *A. catenella* (Acat: W08/056/01; 1119/28) and non-toxic *A. tamarense* (Atam: W07/069/01; 1119/33). All phytoplankton were grown in a temperature-controlled room at 15 °C with 24 h photoperiod, using seawater from Stonehaven, UK which was filtered (4.7 mm Whatman GFF filters) and amended with L1 medium.

5.3.3 Examining how the relative abundances of toxic and non-toxic *Alexandrium* spp. affect feeding, egg production and egg viability in *C. helgolandicus*

Food removal experiments (Båmstedt et al., 2000) were used to examine how, for a given total quantity of food, ingestion rates for *C. helgolandicus* changed in response to changes in the relative abundances of Acat and Atam. Adult female *C. helgolandicus* were carefully transferred via pipette into a 10 L bucket of 0.2 µm filtered seawater (FSW hereafter) and incubated for 24 h to clear their guts. Experiments were conducted in 100 mL beakers, each containing a total of 400 µg C L^{-1} of algae (see Section 2.4) to ensure that feeding conditions were always saturating. The five experimental treatment levels contained 0 %, 25 % 50 %, 75 % and 100 % of Acat-derived carbon, with the

remainder being provided via Atam. These concentrations were achieved by determining the cell concentrations of the *A. catenella* stock cultures, adding the required amounts of Acat and Atam to the incubation bottles, and subsequently topping them up with an appropriate volume of FSW. Nine bottles at each of the five treatments level were initially set up (total n = 45), with 50 mL samples from each of three bottles from each treatment level being immediately preserved with acidified Lugol's iodine to enumerate the number of cells at the start of the experiment. Three female *C. helgolandicus* were added to each of three grazing bottles at each treatment level and incubated alongside triplicate control bottles at each treatment level for four consecutive 24 h periods. At the end of each 24 h period, the copepods were removed from the grazing bottles using a 200 µm mesh and transferred into FSW. The number of motile copepods observed after mechanical stimulus was recorded before they were transferred into new beakers containing fresh medium at the experimental conditions they had previously experienced. The eggs left in the beakers were collected with a 63 µm mesh, washed with FSW and counted using a binocular microscope (Wild M3) before determining their viability (Section 2.4). Ingestion was determined at the end of days 1 and 3 by preserving 50 mL water samples from each of the grazing and control bottles with acidified Lugol's iodine. At the end of day 4, the experimental copepods from each treatment level were grouped together, transferred into single 1.5 mL Eppendorf tubes and frozen at -80 °C for toxin content analysis (Section 2.4).

5.3.4 Analytical methods

The carbon contents and toxin concentration profiles were determined from the stock cultures of Acat and Atam prior to their exposure to copepod grazing. The density of cells in each culture was counted from a 1 mL subsample using a Sedgewick rafter cell and a ZEISS X200 inverted microscope. The average volume of a cell in each culture was calculated using the diameter of 30 cells, measured using a calibrated eyepiece graticule, and the equation for calculating the volume of a sphere. Carbon content of an average cell was calculated using the C:volume relationship for protist plankton excluding diatoms (Menden-deuer & Lessard, 2000; $pgC\ cell^{-1} = 0.216 \times volume^{0.939}$). Cells in experimental bottles with acidified Lugol's iodine were counted from a 50 mL subsample settled for 48 h in a Utermöhl chamber. The copepod daily clearance and ingestion rates were calculated using established equations (Frost, 1972) and expressed as mL $copd^{-1}\ day^{-1}$ and µg C $copepod^{-1}\ day^{-1}$, respectively.

Egg viability was examined using SYTOX® Green (Buttino et al., 2004); live cells are impermeant to this stain, making it a useful indicator of dead cells and hence eggs that will not hatch.

In brief, the eggs were incubated for 50 minutes in chitinase solution (final concentration 1 mg mL^{-1} in FSW) at room temperature and subsequently stained using SYTOX® Green nucleic acid stain (final concentration 20 µM in DMSO) for 50 minutes in the dark at room temperature. The number of fluorescent eggs was counted using a Zeiss Axiovert 200 inverted fluorescence microscope and used to calculate the percentage of viable eggs likely to hatch.

The samples of *C. helgolandicus* and the Acat and Atam cultures that were collected during the feeding experiment were analysed for PSTs using the PCOX method (Van de Riet et al., 2011). The Acat and Atam culture samples (Table 6) were centrifuged at 3,000 rpm for 20 minutes (multiple stages) to form pellets of 250,000 cells in 2 mL Eppendorf tubes. The supernatants were removed using a pipette and the pellets were then stored at -20 ºC until extraction. Glass beads (180 µm, 100 ± 20 mg) were acid-washed and added to the Eppendorf tubes. The extraction solvent (0.5 M acetic acid, 100 µL) was then added with a calibrated pipette. The cells were extracted for two minutes at 25 Hz using a TissueLyser 2. Following microscopic confirmation that the cells had ruptured, they were centrifuged at 14,000 rpm for 5 minutes. The supernatants were transferred to 0.2 µm Ultrafree-MC centrifugal filters using a pipette fitted with a long tip and this was followed by centrifugation for five minutes at 10,000 rpm. The *C. helgolandicus* samples (6 – 9 individuals per sample: Table 7) were extracted using the same techniques. All extracted filtrates were transferred to pre-insert amber vials and were immediately analysed by HPLC following the PCOX method (Van de Riet et al., 2011). This method provides a very low limit of detection for the N-sulphocarbomoyl I toxins, whereas the limits of detection for the other PSTs are ~ 10 – 80 fold higher.

5.3.5 Data analyses

The gross retention efficiency of toxins was calculated as:

$$\text{Retention efficiency (\%)} = \frac{\text{Body toxin concentration (pg STX diHCl eq.)}}{\text{Total toxicity ingested (pg STX diHCl eq.)}} * 100 \qquad \text{Equation 1}$$

This assumes that the diet is proportionally consistent with the food available. The influence of the relative abundance of Acat and Atam and sampling day on rates of 1) clearance, 2) ingestion, 3) egg production and 4) egg viability in *C. helgolandicus* were all examined using analysis of variance (ANOVA) using backwards selection. The treatment levels '% Acat' and 'Day' were treated as continuous and categorical variables, respectively. All statistical analyses were carried out using the software Prism Graphpad (v.9.2).

5.4 Results

5.4.1 *A. catenella* toxin analyses and body toxin concentrations of *C. helgolandicus*

Toxin profile concentrations of Acat, Atam, and *C. helgolandicus* are presented in Tables 6 & 7, respectively. STX, NEO, GTX3 and C2 were the four main toxins present. No PSTs were detected in the Atam culture used in this experiment (Table 6). The total toxicity retained in *C. helgolandicus* ranged between 3.6-356.6 pg STX diHCl eq. copepod^{-1}. Only NEO (sample day 4: 25% Acat), C1 (samples day 4: 50 and 75 % Acat) and C2 (all 4 samples) toxins were detected in the copepods fed on mixtures of Acat and Atam, demonstrating that the copepods had ingested the toxic *Alexandrium* cells.

Table 6. Paralytic Shellfish Toxins content of the *Alexandrium catenella* (Acat) and *Alexandrium tamarense* (Atam) cultures used during the feeding and egg production experiment.

Sample type	Time of Analysis (d)	PST (fg STX diHCl eq. cell^{-1})												
		GTX1	GTX2	GTX3	GTX4	GTX5	dcGTX 2	dcGTX 3	STX	NEO	dcSTX	C1	C2	Overall Toxicity
Acat (W08/056/01: 1119/28)	0	0	36.9	615	0	28.4	0	15.8	1135	1236	0	5.7	846	3918.8
	1	0	46.6	555	0	19.2	0	26.2	1049	928	0	4.6	381	3009.6
	2	0	14	391	0	18.2	0	3.6	924	846	0	2.2	299	2498
	3	0	27.3	525	0	20.9	0	17.6	1068	1024	0	3.5	408	3094.3
Atam (W07/069/01: 1119/33)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 7. Toxin concentration profiles of experimental *Calanus helgolandicus* adult females. % *Alexandrium catenella* (Acat) toxin retained calculated as shown in equation 1.

No. animals analysed (survived after experiment)	Exposure time (day)	% Acat in diet	PST (pg STX diHCl eq. copepod^{-1})													
			GTX 1	GTX 2	GTX 3	GTX 4	GTX 5	dcGTX 2	dcGTX 3	STX	NEO	dcSTX	C1	C2	Overall Toxicity	% Toxin Retained
9	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-
9	**0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-
9	**4**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-
6	**4**	25	0	0	0	0	0	0	0	0	102	0	0	2.4	104.4	1.7 %
8	**4**	50	0	0	0	0	0	0	0	0	0	0	8.6	348	356.6	3.3 %
9	**4**	75	0	0	0	0	0	0	0	0	0	0	1.2	51	52.2	2.9 %
9	**4**	100	0	0	0	0	0	0	0	0	0	0	0	3.6	3.6	0.02 %

5.4.2 Feeding of *C. helgolandicus* in the presence of toxic and non-toxic *Alexandrium* spp.

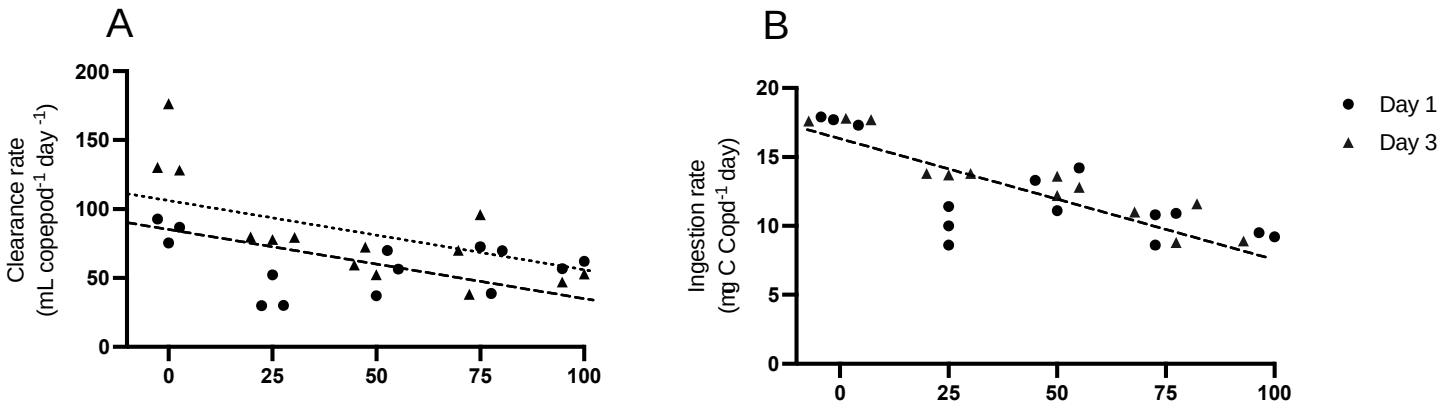


Figure 13. Clearance (A) and ingestion (B) rates of Calanus helgolandicus fed 400 µg C L^{-1} with a variable contribution of toxic *Alexandrium catenella* and non-toxic *Alexandrium tamarense* after day 1 (circles) and day 3 (*triangles*).

