Chemical Communication in Microbial Phytoplankton Communities

Allelopathy and Algicidal Bacteria

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Contents

List of Abbreviations

ASW	artificial seawater
chl *a*	chlorophyll *a*
DMSP	Dimethylsulfoniopropionate
DOC	dissolved organic carbon
DOM	dissolved organic matter
Dt	*Dunaliella tertiolecta*
EDTA	Ethylenediaminetetraacetic acid
Eh	*Emiliania huxleyi*
Fp	*Florenciella parvula*
HAB	Harmful Algal Bloom
HLB	Hydrophilic-lipophilic balanced
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
MB	marine broth
MeOH	methanol
Ot	*Ochromonas triangulate*
Pg	*Pavlova gyrans*
POC	particulate organic carbon
POM	particulate organic matter
Pp	*Prymnesium parvum*
RFU	relative fluorescence unit
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction

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Summary

Phytoplankton fuel the marine food web and play a role in all major biogeochemical cycles in our ecosystem. They are responsible for over half of the world's CO_2 fixation as well as the production of half the global oxygen supply annually. Humans rely on phytoplankton to not only sustain life, but in the production of goods and services as well. Though these communities are generally considered beneficial to all living organisms, a subset of these phytoplankton can produce toxins which can harm other members of the local community and bioaccumulate through higher trophic levels, affecting larger marine animals and even humans. The over proliferation of these toxic and other non-toxic species can cause additional harm to the local environment by inducing anoxic conditions, suffocating the surrounding organisms, termed harmful algal blooms (HABs). Phytoplankton communities are comprised of a complex consortium of interacting microorganisms, grazers, and layers of higher trophic level predators which communicate through the production and reception of chemical cues in the environment. Biotic factors influencing microbial community interactions include predation by herbivores, chemical defenses, intraspecific signaling, competition, and predation by other microorganisms ie algicidal bacteria, viruses and parasites. Microbial communication in the form of allelopathic communication between phytoplankton species and lysis or inhibition of phytoplankton by algicidal bacteria is thought to be key elements in the determination of species succession and bloom control within phytoplankton communities.

Allelopathic chemicals can facilitate competition between phytoplankton species and influence community composition and succession. Much is still unknown about the identities of these allelochemicals, and the ecological relevance of laboratory derived results are often unclear. We sought to evaluate the sensitivity of this relationship by employing a systematic method to investigate phytoplankton allelopathy. We used *Prymnesium parvum* as a model for assessing the influence of media, evaluation methods (chl *a* versus cell density), and species-specific interactions on observed allelopathy in laboratory co-cultivations with five ecologically relevant phytoplankton species. Our study demonstrated the species-specific nature of allelopathic interactions, and the pronounced effects derived from slight variations of laboratory parameters. This variability may impact the ecological insight gained from co-culturing experiments with implications on the interpretation of internal regulation within diverse phytoplankton communities. We support our findings through the identification of interaction patterns from mining the TaraOceans database for trends in natural observations relevant to our studies.

Interest in algicidal bacteria has risen due to their potential as biotic regulators of harmful algal blooms. *Kordia algicida* strain OT-1 is a model marine algicidal bacterium that was isolated from a bloom of the diatom *Skeletonema costatum*. Previous work has suggested that the algicidal activity is mediated by secreted proteases. We used a transcriptomics-guided approach to identify the serine protease gene *KAOT1_RS09515*, hereby named *alpA1* as a key element in the algicidal activity of *K. algicida*. AlpA1 was then expressed and purified from a heterologous host and used *in vitro* bioassays to validate its activity. Finally, we showed that *K. algicida* was the only algicidal species in the *Kordia* genus. The identification of this algicidal protease opens up the possibility of real-time monitoring of the ecological impact of algicidal bacteria in natural phytoplankton blooms.

Algicidal bacteria used for bioengineering may not necessarily require ecological relevance within natural blooms, but rather target specificity for the control of bloom species. Investigating the target range of algicidal bacterium would therefore benefit an ecological understanding of the impact these organisms have on phytoplankton communities, improving the predictability of the succession of species, potential bloom control, and provide insight to algicidal resistance. We surveyed 37 species of phytoplankton and a total of 48 isolates for susceptibility towards *K. algicida* attack. We observed high species-specificity for *K. algicida* and identified 23 resistant phytoplankton species. Variability in repeat measures indicated that the condition of the phytoplankton also plays an important role in determining the susceptibility to algicidal attack. Comparative analysis of these susceptible and resistant species may provide insight into the metabolic characteristics which determine algal response to *K. algicida*.

A common theme which arose in the investigation of these chemically mediated interactions was the prominence of species-specificity. Despite the complexity of phytoplankton communities, targeted interactions seem to play a role in the internal regulation of these populations. The sum of these interactions likely determines the fate of these communities, influencing species succession and bloom termination. These interactions can be influenced by parameters that are chosen within controlled laboratory conditions, which makes translation to natural environments difficult. To gain meaningful ecological insight, laboratory studies must consider the various environmental and biological factors which regulate these communities and mimic the natural environment to the fullest extent. We may then unravel the complex chemical signaling which governing the interactions of bloom dynamics.

Zusammenfassung

Phototrophes Phytoplankton ist die Grundlage des marinen Nahrungsnetzes und spielt eine Rolle in allen wichtigen biogeochemischen Kreisläufen in unserem Ökosystem. Es ist für mehr als die Hälfte der weltweiten CO2-Fixierung sowie für die Produktion der Hälfte des weltweiten Sauerstoffs pro Jahr verantwortlich. Der Mensch ist auf das Phytoplankton nicht nur zur Erhaltung des Lebens angewiesen, sondern auch bei der Produktion von Waren und Dienstleistungen. Obwohl diese Gemeinschaften im Allgemeinen als vorteilhaft für alle lebenden Organismen angesehen werden, kann eine Untergruppe dieses Phytoplanktons Giftstoffe produzieren, die andere Mitglieder der lokalen Gemeinschaft schädigen und sich über höhere trophische Ebenen bioakkumulieren können, was wiederum größere Meerestiere und sogar den Menschen beeinträchtigt. Die übermäßige Vermehrung dieser giftigen und anderer ungiftiger Arten kann der lokalen Umwelt zusätzlichen Schaden zufügen, indem sie anoxische Bedingungen hervorruft, die die umliegenden Organismen ersticken, was als schädliche Algenblüte (HAB) bezeichnet wird. Phytoplankton-Gemeinschaften bestehen aus einem komplexen Konsortium von interagierenden Mikroorganismen, Zooplankton und Räubern höherer Trophiestufen, die durch die Produktion und den Empfang chemischer Signale in der Umwelt miteinander kommunizieren. Zu den biotischen Faktoren, die die Interaktionen zwischen den mikrobiellen Gemeinschaften beeinflussen, gehören Prädation durch Pflanzenfresser, chemische Abwehrprozesse, intraspezifische Signalübertragung, der Wettbewerb und die Prädation durch andere Mikroorganismen, d. h. algizide Bakterien, Viren und Parasiten. Man geht davon aus, dass mikrobielle Kommunikation in Form von allelopathischer Kommunikation zwischen Phytoplanktonarten und Lyse oder Hemmung von Phytoplankton durch algizide Bakterien Schlüsselelemente bei der Bestimmung der Artensukzession und der Kontrolle der Blüte in Phytoplanktongemeinschaften sind.

Allelopathische Chemikalien können den Wettbewerb zwischen Phytoplanktonarten fördern und die Zusammensetzung und Abfolge der Gemeinschaften beeinflussen. Vieles ist noch unbekannt über die Identität dieser Allelochemikalien, und die ökologische Relevanz der im Labor gewonnenen Ergebnisse ist oft unklar. Wir haben versucht, die Empfindlichkeit dieser Beziehung zu bewerten, indem wir eine systematische Methode zur Untersuchung der Phytoplankton-Allelopathie eingesetzt haben. Wir verwendeten *Prymnesium parvum* als Modell, um den Einfluss von Medien, Bewertungsmethoden (chl a versus Zelldichte) und artspezifischen Wechselwirkungen auf die beobachtete Allelopathie in Labor-Co-Kulturen mit fünf ökologisch relevanten Phytoplanktonarten

zu untersuchen. Unsere Studie zeigte die artspezifische Natur der allelopathischen Interaktionen und die ausgeprägten Effekte, die sich aus geringen Variationen der Laborparameter ergeben. Diese Variabilität kann sich auf die ökologischen Erkenntnisse auswirken, die aus Co-Kultivierungsexperimenten gewonnen werden, und hat Auswirkungen auf die Interpretation der internen Regulierung innerhalb verschiedener Phytoplanktongemeinschaften. Wir untermauern unsere Ergebnisse durch die Identifizierung von Interaktionsmustern, die sich aus der Durchsicht der TaraOceans-Datenbank auf Trends in natürlichen Beobachtungen ergeben, die für unsere Studien relevant sind.