Clearance rates of *C. helgolandicus* ranged between 30 – 176 mL $copepod^{-1}$ day^{-1}. The observed rates decreased significantly as the percentage of Acat in the diet increased and also differed between the experimental days (Fig. 13A, % Acat: $F = 12.25$, $p = 0.002$; Day: $F = 6.11$, $p = 0.02$). Total ingestion rates ranged between 8.6 – 17.9 µg C $copepod^{-1}$ day^{-1}, and declined significantly as a function of the relative abundance of Acat in the available prey field but was not affected by the experimental day on which the observations were made (Fig. 13B, % Acat: $F = 61.68$, $p < 0.001$; Day: $F = 1.82$, $p = 0.189$).

5.4.3 Egg-production and viability of *C. helgolandicus* in the presence of toxic and non-toxic *Alexandrium* spp.

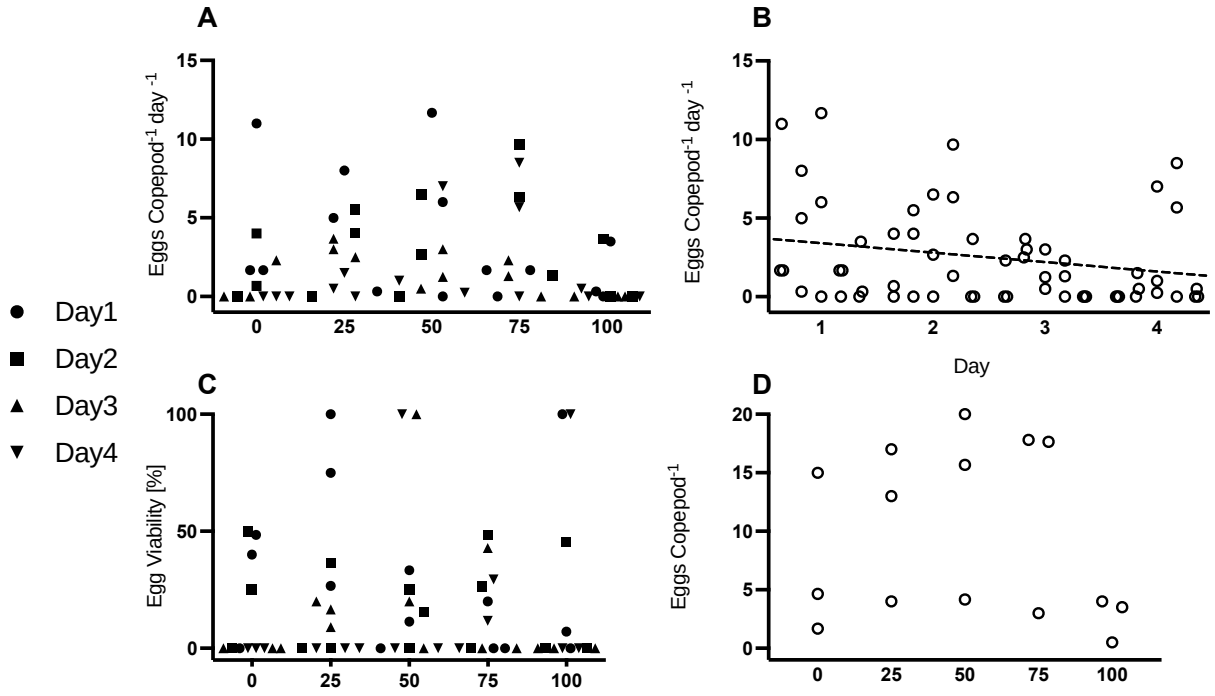


Figure 14. Daily (A, C) and total over the whole duration (B, D) egg production and viability of *Calanus helgolandicus* fed 400 µg C L^{-1} with a variable contribution of toxic *Alexandrium catenella* and non-toxic *Alexandrium tamarense* over four consecutive 24 h incubation periods. See inset legends for symbols used.

The daily egg production rate of *C. helgolandicus* across all treatments ranged between 0 – 11.7 eggs $copepod^{-1}$ day^{-1} (Fig 14A). The observed rates were not significantly affected by the relative abundance of Acat in the available prey field (Fig. 14A, % Acat: F = 0.465, p = 0.498) but there was some evidence to indicate that they did decline as a function of time (Fig. 14B, Day: F = 4.416, p = 0.040). The viability of the eggs produced over the duration of the experiment ranged between 0 – 100 %, and was not significantly influenced by either of the treatments examined (Fig. 14C, % Acat: F = 0.049, p = 0.8266; Day: F = 0.956, p = 0.420).

The total numbers of eggs produced across the four days in the 100 % Atam and 100 % Acat treatments ranged between 1.7 – 15 eggs $copepod^{-1}$ day^{-1} and 0.5 – 4 eggs $copepod^{-1}$, respectively, and reached a maximum of 20 eggs $copepod^{-1}$, occurred in the 50 % Acat treatment (Fig. 14C). Total egg production over the four-day experiment was not influenced by the relative abundance of Acat in the diet (Fig. 14D, % Acat: F = 0.306, p = 0.590).

5.5 Discussion

This study examined how the feeding, egg production and egg viability in *C. helgolandicus* were influenced by the relative abundance of toxic- and non-toxic dinoflagellates, *Alexandrium catenella* (Acat) and *Alexandrium tamarense* (Atam), respectively. It also presents a full toxin profile for Acat, as well as body toxin concentrations of *C. helgolandicus* after feeding on Acat for four days.

5.5.1 Feeding of *C. helgolandicus* in the presence of toxic and non-toxic *Alexandrium* spp.

C. helgolandicus appeared capable of consuming a typical food ration over the duration of our experiment, even when offered a diet consisting of 100 % Acat. The clearance and ingestion rates observed in this study agree with those previously reported for copepods of the genus *Calanus* when feeding upon natural microplankton assemblages (Irigoien et al., 2000; Wexels Riser et al., 2003; Mayor et al., 2006, 2009; Castellani et al., 2008) and toxic dinoflagellates (Turriff et al., 1995; Campbell et al., 2004; Teegarden et al., 2008; Roncalli et al., 2016). Nevertheless, both clearance- and ingestion rates decreased as the percentage of Acat increased in the prey field (Fig.13). *C. finmarchicus* is reported to be capable of distinguishing between toxic and non-toxic dinoflagellates (Campbell et al., 2004), and reduced food intake may have resulted because the animals were actively selecting against Acat. However, cells were clearly ingested in the 100 % Acat treatment and hence this explanation seems unlikely, particularly as the Acat and Atam in our experiments were physically identical. We therefore suggest that the negative effect of increasing the relative abundance of Acat in the food on clearance and ingestion rates did not result from *C. helgolandicus* selecting against Acat cells on the basis of their physical attributes. It was not possible to discern between Acat and Atam when counting the Lugol's samples from the grazing experiment, and thus we cannot investigate this suggestion further. However, other studies indicate that *Calanus* spp. consume both toxic and non-toxic prey with little or no selectivity (Turner & Borkman 2005; Teegarden et al., 2008; Roncalli et al., 2016), and this is consistent with the understanding that the diet of *Calanus* is often proportionally equivalent to that of the food environment (Mayor et al., 2006, 2009; Castellani et al., 2008; Teegarden et al., 2008; Djeghri et al., 2018). We therefore suggest that the negative relationship between % Acat and food intake was attributable to a noxious effect of the ingested toxins. This effect could have been exacerbated by the experimental animals exuding copepodamides (also known as taurine-containing lipids; Mayor et al., 2015), which are reported to significantly increase the production of PSTs in dinoflagellates and their

release into the water column (Wohlrab et al., 2010; Selander et al., 2015, 2019; Griffin et al., 2019).

Copepods are known to be able to feed on toxic algae with no ill-effects for several days (Roncalli et al., 2016), although ingestion rates have been shown to decrease over time (Guisande et al., 2002; Colin & Dam, 2003). We found that clearance rates were significantly higher during day 3, relative to those during day 1 of the experiment, although this result appears to be driven primarily by the 100 % Atam treatment and hence seems unlikely to be related to any toxin-related effect. Total ingestion rates remained unchanged across the two time points examined and our results therefore suggest that the negative impact of Acat on feeding began at the onset of exposure, and did not increase over the duration of the experiment.

Acat negatively affected ingestion, and could therefore impact upon the ability of *Calanus* spp. to successfully complete their lifecycle and contribute to vital ecosystem processes, including the transfer of biomass to higher trophic levels. However, realistically, in the natural environment there are multiple species of prey to feed upon, and given that HA cells typically form a relatively small proportion of the available prey biomass (e.g. Harris et al., 2000; Bresnan et al., 2008; Fehling et al., 2012), their impact on the feeding of *C. helgolandicus* and its congeners seems likely to be low (Turner & Borkman 2005; Leandro et al., 2010; this study). Overall, our results suggest that this might not be a significant threat at this stage, given that Acat forms only low biomass HA blooms in the North East Atlantic. However, there have been records of big *Alexandrium* blooms and resting cysts in the Chukchi and Beaufort Seas in the Pacific Ocean (Natsuike et al., 2013; Anderson, 2021), which could potentially impact any copepod there, especially during summer where there is a mass occurrence of copepods in the Southern Chukchi Sea (Kim et al., 2020).

5.5.2 Egg-production and viability of *C. helgolandicus* in the presence of toxic and non-toxic *Alexandrium* spp.

The egg production rates of *C. helgolandicus* feeding on Acat across the 4-day exposure experiments, 0 – 11.7 eggs copepod^{-1} day^{-1} (Fig. 14A), agree well with values previously reported for *Calanus* spp. feeding on natural microplankton (Pond et al., 1996; Jónasdóttir et al., 2005; Mayor et al., 2006, 2009; Castellani et al., 2008), and toxic *Alexandrium sp.* (Niehoff et al., 2000; Jansen et al., 2006; Madsen et al., 2008; Roncalli et al., 2016). The observed range of egg viability over the duration of the 4-day experiment, 0

– 100 % (Fig. 14C), did not differ significantly between the treatments, and agrees well with values previously reported for *C. helgolandicus* and *C. finmarchicus* (Jónasdóttir et al., 2005; Mayor et al., 2007).

Increasing the toxin concentration in the diet has previously been shown to negatively affect copepod egg production rates (Båmstedt et al., 1999; Roncalli et al., 2016; Abdulhussain et al., 2021), but our results suggest that egg production of *C. helgolandicus* was not affected by the consumption of Acat (Fig. 14A & D). *Calanus* spp. are known to accumulate significant biomass reserves, and may adopt a spectrum of reproductive strategies, from capital breeding (from internal reserves), to income breeding (from ingested food) (Mayor et al. 2009; Sainmont et al., 2014). The available evidence suggests that *C. helgolandicus* is typically an income breeder, with egg production being positively associated with various descriptors of food availability (e.g. Pond et al., 1996). However, we did not determine if/how the biomass of *C. helgolandicus* changed throughout our experiments, and therefore cannot exclude the possibility that they were able to offset the negative effects of toxin ingestion and/or reduced food intake by producing eggs from stored reserves. Future experiments that use longer incubation periods and/or monitor the biomass of females over time are required before any negative effects of Acat on egg production in *C. helgolandicus* can be dismissed. In addition, Acat blooms are common on the West Coast of UK (specifically: Stonehaven, UK) throughout the range of experimentally collected *C. helgolandicus* (Bonnet et al., 2005; Bresnan et al., 2008; Brown et al., 2010; Bresnan et al., 2021), and it has been suggested that copepods that co-exist with HA may develop tolerance to their toxins (Turner and Borkman 2005; Teegarden et al., 2008). Thus, our observed result may be because *C. helgolandicus* has evolved tolerance to the toxins of Acat.

The marginally significant decline in egg production rate as a function of time observed across all treatments ($p = 0.04$: Fig. 14B) is not uncommon for *Calanus* spp. when incubated over several days. This could have occurred because the animals were not consuming enough food to sustain normal egg production rate (Mayor et al., 2007), or because the available food was lacking essential dietary components (Pond et al., 1996). Alternatively, if the eggs were produced from previously ingested food and/or stored reserves, the decrease in egg production through time may indicate that these reserves were becoming depleted. Regardless, these effects were apparent across all treatments, and were therefore not a response to the presence of Acat. Similarly, although egg viability was highly variable, it was not influenced by the relative abundance of Acat over the 4-day experimental period. It has been shown that both toxic and non-toxic species of *Alexandrium* produce

extracellular allelochemicals, secondary metabolites that limit the growth of microalgae and heterotrophic protists (Tillmann & John, 2002; Tillmann et al., 2008), and these are also suggested to dramatically decrease egg production and hatching success in *Temora stylifera* and *C. finmarchicus* by interfering with fertilization or egg viability (Ianora et al., 2004, Roncalli et al., 2016). Thus, the variable egg viability observed in the Acat and Atam treatments is not surprising. However, there are other physiological reasons that might have resulted in egg viability being highly variable. For example, it could have resulted from the eggs being produced from nutritionally-deficient food ingested prior to experimentation (e.g. Pond et al., 1996; Jónasdóttir et al., 2002), or because the incubated females were at different phases of their spawning cycle. We made no attempt to assess if the females had previously mated, and hence some of the unhatched eggs could have also been attributable to individuals that had not previously mated. It is important to note that variability in egg viability in the real environment is often observed, e.g. Jónasdóttir et al. (2005) showed that the hatching success of *C. helgolandicus* in the North Sea varies greatly throughout the year at different sampling stations and egg viabilities < 50 % in this species are not uncommon. Similarly, Miralto et al. (2003) showed that during diatom blooms in the northern Adriatic Sea, copepod egg production rates were high, but only 10 % of the eggs produced by C. *helgolandicus* were viable. *C. helgolandicus* egg viability is positively related to the total and relative contribution of specific taxa to the diet due to seasonal variability (Harris et al., 2000; Irigoien et al., 2000).

5.5.3 Body toxin concentrations of *C. helgolandicus*

The toxin profile analysis showed that the bodies of *C. helgolandicus* retained 0.02 – 3.3 % of the ingested toxins after feeding on Acat for four days, assuming that the diet is proportionally consistent with the prey field (Table 7). Retention efficiencies for *A. tonsa, A. hudsonica, Centropages hamatus, C. finmarchicus, and Eurytmora herdmani* fed on *Alexandrium* spp. are also reported to vary between 0.2 – 10 % (White, 1981; Teegarden and Cembella, 1996; Teegarden et al., 2003; Campbell et al., 2004). These low retention efficiencies suggest that marine copepods are either able to metabolise toxins, or void them from their bodies via excretion, egestion or regurgitation (White 1981; Sykes & Huntley 1987; Guisande et al., 2002; Teegarden et al., 2003; Wexels Riser et al., 2003; Maneiro et al., 2002, 2005). Guisande et al. (2002) found low concentrations of PSTs in tissues and fecal pellets of *A. clausi* feeding on *Alexandrium minutum*, and suggested detoxification and excretion of dissolved toxins as the mechanisms of toxin loss. This interpretation would result in time-dependent increases in toxin degradation products within copepod tissues (e.g. metabolism of sulphocarbomoyl toxins to gonyautoxins and eventually to saxitoxins), as

well as increased overall levels of toxins within their tissues. However, Teegarden et al., (2003) questioned this 'metabolic detoxification' pathway as studies suggesting detoxification (e.g. Teegarden & Cembella 1996; Guisande et al., 2002) did not observe these effects. An alternative explanation for the lower impact of toxins is that copepods feeding on HA lower the efficiency with which they absorb materials from their food. Teegarden (1999) suggested that the absorption of both toxins and carbon may be lower for copepods feeding on toxic *Alexandrium* cells, relative to a non-toxic diet. For example, *A. tonsa*, *C. hamatus*, and *E. herdmani* fed non-toxic Atam over the course of 24 h showed an increase in total body carbon (14-28 %), whereas copepods fed toxic *A. fundyense* either had no significant gains in body carbon (*C. hamatus*) or lost a significant amount of body weight (*A. tonsa*, and *E. herdmani*), despite the fact that the total carbon 'ingested' (32–63 % body weight day^{-1}) was not significantly different between the two diets offered (Teegarden, 1999). These results suggest that not absorbing carbon from toxin-producing *Alexandrium* sp. may help lower the impact of toxins. Alternatively, Sykes & Huntley (1987) suggested that toxic dinoflagellates may cause acute physiological reactions when ingested, inducing regurgitation. This idea was also supported by Teegarden et al., (2003), who suggest that regurgitation or sloppy feeding could explain the observed low efficiencies with which toxins are retained.

Regardless of the low toxin retention efficiencies in copepods, species at higher trophic levels may still be at risk due to chronic exposure (Kershaw et al., 2021). Forty different species of fish tested for toxin accumulation in Scottish waters found PST in 96.2 % of the samples. Since there were no HA recorded during the months when the fish were sampled, the concentrations reported in the study are unlikely to represent the acute doses ingested by fish during HA events (Kershaw et al., 2021). Therefore, even low toxin retention efficiencies may still enable toxins to be passed up the food chain and accumulate in higher trophic levels at discernible levels. Most previous experiments examined toxin accumulation in copepods over 24 h, and little is known about how increasing the exposure time will affect rates of detoxification, acclimatization or toxin accumulation in copepods. This considerable knowledge gap hinders our ability to understand how the transfer of toxins to higher trophic levels will change as the frequency and magnitude of toxin-producing HA blooms change in the future.

5.6 Conclusion

Blooms of the toxin-producing dinoflagellate, *A. catenella*, may have a direct or indirect impact on *C. helgolandicus*. Food ingestion by *C. helgolandicus* declined as the relative abundance of the *A. catenella* in the available food increased. This may decrease the transfer of biomass from *C. helgolandicus* to higher trophic levels. Egg production and egg viability in *C. helgolandicus* were not affected by increasing the relative abundance of *A. catenella* in the available food. This may indicate that *C. helgolandicus* is able to tolerate the toxins produced, but could also suggest that eggs released within the 4-day experiments were produced either from food ingested prior to experimentation, or from maternal reserves. The body toxin concentrations within *C. helgolandicus* after 4 days of feeding on *A. catenella* were low, indicating that toxin retention efficiencies were also low. These results suggest that the impact of *A. catenella* blooms on *C. helgolandicus* are likely to be minimal; however, even low toxin retention efficiency in copepods may still result in the transfer of toxins to higher trophic levels.

Chapter 6

Discussion

6.1 Impact of harmful algae on copepods

If HA toxins are primarily used as grazing deterrents, copepods should avoid consuming algae that produce them. However, numerous copepod species have been found to ingest a variety of toxic HA species without negative influence. There were no obvious negative effects from grazing on toxic algae in several cases (Turner & Tester, 1989; Colin & Dam, 2002; Teegarden et al., 2001; Hassett, 2003; Turner & Borkman, 2005; Appendix A). However, there are number of cases where negative effects were reported, including reduced rates of feeding egg production, egg hatching success, survival, delayed development, and behavioral alterations (Turriff et al., 1995; Barreiro et al., 2007; Jiang et al., 2009; Sopanen et al., 2011; Costa et al., 2012; Appendix A). Varied amounts of HA consumed as food, different levels of toxins in the ingested cells, and different tolerance to various toxins by different copepod species from different waters are all believed to contribute to the differences observed between different studies.