Das Interesse an algiziden Bakterien ist aufgrund ihres Potenzials als biotische Regulatoren von schädlichen Algenblüten gestiegen. Kordia algicida Stamm OT-1 ist ein Modellbakterium, das aus einer Blüte der Kieselalge Skeletonema costatum isoliert wurde. Frühere Arbeiten deuten darauf hin, dass die algizide Aktivität durch sekretierte Proteasen vermittelt wird. Mit Hilfe eines transkriptomischen Ansatzes identifizierten wir das Serinprotease-Gen KAOT1_RS09515, hier alpA1 genannt, als Schlüsselelement für die algizide Aktivität von K. algicida. AlpA1 wurde dann in einem heterologen Wirt exprimiert und gereinigt und seine Aktivität in In-vitro-Bioassays validiert. Schließlich konnten wir zeigen, dass K. algicida die einzige algizide Spezies in der Gattung Kordia ist. Die Identifizierung dieser algiziden Protease eröffnet die Möglichkeit einer Echtzeitüberwachung der ökologischen Auswirkungen algizider Bakterien in natürlichen Phytoplanktonblüten.

Algizide Bakterien, die für das Bio-Engineering eingesetzt werden, müssen nicht unbedingt ökologisch relevant sein, sondern eher spezifisch auf die Bekämpfung von Blütenarten ausgerichtet sein. Die Untersuchung des Zielspektrums algizider Bakterien würde daher ein ökologisches Verständnis der Auswirkungen dieser Organismen auf Phytoplanktongemeinschaften fördern, die Vorhersagbarkeit der Sukzession von Arten und die potenzielle Kontrolle von Blüten verbessern und einen Einblick in die Algizidresistenz geben. Wir untersuchten 37 Phytoplanktonarten und insgesamt 48 Isolate auf ihre Anfälligkeit gegenüber K. algicida. Wir stellten eine hohe Artenspezifität für K. algicida fest und identifizierten 23 resistente Phytoplanktonarten. Die Variabilität der Wiederholungsmessungen deutet darauf hin, dass der Zustand des Phytoplanktons ebenfalls eine wichtige Rolle bei der Bestimmung der Anfälligkeit für den Algizidbefall spielt. Eine vergleichende Analyse dieser anfälligen und resistenten Arten könnte Aufschluss über die metabolischen Merkmale geben, die die Reaktion der Algen auf K. algicida bestimmen.

Ein gemeinsames Thema, das sich bei der Untersuchung dieser chemisch vermittelten Wechselwirkungen herauskristallisierte, war die herausragende Bedeutung der Speziesabhängigkeit. Trotz der Komplexität von Phytoplanktongemeinschaften scheinen gezielte Wechselwirkungen eine Rolle bei der internen Regulierung dieser Populationen zu spielen. Die Summe dieser Wechselwirkungen bestimmt wahrscheinlich das Schicksal dieser Gemeinschaften und beeinflusst die Artenfolge und das Ende der Blüte. Diese Wechselwirkungen können durch Parameter beeinflusst werden, die unter kontrollierten Laborbedingungen gewählt werden, was eine Übertragung auf natürliche Umgebungen erschwert. Um aussagekräftige ökologische Erkenntnisse zu gewinnen, müssen Laborstudien die verschiedenen Umwelt- und biologischen Faktoren berücksichtigen, die diese Gemeinschaften regulieren, und die natürliche Umgebung so weit wie möglich nachahmen. Auf diese Weise können wir die komplexe chemische Signalübertragung entschlüsseln, die die Wechselwirkungen der Blütendynamik bestimmt.

Translated by DeepL

1) Introduction

1.A.   Phytoplankton contributions to biogeochemical cycles and ecosystem health

Phytoplankton are a collection of diverse microscopic photosynthetic protists and cyanobacteria that live in aquatic environments, fueling food webs and driving biogeochemical cycling (Rousseaux and Gregg 2015). Phytoplankton communities are the primary producers within aquatic food webs and support the biosphere in nutrient cycling and redistribution (Naselli-Flores and Padisák 2022). Approximately half of Earth's total global oxygen production is derived from phytoplankton sources, and about half of the global net primary production is derived from photosynthetic activity of these phytoplankton communities (Behrenfeld et al. 2001, Field et al. 1998). The dissolved organic matter (DOM) and particulate organic matter (POM) that is released from lysed or damaged biomass is transformed and used as an energy source for bacteria in the water column, which is eventually returned to the food web through predation by successive trophic levels (Azam et al. 1983, Worden et al. 2015). This microbial sequestration and cycling of carbon are commonly referred to as the microbial-loop, that is only possible by the initial primary production of diverse phytoplankton species in the ecosystem. The complex interactions involved in the bacterial transformation of phytoplankton primary production were summarized by Buchan and colleagues in Figure 1.

Nitrogen is the most abundant element in the air. Half the estimated global biological N_2 comes from planktonic cyanobacteria in the oceans (Bergman et al. 2013, Monteiro et al. 2010). These diazotrophic cyanobacteria, termed for their ability to fix inorganic nitrogen into organic nitrogen, making it available to other non-diazotrophic phytoplankton and secondary producers (Jabir et al. 2013). The availability of organic nitrogen can be a significant limiting factor for marine primary production (Falkowski 1994), thus the diazotrophic nature of some members of the phytoplankton community provides a community service to the benefit of all non-diazotrophic members.
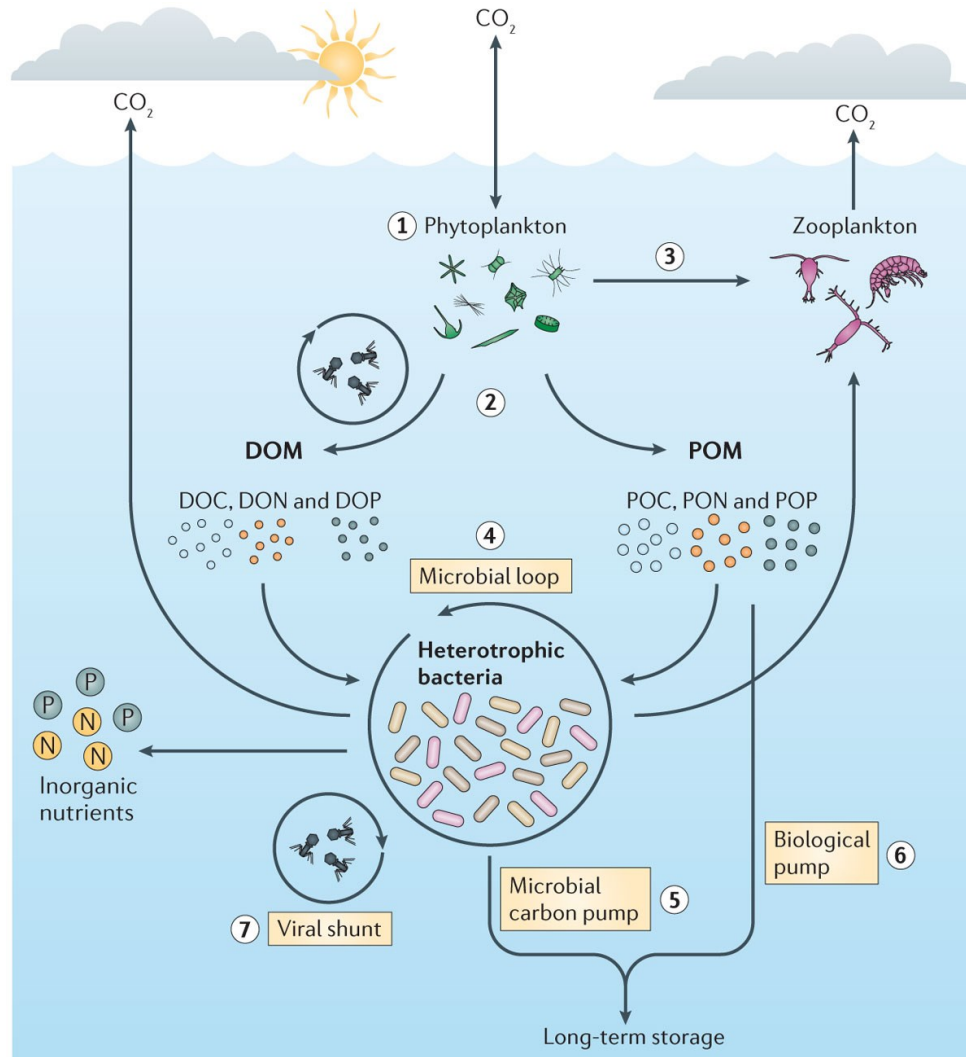
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Figure 1. Depiction of marine carbon cycle. The (1) phytoplankton convert inorganic carbon into organic carbon via photosynthesis which is (2) release of dissolved organic matter (DOM) or particulate organic matter (POM) or (3) further consumed by grazers. Released organic matter is transformed (4) by heterotrophic bacteria via the microbial loop and re-mineralized. Some of the recalcitrant dissolved organic carbon (DOC) that cannot further degrade is (5) sequestered to the deep sea for long terms. Similarly (6) shows the sinking effect of POM that is not transformed by the microbial loop. Additionally, viruses (7) contribute to the lysis of phytoplankton biomass releasing DOM and POM (Buchan et al. 2014).