6.1.1 Impact of harmful algae on copepod feeding (mono-specific diet experiments)

In my studies, *A. tonsa* and *C. helgolandicus* showed different feeding behaviours when exposed to mono-specific diets of toxic cells. *A. tonsa* consumed more toxic *A. catenella* than the control algae *Rhodomonas* sp. (Chapter 3), suggesting that they feed to compensate for metabolic costs associated with detoxification or that *A. catenella* is somehow nutritionally insufficient as a food source. On the other hand, *C. helgolandicus* consumed less toxic *A. catenella* than the control, non-toxic *A. tamarense* (Chapter 5), suggesting that HA toxins may impair or suppress their ability to feed. Mono-specific feeding experiments are important for understanding how HA affect the feeding behaviour of copepods, but they are often not representative of the real environment. HA blooms do not happen in the absence of other phytoplankton species, and grazers do not just eat HA during their blooms. In order to observe negative HA impacts in the field, it is likely that there must be a high concentration of toxin-producing cells, few prey alternatives, and active feeding on the toxic cells (Pierson et al., 2005); therefore, the design of laboratory experiments should include mixed food choices to represent the real environment. In addition, it has been shown that the adverse effects of HA can be reduced by alternate food choices or by dilution (Lee et al., 1999; Turner et al. 2001; Colin & Dam, 2002; Frost, 2005; Leising et al., 2005; Li et al., 2006; Li et al., 2008; Poulet et al., 2006; Sommer, 2009).

6.1.2 Impact of harmful algae on copepod feeding (mixed-diet experiments)

When grazing on a mixed diet, *A. tonsa* was found to discriminate against toxic *A. catenella* and showed a preference for the non-toxic *Rhodomonas* sp. which is considered high quality food for *A. tonsa* (Chapter 3). Total ingestion in the mixed diet treatment was not influenced by changing the proportion of toxic cells in the prey field between 25, 50, and 75 % toxic cells. The ability of *A. tonsa* to discriminate between *Alexandrium* with different toxin contents is well known; preference for the least toxic species have been well documented (Turner & Tester, 1989; Teegarden, 1999; Colin & Dam, 2003; Selander et al., 2006). Thus, the influence of toxic *A. catenella* on *A. tonsa* feeding appears to be minimal when alternative food is available. By contrast, *C. helgolandicus* reduced their total ingestion rate in the mixed diet treatments, relative to the non-toxic control (Chapter 5). The decreased ingestion rates likely reflect physical incapacitation due to increased toxicity in the treatments (Colin & Dam, 2003; Sopanen et al., 2011). The apparently contradictory results for *A. tonsa* and *C. helgolandicus* are consistent with previous studies. Differences in the feeding behaviours of copepod have also been observed amongst other inshore species such as *A. hudsonica*, *Centropages hamatus*, and *Eurytemora herdmani* and offshore species such as *Calanus finmarchicus* (Teegarden et al., 2008). In some cases, copepods appear to graze non-selectively on toxic algae whereas other copepod species appear to select against the toxic algae during the same bloom (Turner & Tester, 1989; Teegarden et al., 2001; Teegarden et al., 2008). Non-selective grazing on toxic phytoplankton taxa has been found in previous studies of copepods grazing during natural HA blooms (Turner & Anderson, 1983; Turner & Tester, 1989; Teegarden et al., 2001; Teegarden et al., 2008). There have been reports of selective and non-selective feeding, physiological incapacitation, feeding deterrents, genomic toxin resistance, and HA-copepod co-occurrence tolerance (Teegarden et al., 2008; Cook et al., 2010; Turner, 2014; Appendix A). The cellular toxicity of the individual prey items, as well as their cell/toxin concentrations relative to other non-toxic prey, might be key factors determining the feeding response, and therefore generating the relatively wide variety of observed behaviours. The findings of the experiments presented herein (Chapters 3 & 5; Appendix C) support the idea that both factors have a role in the observed feeding response, or, more precisely, that neither component alone will result in a predicted feeding response from copepods.

6.1.3 Impact of harmful algae on egg production and hatching success

Tolerance to survival and grazing might not be the only factors that are indicative of a copepod's fitness. Contrasting responses in grazing (Chapters 3 & 5) and reproduction

were observed between *A. tonsa* and *C. helgolandicus* (Chapters 4 & 5, respectively). Diets of toxic *A. catenella* had no effect on *A. tonsa* survival and grazing activity, but did have a negative influence on their reproductive output (Chapter 4; Appendix C);overall their egg production and hatching success suggested that feeding on the toxic *A. catenella* impacted the reproductive success. That was not the case with *C. helgolandicus*. Diets of *A. catenella* had a negative influence on *C. helgolandicus* grazing activity, but there were no effects on *C. helgolandicus* egg production and egg viability (Chapter 5; Appendix C).

These contrasting results demonstrate that using *A. tonsa* as a model organism to study the effects of HA on copepods is not always appropriate. One of the main differences between coastal species like *A. tonsa* (Fig. 3A) and shelf/oceanic species like *C. helgolandicus* (Fig. 3B) is the carbon-rich biomass reserves that are found in *Calanus* but not *Acartia*. *Calanus* spp. females have been observed to utilise approximately 50 % of their maternal biomass before the release of eggs (Gatten et al., 1980). The quantity of these biomass reserves varies both geographically and seasonally, reflecting the feeding and physiological history (Marshall & Orr, 1955). In addition, similar to *C. finmarchicus*, *C. helgolandicus* may use the internal reserves in sustaining reproduction during periods of food scarcity (Mayor et al., 2006, 2009). As mentioned in Chapter 5, the available evidence suggests that *C. helgolandicus* is typically an income breeder (Pond et al., 1996). However, we cannot categorically say that they are not capable of capital breeding and therefore be able to offset the effect of toxins. Because I did not determine if/how the biomass of *C. helgolandicus* changed throughout our experiments, it is therefore not possible to assess whether or not the eggs were produced from stored reserves. In addition, *C. helgolandicus* had reduced feeding on toxic *A. catenella* compared to non-toxic *A. tamarense*, but they did feed within the range of other laboratory and field studies on toxic *A. catenella*. Thus, it is quite possible that the amount they fed on toxic cells for three days is enough to help them survive the HA bloom and produce eggs, and longer incubation periods are required before any negative effects of *A. catenella* on egg production in *C. helgolandicus* can be completely ruled out.

6.1.4 Toxin accumulation in copepods after a fixed period of exposure

During natural HA blooms, toxins may be ingested and concentrated within the tissues of grazers, which then serve as a vector for the transfer of toxins through pelagic food webs (Turner et al., 2000, 2005; Doucette et al., 2005; Deeds et al., 2014; Petitpas et al., 2014). However, only a small fraction of the ingested toxins are typically retained in the

tissues of zooplankton grazers (Guisande et al., 2002; Lehtiniemi et al., 2002; Hamasaki et al., 2003; Kozlowsky-Suzuki et al., 2003; Teegarden et al., 2003; Spyrakos et al., 2013). Indeed, Chapter 5 demonstrates that *C. helgolandicus* only retains between 0.02 – 3.3 % of the ingested toxins after feeding on toxic *A. catenella* for four days, assuming that the diet is proportionally consistent with the prey field. Other copepod species also show low toxin retention efficiencies (White, 1981; Teegarden & Cembella, 1996; Teegarden et al., 2003; Campbell et al., 2004). However, the accumulated toxins can still be consumed by small fish and passed up the food chain to higher trophic levels such as large fish, seabirds, and marine mammals (reviewed by Turner & Tester, 1997; Turner et al., 1998; Tester et al., 2000; Jensen et al., 2015; Kershaw et al., 2021). For example, the accumulated toxins in large copepods, e.g. *C. finmarchicus*, can still negatively affect North Atlantic right whales, *Eubalaena glacialis* (Doucette et al., 2006; Durbin et al., 2002). Even humpback whales, *Megaptera novaeangliae*, which had fed on *C. finmarchicus*, were killed by the intake of PSTs (Geraci et al., 1989).

6.1.5 Impact of harmful algae during prolonged exposure

It has been widely shown that extended periods of exposure to HA may cause substantial mortality of copepods (Table 1). However, the results presented herein show no differences between the results of short-term and prolonged experiments. Exposure to toxic *A. catenella* did not appear to impact the survival of *A. tonsa* (Chapters 3 & 4); the ingestion of *C. helgolandicus* offered toxic *A. catenella* showed no apparent change between days 1 and 3 (Chapter 5), suggesting that there was no impact over the period of the experiment (Chapter 5). The egg production of *C. helgolandicus* over 4 days showed significant differences between days 1 and 4. However, a progressive reduction in egg production over a five-day period has also been observed before, and suggested to be due to food limitation and food quality during the incubations (Mayor et al., 2007) rather than toxicity. It is possible, however, that significant changes in copepod survival, feeding and reproduction may not have been detectable during the experimental periods used herein for methodological reasons. For example, *C. helgolandicus* may use the internal biomass reserves to sustain themselves and their reproduction. However, to determine how long they can produce eggs from stored reseves may require experiments that last for several weeks or even months (Hirche, 1983, 1984; Hirche & Kwasniewski, 1997). Thus, it is still important to examine how prolonged exposure to HA affects copepods because blooms can last in the marine environment up to several weeks (Bresnan et al., 2005; 2008).

6.1.6 Methodological protocols

Several studies have examined the methods used to study the trophic interactions between copepod and HA toxins (Caldwell et al., 2004; Colin & Dam, 2002; Irigoien et al., 2002; Jónasdóttir et al., 1998; Paffenhöfer et al., 2005). One of the major critical points was that there is no standard published protocol, which means that it is difficult to compare studies (Caldwell et al., 2004; Paffenhöfer et al., 2005). Therefore, it is important to simultaneously measure the level of toxins in the experimental diet as well as the concentration fed to copepods, e.g. carbon and nitrogen contents, in order to produce data that can be compared across different studies.

Several experiments have tested the impact of toxic diatoms on copepod reproduction using extracts or commercially available toxins (Poulet et al., 1994; Ianora et al., 1995; Uye 1996). These studies have been debated for several reasons. For example, negative effects of diatom toxins on copepod reproduction may only occur where the exposure concentrations are an order of magnitude higher than those encountered in the field (Jónasdóttir et al., 1998). Also, female copepods may alter or mediate the toxic effect by not absorbing the toxins (Roncalli et al., 2016), and the commercially available toxins are very different to those encountered in the field by copepods. It has been suggested that the negative impacts in these experiments is due to anoxia from bacteria feeding on the extracts (Jónasdóttir et al., 1998) as the negative effects disappeared when the incubations included aeration (Jónasdóttir & Kiørboe, 1996). Another study found that without aeration, high concentrations of dinoflagellate extracts had no effect on copepod egg hatching success (Miralto et al., 1995). It is important to note these experiments could help in understanding the impact of toxins, yet they are not representative of the real environment, where copepods are exposed to far lower concentrations of toxins (Caldwell et al., 2004). Direct grazing experiments on HA cells are more ecologically relevant because they imitate conditions encountered by copepods in field. Finally, many of the negative effects observed on grazers feeding on cultures of toxic HA may be due to the experimental diets containing larger concentrations of HA cells than those found in natural blooms of the same species; HA species typically make up only a small part of the overall composition of phytoplankton communities in most natural situations, even during HA blooms.