Phosphorus is also considered as a limiting nutrient within phytoplankton communities (Paytan and McLaughlin 2007). Contrary to nitrogen, phosphorus cannot be fixed from the atmosphere and is primarily delivered to the oceans through weathering as it is most abundantly found in the Earth's crust (Paytan and McLaughlin 2007, Toggweiler 1999, Tyrrell 1999). Inorganic phosphorus is generally considered the most bioavailable form of phosphorus, but the concentrations of dissolved organic phosphorus far exceed it in the surface layer of the open ocean, where phosphorus is rapidly cycled by planktonic microbes (Dyhrman et al. 2007, Paytan and McLaughlin 2007). Phytoplankton take up dissolved inorganic phosphorus and transform it into organic phosphorus (Cotner Jr and Wetzel 1992). When they are ingested by grazers, much of the organic phosphorus gets excreted as dissolved inorganic and organic phosphorus (Cotner and Biddanda 2002). Injured or lysed phytoplankton may also release dissolved inorganic and organic phosphorus into the water column (Anderson and Zeutschel 1970). This released phosphorus is then assimilated by other phytoplankton or bacteria and the cycle is continued in the water column (Azam et al. 1983, Cotner and Biddanda 2002). Some of this dissolved phosphorus, in the form of particulate matter may detour from this cycle and fall into a deep sea sink of phosphorus (Delaney 1998, Faul et al. 2005), which may re-enter the cycle upon events such as deep sea upwelling (Conkright et al. 2000).

In addition to metabolic contributions to elemental cycling, the physiology of certain phytoplankton groups makes them large sinks for remineralization. Marine coccolithophores represent a large group of phytoplankton which contribute up to 20% of marine primary production (Poulton et al. 2017). Their namesake derives from the scales of calcium carbonate which cover their cells, which sink to the deep ocean upon death of these cells (Poulton et al. 2017). The formation of these shells contribute to the direct sequestration of 20-35% global CO_2 emissions (Khatiwala et al. 2009) and are the reason for the large impact (60%) of phytoplankton to carbonate flux in the ecosystem (Haidar et al. 2000, Haunost et al. 2021). Another group of phytoplankton with interesting cell wall anatomy are diatoms, which are highly abundant and contribute about 20% of global primary production (Field et al. 1998). Diatom cell walls are formed by template-catalyzed precipitation of silicic acid (Kroger et al. 2002), which drives the silicate cycle due to their abundance in the ecosystem (Tréguer and De La Rocha 2013, Yool and Tyrrell 2003). The large quantity of silica precipitated by diatoms each year makes them a major sink in the global silica cycle (Bondoc et al. 2016).

Many phytoplankton species produce dimethylsulfoniopropionate (DMSP), an important metabolite in the marine sulfur cycle produced at 2 billion tons of sulfur annually (Ksionzek et al.

2016). Enzymatic lysis of DMSP produces volatile dimethyl sulfide (DMS) which is the main source of organosulfur in the atmosphere (Lana et al. 2011), but also potentially affects cloud formation and climate regulation (Charlson et al. 1987). Phytoplankton may release dissolved DMSP into the environment via exudation, injury, or lysis, which are taken up by other marine microbial organisms (Kiene et al. 2000, Todd et al. 2007, Yoch 2002).