6.1.7 Changes in experimental toxin concentrations

The composition, potency and concentrations of toxins can vary significantly between clones or blooms of the same HA species (Turner et al., 1998; Brown et al., 2010;

Waggett et al., 2012). Within a single clone, cellular toxin levels can fluctuate due to the development phase (Taroncher-Oldenburg et al., 1997), turbulence (Juhl et al., 2001), salinity (Grzebyk et al., 2003), or nutritional conditions (John & Flynn, 2002; Leong et al., 2004; Hardison et al., 2012). Different strains of the same HA species cultivated under equal culture conditions can also have different toxin levels (Loret et al., 2002; Etheridge & Roesler, 2005; Collins et al., 2009; Brown et al., 2010). In this study, the same strain of toxin-producing *A. catenella* was used in Chapters 3 and 4 (strain 1119/27), whereas a different strain was used in Chapter 5 (strain 1119/28) because strain 1119/28 was archived and therefore no longer available. The concentrations of toxins of *A. catenella* were highly variable in all experiments, despite being grown under near identical conditions (Appendix C). The total saxitoxin equivalents were 2732.5 fg STX eq $cell^{-1}$, 501.1 – 3575.5 fg STX eq $cell^{-1}$ and 2498 – 3918.8 fg STX eq $cell^{-1}$ in Chapters 3, 4 and 5, respectively (Appendix C). In Chapters 3 and 4, between 9 and 12 of the 19 toxins examined were identified in *A. catenella* (EFSA, 2009), whereas in Chapter 5, 8 out of the 12 analysed toxins were detected. The non-toxic species of *A. tamarense* used in Chapters 3 and 4 (strain 1119/19) and in Chapter 5 (strain 1119/33) were also examined for toxins using LC MS/MS and no toxins were detected. It is important to note that the analysis for Chapter 4 took place at different laboratory and the methodological approach followed the PCOX method (Van de Riet et al., 2011) to extract toxins. All of the employed toxin extraction methodologies have been approved (Van de Riet et al., 2011; Turner et al., 2014, 2015, 2019) and the determination of toxin concentrations in each of the presented experiments enables the resulting data to be more meaningfully compared (Appendix C).

6.1.8 Toxicity vs. nutritional insufficiency

It is important to differentiate between toxic and nutritional effects during HA-copepod experiments because both have been suggested to cause reduced egg production (Dutz, 1998; Collumb & Buskey, 2004; Prince et al., 2006; Costa et al., 2008). Also, copepod growth and development have been found to be influenced by food quality. N-depleted algae result in slower copepod growth rates, lower C growth efficiency, and lower egg production rates relative to those measured on animals fed N-replete algae (Jones et al., 2002; Jones & Flynn, 2005). These responses to nutritionally insufficient food could be be mistaken for toxic effects on copepods. For example, adult female *A. tonsa* fed on a monospecific diatom diet produced fewer, smaller eggs and exhibited greater death rates relative to aminals offered dinoflagellate prey or a mixed diet (Jones & Flynn, 2005). This was linked to nutritional insufficiency of a single species diet, and not to toxicity.

In Chapter 3, the total ingestion rate of *A. tonsa* fed on toxic *A. catenella* was higher than the control species *Rhodomonas* sp. Copepods are thought to increase their intake to compensate for the extra energy required for detoxification (Dutz, 1998; Costa et al., 2008). Therefore, if the nutritional value of *A. catenella* is lower than that of the control algae, *Rhodomonas* sp., compensatory feeding may have resulted in their increased food intake to compensate for a nutritional deficient diet. This was observed when *A. tonsa* was fed a nutritionally deficient species of toxic algae, *K. brevis*, which lacked various fatty acids and other nutritional components and affected reproduction in a manner similar to starvation (Collumb & Buskey, 2004; Prince et al., 2006). Unfortunately, the data presented in Chapter 3 did not allow for a reliable distinction between these two mutually exclusive mechanisms. Nutritionally deficient food and the presence of toxins have both been suggested to cause reduced egg production in *Acartia* sp. (Dutz, 1998; Collumb & Buskey, 2004; Prince et al., 2006; Costa et al., 2008). Therefore, in Chapter 4, adult female *A. tonsa* were exposed to mono-specific diets of toxic *A. catenella* and non-toxic *A. tamarense*, and the high-quality algae *Rhodomonas* sp. as a control diet to examine whether the impact of toxic *A. catenella* (strain 1119/27) was different from non-toxic *A. tamarense* (1119/19). The egg production and hatching success rates were distinguishable between toxic and non-toxic *Alexandrium* spp., and as discussed in Chapter 4, suggesting a toxic impact on *A. tonsa*. Choosing a high-quality control is necessary to determine if any observed effects are related to the toxin- or nutritional content of the prey.

6.2 Prognosis for the future

6.2.1 Climate change, copepod, and harmful algae

HA have direct and indirect effects on copepods (Appendix A & C), and copepods have to cope with the increasing stress of HA whilst also dealing with other environmental stressors including ocean warming, ocean acidification, and changing spatial and temporal patterns of food. Climate change is the main driver of environmental stressors (Beaugrand et al., 2002, 2009; Beaugrand, 2012; Chivers et al., 2017; Murphy et al., 2020), and evidence of how climate change affects planktonic species is growing, particularly in terms of increased ocean temperature and ocean acidification (George & Harris, 1985; Costello et al., 2006; Edwards et al., 2006; Mayor et al., 2007, 2012; Cripps et al., 2014). Direct effects of warming and acidification to copepods include changes in physiology and behavior that can alter growth (McFeeters & Frost, 2011; Doan et al., 2019), body size (Escribano & Mclaren, 1992; Garzke et al., 2015; Sommer et al., 2016), reproductive output (Cook et al., 2007; Mayor et al., 2012; Weydmann et al., 2012), naupliar development (Cook et al., 2007), and

survival (Sommer et al., 2007; Kroeker et al., 2010; Cripps et al., 2015). Understanding the impact of climate change on the planktonic phenology and copepod physiology is important to understand the future ocean system.

6.2.1.1 Impact of ocean warming on HA

One of the most significant stressors influencing future changes in marine ecosystems is global warming (Boyce et al., 2010; Mackas et al., 2012). Climate temperatures have been increasing and, based on climate projections, will continue to increase in the future (IPCC, 2021). This increase has been associated with increased impact of HA blooms (Hallegraeff, 1993; Hallegraeff et al., 2010; Anderson et al., 2012; Fu et al., 2012; Berdalet et al., 2016; Gobler et al., 2017; Dees et al., 2017; Nohe et al., 2020; Hallegraeff et al., 2021; Marampouti et al., 2021). For example, two algae that produce strong biotoxins, *A. fundyense* and *Dinophysis acuminata*, were studied using high-resolution sea-surface temperature records (1982 to 2016) and temperature-dependent growth rates (Gobler et al., 2017). The study concluded that increasing ocean warming is playing a key role in the intensification of toxic HA blooms in the North Atlantic and North Pacific Ocean. Moreover, in recent years, HA blooms were not only detected in the warm-temperate region, but also have been spreading to the Arctic (Bresnan et al., 2021), which could be due to global warming (Hallegraeff et al., 2021), or genetically changed HA that are adapted to cold regions (Wang et al., 2021). According to satellite-based remote sensing data, annual pelagic net primary production in shelf regions of the Arctic Ocean increased by 20 % between 1998 to 2009, as a result of a longer pelagic growth season associated with the increased duration of the ice-free period (Arrigo et al., 2011). Several studies have shown that enhanced under-ice irradiance due to melting sea ice, refrozen leads, and/or melt pond formation (i.e. increased transmittance) generated diatom- and *Phaeocystis*-dominated under-ice blooms, which were fuelled by an excess of nutrients in the under-ice waters (Arrigo et al., 2012; Assmy et al., 2016). In addition, a study from North East Greenland found that the bloom concentration of haptophytes (~ 2,000,000 cells L^{-1}) was similar to bloom concentrations recorded during harmful *Chrysochromulina* blooms in the Skagerrak, Southern Norway (Søgaard et al., 2021). As evidenced by records of *Chrysochromulina* spp. in the low salinity waters (5–6 PSU) of the Baltic Sea and the Kattegat, Norway, brackish environments may increase the abundance of haptophytes (Nielsen et al., 1990; Hansen et al., 1995). Thus, future Arctic Ocean blooms dominated by potentially toxic mixotrophic algae (*Prymnesium* and *Chrysochromulina*) will grow more prevalent and widespread (Søgaard et al., 2021). In summary, even though blooms of mixotrophic organisms during the Arctic spring show that

they may be essential in driving ecosystem- and carbon cycling dynamics in this region (Arrigo et al., 2012; Assmy et al., 2016; Søgaard et al., 2021), toxic blooms in the Arctic require further investigation to understand their impact in marine ecosystems.

Quantifying how interactions between climate change and nutrients regimes in coastal areas, caused by growing human population and nutrients from agriculture, affect the frequency and magnitude of HA blooms is important for understanding what the future holds in the context of HA. The lack of fundamental understanding of the mechanisms that encourage the growth of toxic algal blooms make anticipating their future prevalence difficult. Understanding the patterns of toxic HA bloom in the next few decades will be critically dependent on taking HA blooms into account within the competitive context of plankton communities and connecting these insights to ecosystem, oceanographic, and climate models (Wells et al., 2015, 2020).

6.2.1.2 Impact of ocean warming on copepods

Increasing temperatures have a direct effect on marine copepods. Animals are adapted to live within upper and lower limits of thermal tolerance and changes in temperature may lead to reduced performance and eventually mortality unless certain adaptations are present, migration occurs, or the change rate is slow enough to allow adaptation (Pörtner & Farrell, 2008). Negative impacts of increasing temperature on copepods have been well documented (Escribano & Mclaren, 1992; Ban, 1994; Holste & Peck, 2006; Cook et al., 2007; Bonnet et al., 2009; Mayor et al., 2012; Edwards et al., 2013; Mayor et al., 2015) with experiments showing that increasing temperature negatively impact *Calanus* spp. survival, egg production, egg hatching times and naupliar development (Cook et al., 2007; Bonnet et al., 2009; Mayor et al., 2012). In addition, the 70 % decrease in the overall *Calanus* spp. biomass in the North Sea and North East Atlantic since the 1960's (Edwards et al., 2013) has been attributed to the North Atlantic Oscillation and high sea surface temperature (Beaugrand et al., 2014). On the other hand, the warm waters (>3 °C) observed in the Bering Strait during 2015 showed a high abundance of Pacific copepod species (*Eucalanus bungii*, *Metridia pacifica*, and *Neocalanus* spp.), relative to cooler periods in 2014 and 2016 (Kim et al., 2020). The Bering Strait is the only point of entry from the Pacific Ocean to the Chukchi Sea, and high-temperatures of the Bering Summer Water inflow may therefore have a key influence on the abundance of Pacific copepod species in the southern Chukchi Sea. Finally, the effects of changing temperature on copepods appear to be species- and/or population-specific (Richardson, 2008).