1.B.   Anthropogenic exploitability of phytoplankton

We have shown that phytoplankton are vital to the function of the biosphere and implicated in the formation of clouds, thus impacting weather conditions and climate regulation. It is not surprising that monitoring these communities can infer the health of the marine ecosystem. The rapid growth rates and adaptability to changing environmental conditions makes them suitable early warning signals for ecosystem changes for human benefit (Salmaso et al. 2012). Biological monitoring of phytoplankton in aquatic ecosystems is included in the assessment of ecological quality of lake and river surface waters as part of the EU Water Framework directive (Birk et al. 2012).

The great diversity of species in phytoplankton communities provides a wealth of chemicals and potentially bioactive compounds. The relatively rapid growth and ease of cultivation make it appealing to the exploitability for human benefit. Historically, phytoplankton have been used as a food source by humans for centuries (Spolaore et al. 2006). In the last 60 years, more systematic approaches have been used to consider phytoplankton biomass as a widespread new food source (Becker 2004). Nutritionally, algae contains many biologically active metabolites including proteins, carbohydrates, lipids, trace elements, vitamins, antioxidants, carotenoids, sterols, inorganic and organic minerals, chlorophyll and enzymes which may be commercially exploitable and make them appealing to many industries (Napiórkowska-Krzebietke 2017, Yaakob et al. 2014).

The use of microalga in aquaculture feed has been used for many years. The marine cyanobacteria *Phormidium valderianum* was successfully used as a complete aquaculture feed source, based on nutritional qualities and non-toxic nature (Thajuddin and Subramanian 2005). The diatom *Thalassiosira pseudonana* is cultivated to feed a variety of mollusks, and the addition of algae in fish meal for shrimp cultivation increased shrimp growth rates (Yaakob et al. 2014). The value of algal nutritional supplementation extends beyond aquaculture feeds, as studies have demonstrated increased weight in different livestock animals including sheep and pigs, with algal supplementation (Yaakob et al. 2014). The Institute für Getreideverarbeitung (Bergholz-Rehbrücke, Germany) produces a natural feed with algae called Algrow for poultry (Pulz and Gross 2004).

The large amounts of lipids found in microalgal cells make them particularly interesting to the cosmetic and biofuel industries (Jacob-Lopes et al. 2019, Lupette and Maréchal 2018, Pienkos and Darzins 2009, Vanthoor-Koopmans et al. 2013). Lipids in cosmetics perform different functions from moisturizing agents, emollient and softening agents, surfactants and emulsifiers, texturizers, preservatives, to product integrity maintenance (De Luca et al. 2021). The unsustainable burning of fossil fuels has contributed to the climate change crisis in our current environment. In an effort to reduce fossil fuel consumption, efforts have been invested in biofuel production from microalgae (Pienkos and Darzins 2009, Vanthoor-Koopmans et al. 2013), as the many species produce large amounts of hydrocarbons which may be suitable for biodiesel production, and are relatively quickly cultures at large quantities (Mucko et al. 2020, Razeghifard 2013). In line with the investigation of sustainable alternatives for biofuel, microalgae have also been implicated in the "green chemistry" space, for production of bioplastics and textiles (Onen Cinar et al. 2020). The range of interesting biomolecules and culturability sparks potential as biorefineries for the sustainable production of compounds for the many industries of interest (Vernès et al. 2019).

Of the many bioactive compounds produced by phytoplankton, the toxins which characterize harmful algal blooms are especially interesting for pharmaceutical, nutraceutical and biotechnological industries due to their potential as medicinal remedies and biological exploratory probes (Pradhan and Ki 2022). Though these toxins are harmful to other organisms, the apoptotic effects are reminiscent of anticancer properties and currently explored as treatment options for various cancer types (Pradhan and Ki 2022). Their large range of activity profiles shows potential as therapeutic agents for other disorders like dyslipidemia, obesity, diabetes, and hypertension (Pradhan and Ki 2022). Okadaic acid is a predominantly dinoflagellate derived toxin that is naturally abundant and accumulated by shellfish (Valdiglesias et al. 2013). It has been implicated in a wide range of potential therapeutics including cancer, AIDs, fungal disease, and neurodegenerative disease such as Alzheimer's and schizophrenia (Borowitzka 1995, He et al. 2005, Kamat et al. 2013, Liu and Sidell 2005, Nagai et al. 1990, Re et al. 1995). Gonyautoxins have been implicated in pain management (Garrido et al. 2005), while karlotoxins were investigated in alleviation of coronary heart disease (Waters et al. 2010). Cyanobacteria as a group produce many bioactive secondary metabolites in addition to cyanotoxins which endanger aquatic organisms as well as humans when exposed (Zanchett and Oliveira-Filho 2013). The natural cytotoxicity of cyanotoxins are of particular interest due to the effects on multiple tumor cell lines (Mondal et al. 2020). Additionally, the bioactive compounds in cyanobacteria have antibacterial, antifungal, antituberculosis, immunosuppressive,