6.2.1.3 Impact of ocean acidification on copepods

The atmospheric concentration of carbon dioxide (CO_2) has continued to increase, reaching annual averages of 410 ppm for CO_2 (IPCC, 2021). Rising CO_2 concentrations in the atmosphere are causing global warming and ocean acidification (Caldeira & Wickett, 2003, 2005; Feely et al., 2004; Orr et al., 2005; Fabry et al., 2008). The HA blooms have been linked to ocean acidification where the stimulating effects of ocean acidification on growth and toxicity may result in the expansion and intensification of toxic HA blooms (Glibert et al., 2014; Riebesell et al., 2018). In addition, the direct effects on copepods of increased pressure of CO_2 have been reported to vary between species (Zhang et al., 2011), populations (Thor & Oliva, 2015), and developmental stages within a species (Lewis et al., 2013; Cripps et al., 2014; Pedersen et al., 2014). Based on future scenario predictions (2000–10,000 ppm CO_2), studies reported the impact of CO_2 induced acidification resulted in a negative impact on survival, egg production, and hatching success on different copepods including *Acartia pacifica*, *Acartia spinicauda*, *C. finmarchicus*, *C. glacialis*, *Calanus sinicus*, and *Centropages tenuiremis* (Mayor et al., 2007; Zhang et al., 2011; Weydmann et al., 2012; Pedersen et al., 2013). Therefore, the impact of CO_2 concentrations on copepods found in laboratory experiments is an order of magnitude higher than encountered in situ with no/or minimum impact at concentrations found in the real environment. However, Cripps et al., (2015) showed a 35 % decline in *A. tonsa* nauplii recruitment at CO_2 concentrations of 1000 μatm, and an LC_{50} at 1084 μatm pressure CO_2. The study argues that research that attempts to report consequences to future ocean acidification situations merely based on exposure of single life history stages might result in significant misrepresentation (Cripps et al., 2015).

6.2.1.4 Combined environmental stressors

The majority of research has focused on the single effects of ocean warming (Richardson, 2008), ocean acidification (Fabry et al., 2008), or toxic algae (Appendix A), while some have looked at interactions between a variety of biotic and abiotic climate-related variables in combination (Byrne et al., 2009; Gao & Zheng, 2010; Vehmaa et al., 2012; Kroeker et al., 2013; Reymond et al., 2013; Garzke et al., 2016). *C. finmarchicus* have been studied with combined stressors of future global warming and ocean acidification scenarios (Mayor et al., 2012) showing negative impact on *C. finmarchicus* reproduction due to high temperature but not ocean acidification. Another study examined the combined effects of toxic cyanobacteria, ocean warming, and ocean acidification levels predicted for the year 2100 on *Acartia bifolsa* (Vehmaa et al., 2013). Antioxidant capacity of *A. bifolsa* was

lowered as a result of acidification and increased temperature. Egg viability, nauplii development, and oxidative status all declined as the temperature increased. Exposure to toxic cyanobacteria reduced egg production but increased oxidative status and egg viability, resulting in no net effects on the production of viable eggs. Furthermore, the presence of cyanobacteria enhanced nauplii development, substantially alleviating the generally unfavorable impacts of increasing temperature and lower pH on copepod recruitment (Vehmaa et al., 2013). In summary, there is a need for cautious interpretation of data from multiple stressors because observed effects could be related to any of the combined effect on copepods.

6.2.1.5 Plankton phenology

Environmental conditions and climate change are essential drivers of marine plankton distribution (Beaugrand et al., 2002; Parmesan & Yohe, 2003; Hays et al., 2005; McMahon & Hays, 2006; Parmesan, 2006; Thomas et al., 2012; Chivers et al., 2017). The study of biological seasonal cycles and how they are affected by climate and weather is known as phenology (Thackeray, 2012; Thackeray et al., 2016), and it is highly sensitive to increasing ocean temperature (Richardson, 2008). Many studies have reported changes in phenological rates and patterns, which also vary greatly among co-existing and interacting planktonic taxa from the same aquatic ecosystem (Edwards & Richardson, 2004; Winder & Schindler, 2004; Adrian et al., 2006; Costello et al., 2006; Thackeray et al., 2008; Beaugrand et al., 2009; Feuchtmayr et al., 2012; Chivers et al., 2017). Edwards and Richardson (2004) studied whether climate warming signals develop across all trophic levels and functional groups within an ecological community using long-term data from 66 plankton taxa between 1958 to 2002. The study evaluated changes in marine pelagic phenology in the North Sea across three trophic levels: diatoms and dinoflagellates (main producers), copepods (secondary producers), non-copepod holozooplankton (secondary and tertiary producers), and meroplankton, which includes fish larvae, (secondary and tertiary producers). The study found that many pelagic organisms are responding to climate change, and the intensity of the response varies greatly between pelagic assemblages (Edwards & Richardson, 2004). On the other hand, a 25-year time-series of weekly samples at the Plymouth L4 site was applied to examine mismatch theory, comparing 57 plankton taxa across 4 trophic levels (Atkinson et al., 2015). According to the study, extreme cold or warm years had no effect on the phenology in terms of annual mean abundance or egg production rates. In fact, other factors, allow extended production periods including, inter-annual variability in food quantity, high food baseline levels, turnover rates, and prolonged seasonal availability. Therefore, ocean

warming alone might not always results in "mismatch" in timing between trophic levels, and the theory does not always apply to systems with multiple compensating factors. In conclusion, climate change pressure will influence marine plankton distribution, including the impact of toxic HA blooms, which may change the patterns of food availability to copepods.

6.2.1.6 Future physiological experiments

It is currently unclear how well copepods can adapt to multiple environmental stressors, and hence we need to learn more about the physiological responses of copepods to the simultaneous effects of, for example, ocean warming and acidification, changing patterns of food availability and increased prevalence of HA blooms. Furthermore, different copepods respond to environmental stressors differently (e.g. coastal and oceanic/shelf copepods), and therefore comparable experiments across a range of species are required. The rates of copepod development are dependent on food resources, and therefore the seasonal availability of those resources are likely to influence the outcome of experiments. Therefore, in order to understand the physiological responses in copepods, food resource windows should be defined and seasonal variations considered over a long term (Ban, 1994; Campbell et al., 2001). It is important to note that the lifecycle of some copepods such as *Calanus* spp. can be interrupted by long periods of diapause which may result in lags between resource availability and copepod development, and make physiological responses complex to examine. Therefore, future experiments should identify the HA trends and their impact on copepod dynamics by understanding the copepod physiology data derived from laboratory experiments.

6.2.2 Cyclopoid copepods

Almost all studies of copepod interactions with HA have focused on calanoid copepods. However, cyclopoid copepods, especially those of the genus *Oithona*, are far more common than calanoids (Gallienne and Robins, 2001), especially in the near-surface waters where most HA blooms occur (Fig. 15). *Oithona similis*, a widespread cyclopoid, is known to eat microzooplanktonic protists such ciliates and heterotrophic dinoflagellates (Nakamura & Turner, 1997; Lonsdale et al., 2000; Grane li & Turner, 2002). Thus, grazing on toxic dinoflagellates and other HA taxa by copepods of the genus *Oithona* could account for a significant portion of copepod grazing influence on HA blooms, but there are only a very limited number of studies on such interactions (Sevensen & Kiørboe, 2000; Wolhrab et al., 2010). Further work is required to assess if/how cyclopoids are affected by HA toxins.

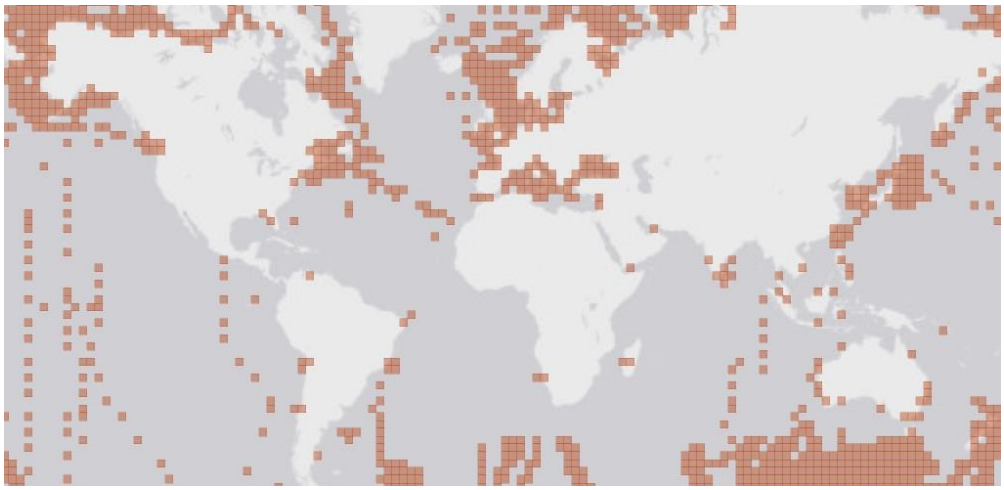


Figure 15. Distribution map of *Oithona similis* (OBIS, 2021). (Ocean biodiversity information system. Intergovernmental oceanographic commission of UNESCO, 2021); open access agreement.

6.3 Conclusions

The research presented in my thesis furthers our understanding of the survival, feeding, egg production, hatching success, toxin accumulation, algal toxin profile, as well as trophic interactions between copepods and HA, an aspect that has been repeatedly highlighted as a key knowledge gap (Cook et al., 2010; Turner, 2014). My observations have shown:

- *A. tonsa* and *C. helgolandicus* survival was unaffected by the exposure to toxic *A. catanella* at reported bloom concentrations during short-term (1 day) and prolonged experiments (4–10 days). Survival of *A. tonsa* only decreased at exposure levels two orders of magnitude higher than encountered in the environment. The observed lethal median concentration (LC_{50}) for *A. tonsa* exposed to *A. catenella* was 12.45 ng saxitoxin equivalents L^{-1}.

- *A. tonsa* ingestion rates increased with prey availability for both toxic- (*A. catenella*) and non-toxic (*Rhodomonas* sp.) prey. Ingestion rates were higher when offered *A. catenella* only, potentially suggesting compensatory feeding, and *A. tonsa* actively selected non-toxic prey over *A. catenella* when offered a mixed diet. *C. helgolandicus* ingestion rates declined significantly as the percentage of toxin-producing *A. catenella* within their diet increased suggesting physical incapacitation due to toxicity.

- Egg production rates and hatching success in *A. tonsa* exposed to toxic *A. catenella* at 1000 μg C L^{-1} were not significantly different from starved animals, but were significantly lower than animals fed non-toxic *A. tamarense*, *Rhodomonas* sp., or a 50:50 mix of toxic *A. catenella* and *Rhodomonas* sp. Egg production rates and hatching success of eggs produced by females that were fed either *Rhodomonas* sp., toxic *A. catenella* or a 50:50 mix of toxic *A. catenella* and *Rhodomonas* sp. at 100 μg C L^{-1} were not significantly different. *C. helgolandicus* egg production and egg viability were unaffected by the relative abundances of toxic *A. catenella* and non-toxic *A. tamarense* in the diet during a 4-day experiment.

- The concentrations of toxins of *A. catenella* were highly variable in all experiments with the total saxitoxin equivalent 501.1 – 3918.8 fg STX eq $cell^{-1}$. The body toxin concentrations of *C. helgolandicus* indicate that this species may bioaccumulate HA toxins; however, the retention efficiency is only 0.02 % when fed 100 % toxic *A. catenella*.

Rapid climate change may be increasing the prevalence of toxic blooms in marine ecosystems and their effects on copepods. The emergence and increasing impact of toxic blooms indicates that HA taxa can, at times, outgrow or otherwise hinder the ability of zooplankton grazers to manage their blooms due to a variety of interacting variables. Future research into copepods should focus on the physiological impact of HA toxins as well as other environmental stressors caused by climate change because different copepod species from different waters are all believed to respond differently to multiple environmental stressors. Understanding how copepods respond to combined environmental stressors can help to predict how copepods will fare in the future. Even though it is complex, it is needed to produce a realistic community level predictions of copepod responses to environmental stressors.

Table: review of published effects of copepods feeding upon toxic dinoflagellates

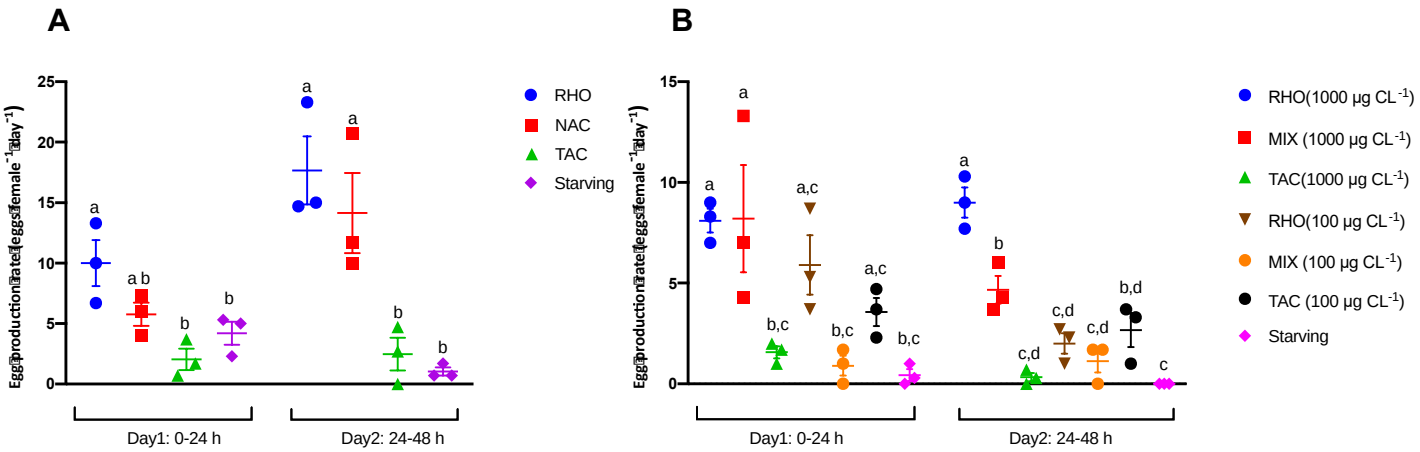
Species	Cultures/ Collected Copepod	Harmful Algae	Non toxic Algae/ control used in experiment	Toxins	Methodology	Toxicity test/ Toxin Profile	Toxic Accumulation	Duration of feeding experiment	Impact	Region	Citation
Acartia bifilosa	Collected species	*Alexandrium ostenfeldii*	*N/A*	PST	Feeding, Behavioural experiment	HPLC/ FD	Yes	24 h	Behavioural disturbance and Incapacitation	Northern Baltic Sea	Sopanen et al., 2011
Acartia clausi	Cultured species	*Alexandrium lusitanicum*	*Rhodomonas baltica*	PST	Survival Feeding, Egg production	HPLC	N/A	-24 h -9 days	-No apparent impact on survival and feeding -Reduced egg	Laboratory exp.	Dutz (1998)
Acartia clausi	Collected species	*Alexandrium tamarense*	*N/A*	PST	Feeding experiment, Behavioural response, Gene expression and copepod-cues	HPLC/ FD	N/A	48 h	-No impact on feeding -50% affected in behavioral response -Response to gene expression and increase PST due to Copepod-cues	Northwest coast of Denmark	Wolhrab et al., 2010
Acartia hudsonica	Collected species	*Alexandrium fundyense*	*Tetraselmis* spp.	PST	Feeding Experiment -Acclimation Experiment	HPLC	N/A	24 h & 14 days	No Apparent impact	Passamaquoddy bay, NB, Canada, Casco bay, ME, Great Pond, MA, Mumford Cove, CT	Colin and Dam 2002
Acartia hudsonica	Collected species	*Alexandrium fundyense*	*Tetraselmis* spp.	PST	Feeding Experiment -Acclimation Experiment	HPLC	N/A	24 h & 14 days	Reduced ingestion rates	Great Bay,New Jersey, USA	Colin and Dam 2002

Acartia tonsa	Collected species	*Cochlodinium polykrikoides*	*Rhodomonas lens*	N/A	Survival Experiment eggs production Grazing Experiment	N/A	N/A	24 h	Increase mortality & Reduced eggs	Long island sound, NY, USA	Jiang et al 2009
Acartia tonsa	Collected species	*Alexandrium* spp.	*Lingulodinium polyedrum.*	PST	Grazing experiment	HPLC/ FD	YES	12 h	No Apparent impact	Damariscotta river estuary, Maine, USA	Teegarden and Cembella 1996
Acartia tonsa	Collected species	*Karenia brevis*	*natural prey*	N/A	Grazing and clearance experiment	N/A	N/A	17.5 to 21.75 h	No Apparent impact	Beaufort, North Carolina	Turner and Tester 1989
Acartia tonsa	Collected species	*Prorocentrum minimum (toxic)*	*Prorocentrum minimum (non toxic strain)*	N/A	feeding experiment, Oyster exposure experiment	N/A	N/A	48 h	No apparent impact (low grazing on toxic and non toxic strains)	York river, USA	Saba et al., 2011
Acartia tonsa	Collected species	*Karlodinium veneficum (toxic)*	*Karlodinium veneficum (non-toxic strain)*	N/A	feeding experiment, Oyster exposure experiment	N/A	N/A	48 h	No apparent impact (low grazing on toxic and non toxic strains)	York river, USA	Saba et al., 2011
Acartia tonsa	Collected species	*Alexandrium fundyense*	*Lingulodinium polyedrum , Gonyaulax cf, Prorocentrum micans*	PST	Grazing experiment	HPLC/ FD	N/A	24 h	Decrease ingestion (grazer-deterrent effects observed)	Damariscotta river estuary, Maine, USA	Teegarden 1999
Acartia tonsa	Cultured species	*Alexandrium minutum*	*Prorocentrum micans*	PST	Selectivity experiment, Density dependence experiment	HPLC/ FD	N/A	3 days	waterborne cues from A. *tonsa* induce PST	Kristineberg Marine Research Station (Sweden	Selander et al., 2006

Acartia tonsa	Collected species	*Karenia brevis*	*Rhodomonas salina*	N/A	feeding and egg production experiments	LC-MS	N/A	17 h	Decrease ingestion, decrease egg production, increase mortality	Beaufort, North Carolina	Waggett et al., 2012
Paracalanus quasimodo	Collected species	*Karenia brevis*	*natural prey*	N/A	Grazing and clearance	N/A	N/A	17.5-21.75 h	No Apparent impact	Beaufort, North Carolina	Turner and Tester 1989
Calanus finmarchicus	Collected species	*Alexandrium fundyense*	*Rhodomonas* spp	PST	feeding and egg production experiments	HPLC	N/A	7 days	Reduced eggs	Gulf of Maine USA	Roncalli et al., 2016
Calanus helgolandicus	Collected species	*Alexandrium tamarense*	*N/A*	PST	Feeding experiment, Behavioural response, Gene expression and copepod-cues	HPLC/ FD	N/A	48 h	-No impact on feeding -29% affected in behavioral response -Response of Gene expression and increase PST due to Copepod-cues	Coast of Scotland	Wolhrab et al., 2010
Centropages typicus	Collected species	*Karenia brevis*	*natural prey*	N/A	Grazing and clearance experiment	N/A	N/A	17.5 to 21.75 h	Reduced ingestion	Beaufort, North Carolina	Turner and Tester 1989
Community Zooplankton	Collected species	*Alexandrium fundyense*	*natural prey*	PST	Grazing Impact	N/A	N/A	13-23.5 h		Gulf of Maine USA	Turner 2010
Eurytemora affinis	Collected species (Lab)	*Alexandrium ostenfeldii*	*N/A*	PST	Grazing and behavioural experiment	HPLC/ FD	Yes	24 h	Behavioral disturbance and Incapacitation	Collected species (Lab)	Sopanen et al., 2011
Eurytemora herdmani	Collected species	*Alexandrium synthesize*	*Lingulodinium polyedrum.*	PST	Grazing experiment	HPLC/ FD	YES	12 h	No Apparent impact	Damariscotta river estuary, Maine, USA	Teegarden and Cembella 1996