antioxidant and anti-inflammatory properties (Pradhan and Ki 2022), and could impact the development of new medications.

The growth of phytoplankton communities is thus not only beneficial for our ecosystem but provides many advantages for the development of human resources addressed here, which is summarized in Figure 2, adapted from Naselli-Flores and Judit (2022).
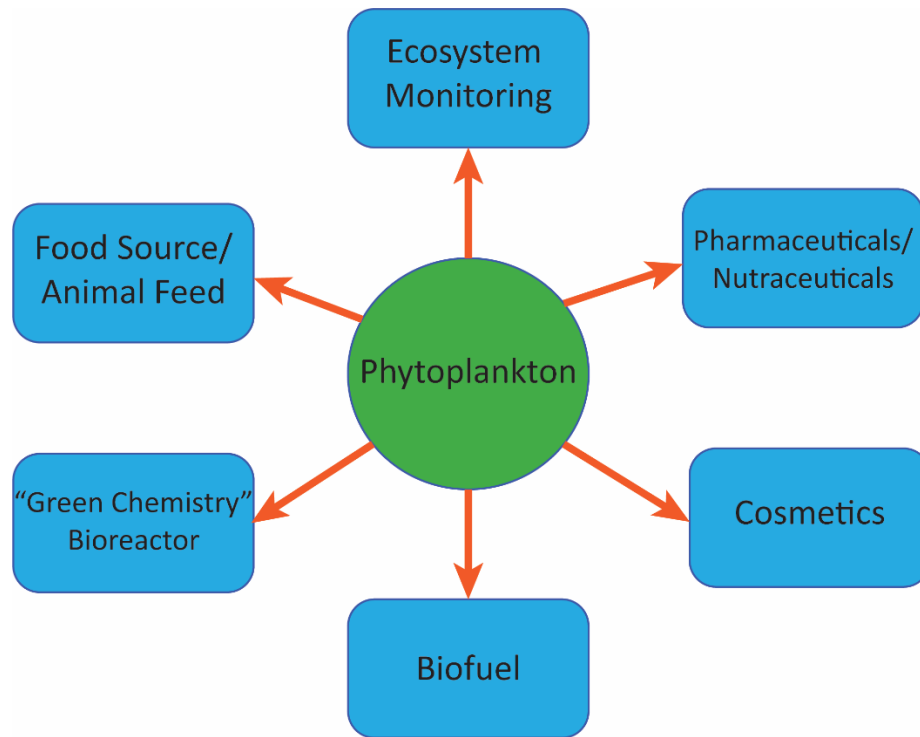
Figure 2. Anthropogenic benefits from phytoplankton

1.C.   Harmful algal blooms

Unfortunately, the phrase "you can have too much of a good thing" also applies in this system. Phytoplankton blooms are a common occurrence where suitable environmental conditions support algal proliferation and accumulation in surfaces of aquatic environments and are generally regarded as beneficial to the ecosystem due to the increased primary production, oxygen production and nutrient cycling. However, as mentioned previously, certain species of phytoplankton produce toxins that harm the environment and bloom are designated harmful algal blooms (HABs). A prominent concern of these types of HABs is the potential harm to local organisms and the danger to human health from exposure to contaminated shellfish or aerosolized toxins (Grattan et al. 2016).