Labidocera aestiva	Collected species	*Karenia brevis*	*natural prey*	N/A	Grazing and clearance experiment	N/A	N/A	17.5 to 21.75 h	No Apparent impact	Beaufort, North Carolina	Turner and Tester 1989
Oithona similis	Collected species	*Alexandrium tamarense*	*N/A*	PST	Feeding experiment, Behavioural response, Gene expression and copepod-cues	HPLC/ FD	N/A	48 h	-No impact on feeding -86 % affected in behavioral response -Response of Gene expression and increase PST due to Copepod-cues	Coast of Scotland	Wolhrab et al., 2010
Oncaea venusta	Collected species	*Karenia brevis*	*natural prey*	N/A	Grazing and clearance experiment	N/A	N/A	17.5 to 21.75 h	No Apparent impact	Beaufort, North Carolina	Turner and Tester 1989
Temora longicornis	Collected species	*Alexandrium fundyense*	*Rhodomonas lens*	PST	Grazing exp. Eggs production experiment & Swimming Behaviour	ELISA/ HPLC	N/A	24 h	Change swimming behavior	Damariscotta river estuary, Maine, USA	Lasley-Rasher et al 2016

Egg production of *Acartia tonsa* on day 1 and day 2 (Chapter 4)



Egg production of *Acartia tonsa* on day 1 (acclimation period) and day 2. The bars show the mean (N=3) ± standard error (SE). The a and b letters above bars show the post-hoc comparisons between the treatments with shared letters denoting no significant difference. A: Egg production of *Acartia tonsa* fed on RHO (*Rhodomonas* sp.; blue circles), TAC (toxic *Alexandrium catenella* 1119/27; green triangles), and NAC (non-toxic *A. catenella* 1119/19; red squares) at a concentration of 1,000 µg C L^{-1} or when starved (purple diamond). B. Egg production of *Acartia tonsa* fed on RHO (*Rhodomonas* sp. ; brown inverted triangles, and blue circles, for 100 & 1,000 µg C L^{-1}, respectively), TAC (toxic *Alexandrium catenella* 1119/27; black circles, and green triangles for 100 & 1,000 µg C L^{-1}, respectively), and MIX (50:50 mixture of both cell types; orange circles and red squares for 100 & 1,000 µg C L^{-1}, respectively) at two concentrations: 100 & 1,000 µg C L^{-1} or when starved (purple diamond).

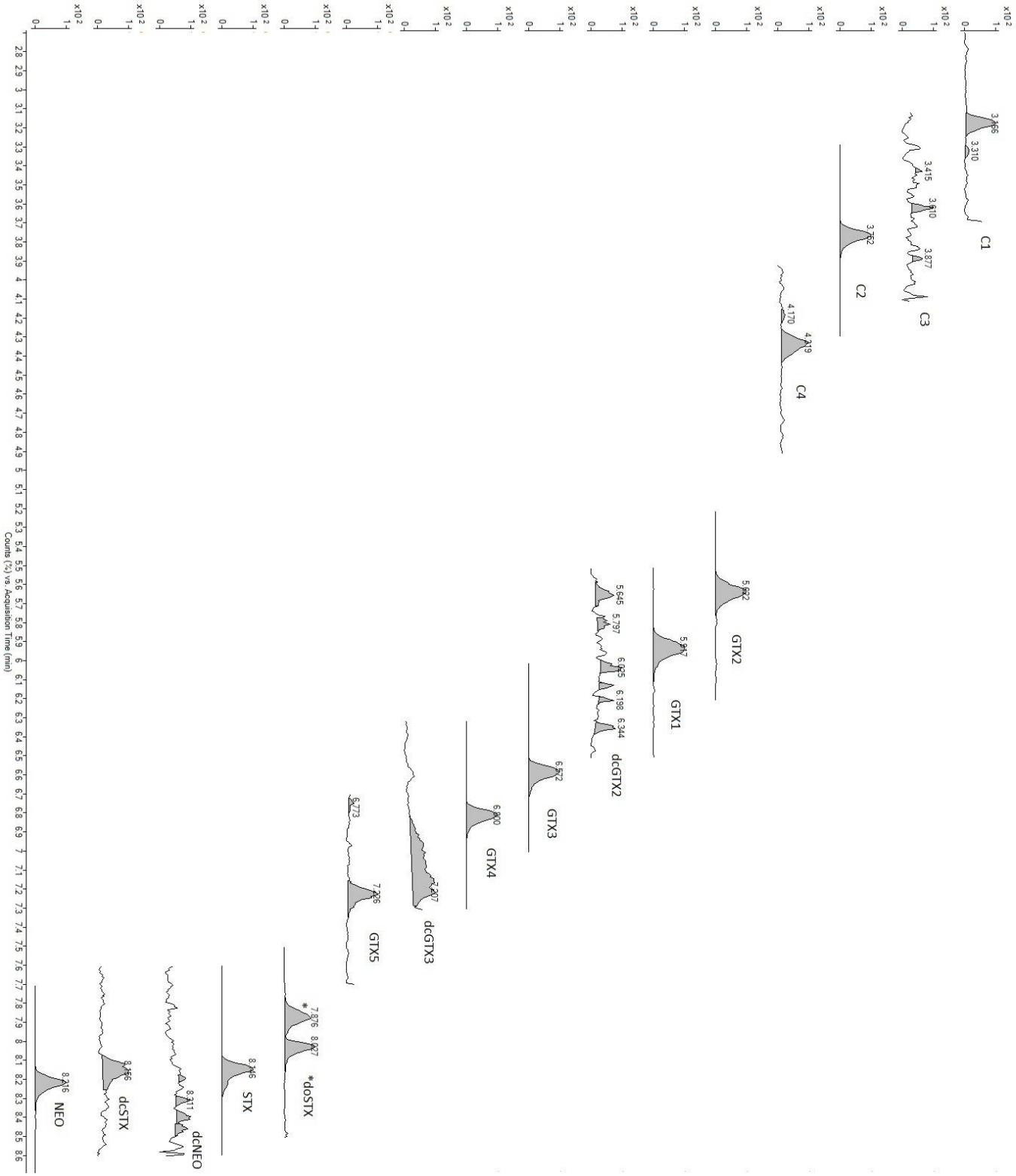
Table: results of experiments in this thesis examining the effects of copepods feeding upon toxic dinoflagellates

Copepod	Algal species	Experiment	Duration (days)	Algae concentration ug C L^{-1}	*Toxic species*	*Toxicity measured STX eq $cell^{-1}$ (fg STX eq $cell^{-1}$)*	Effects	Chapter
A. tonsa	*A. catenella 1119/27*	Survival	1	1000	Toxic	2732.5	No apparent impact (Effects found two order of magnitude higher than encountered in environment	3
A. tonsa	*A. catenella 1119/27*	Mono-specific feeding	1	1000	toxic	2732.5	Increased ingestion	3
A. tonsa	*A. catenella 1119/27 & Rhodomonas* sp.	Mixed-diet feeding	1	1000 (25, 50, 75, and 100% toxic algae)	toxic and non-toxic	2732.5	No apparent impact (feeding selectivity to non-toxic cells)	3
A. tonsa	*A. catenella 1119/27*	Survival	10	1000	toxic	501.1	80% survival in 10 days	4
A. tonsa	*A. tamarense 1119/19*	Survival	10	1000	non-toxic	-	60% survival in 10 days	4
A. tonsa	*Rhodomonas* sp.	Survival	10	1000	non-toxic	-	73% survival in 10 days	4
A. tonsa	Control (No food)	Survival	10	-	-	-	0% survival in 9 days	4
A. tonsa	*A. catenella 1119/27*	Reproduction (exp.2)	2	1000	Toxic	3575.5	Reduced fecundity	4

A. tonsa	*A. tamarense 1119/19*	Reproduction (exp.2)	2	1000	Non- toxic	-	No apparent impact	4
A. tonsa	*Rhodomonas* sp.	Reproduction (exp.2)	2	1000	Non- toxic	-	No apparent impact	4
A. tonsa	Control (No food)	Reproduction (exp.2)	2	-	-	-	Reduced fecundity	4
A. tonsa	*A. catenella 1119/27*	Reproduction (exp.3)	2	1000	toxic	501.1	Reduced fecundity	4
A. tonsa	*A. catenella 1119/27 & A. catenella 1119/19*	Reproduction (exp.3)	2	1000 (50 % toxic)	toxic and non-toxic	501.1	No apparent impact	4
A. tonsa	*Rhodomonas* sp.	Reproduction (exp.3)	2	1000	non-toxic	-	No apparent impact	4
A. tonsa	*No food*	Reproduction (exp.3)	2	-	-	-	Reduced fecundity	4
A. tonsa	*A. catenella 1119/27*	Reproduction (exp.3)	2	100	toxic	501.1	No apparent impact	4
A. tonsa	*A. catenella 1119/27 & A. tamarense 1119/19*	Reproduction (exp.3)	2	100 (50% toxic)	toxic and non-toxic	501.1	No apparent impact	4
A. tonsa	*Rhodomonas* sp.	Reproduction (exp.3)	2	100	non-toxic	-	No apparent impact	4

C. helgolandicus	*A. catenella 1119/28*	Feeding	3	400	toxic	3130	Reduced ingestion	5
C. helgolandicus	*A. catenella 1119/28 & A. catenella 1119/33*	Feeding	3	400 (25, 50, 75% toxic algae)	toxic and non-toxic	3130	reduced ingestion	5
C. helgolandicus	*A. catenella 1119/33*	Feeding	3	400	non-toxic	3130	No apparent impact	5
C. helgolandicus	*A. catenella 1119/28*	Reproduction	**4**	400	toxic	3130	No apparent impact	5
C. helgolandicus	*A. catenella 1119/28 & A. catenella 1119/33*	Reproduction	4	400 (25, 50, 75% toxic algae)	toxic and non-toxic	3130	No apparent impact	5
C. helgolandicus	*A. catenella 1119/33*	Reproduction	4	400	non-toxic	3130	No apparent impact	5

Representative of multiple reaction monitoring (MRM) for PST analogues following analysis of *Alexandrium catenella (*1119/27) extract.



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