Human exposure may lead to some the following toxin-based conditions: Paralytic Shellfish Poisoning, Diarrhetic Shellfish Poisoning, Amnesic Shellfish Poisoning, and Azaspiracid Shellfish Poisoning (Hallegraeff et al. 2021). Toxins are produced by a range of microalgae: dinoflagellates, cyanobacteria, diatoms, haptophytes, raphidophytes, dictyochophytes, and pelagophytes, with dinoflagellates dominating in the contributes of toxic species (Hallegraeff et al. 2021). HABs can also include non-toxic species, that harm the environment due to proliferation at high biomasses that cause anoxia, depriving the local ecosystem of oxygen and harming other organisms within the vicinity, and visibly cause water discoloration (Hallegraeff et al. 2021). A NASA satellite image of a December 2021 HAB on the coast of Rio, Brazil reveals the massive scale these blooms can reach, Figure 3.
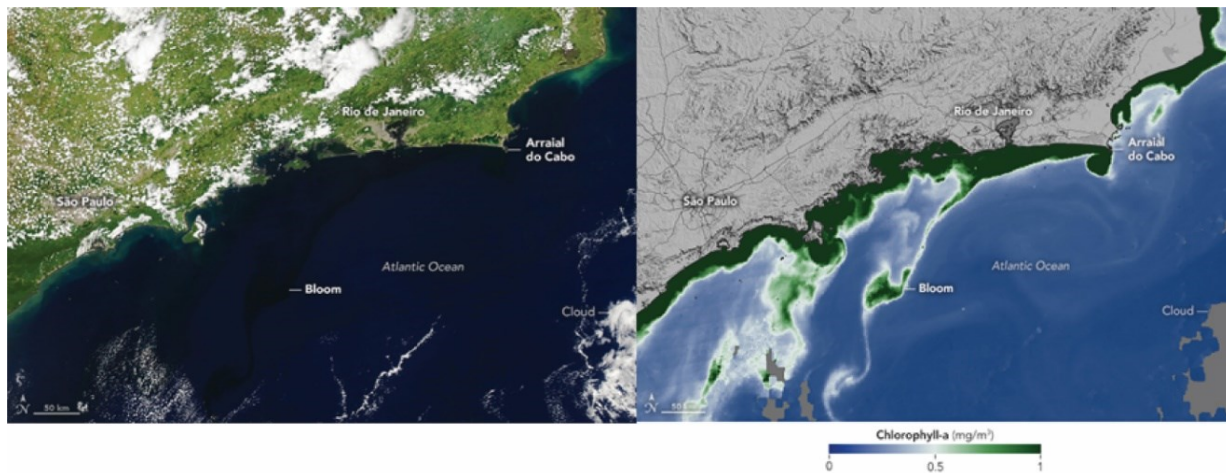
Figure 3. HAB on coast of Rio (NASA). Massive HAB caused by dinoflagellate *Mesodinium rubrum* on the coast of Rio, Brazil. Images taken on NASA Aqua satellite using Moderate Resolution Imaging Spectroradiometer (MODIS) on 26 December 2021. Natural color image on left, depiction of chlorophyll-a concentrations on right (Observatory 2021).

The looming climate crises aroused concerns that the global frequency of HAB events were increasing due to climate change. A recent study by Hallegraeff and colleagues (2021) found no universal trend in the occurrence of HABs over time; but the perception of increase was due to intensified monitoring, partially associated with increased aquaculture production. However, recent studies show a positive correlation on regional occurrences of phytoplankton blooms. Coastal ecosystems experience widespread eutrophication due to increased human activity and pollutants in coastal zones (Anderson et al. 2002, Glibert and Burford 2017, Heisler et al. 2008). In a study

collected over 45 years on the coastal waters of China, researchers found an increase in frequency, duration and extent of HABs, with correlations to eutrophication and ocean warming (Xiao et al. 2019), consistent with projection of increased incidence of HAB frequency and distribution with future climate change (Barton et al. 2016, Gobler 2020). The conflicting data between global and regional activity may be due to anthropogenic influences and scale. Additionally, satellite images have been used to evaluate coastal phytoplankton blooms over a 17-year period (2003-2020) in correlation to sea surface temperatures (SST). The recently published study found a positive correlation between annual mean blooming frequency and increase in SST (Dai et al. 2023). The authors openly acknowledged the limits of detection of blooms based on visible fluorescence signals with potential omissions of low concentration HABs below the detection threshold for this method, and the inability to distinguish between normal and toxic blooms. However, they argue that the conclusions were likely an underestimation of the trends as the metrics were consistent across space and time (Dai et al. 2023).

For context on the catastrophic impact these trends have on anthropogenic institutions, we look at an extreme case of HABs that led to the largest fish farm mortality recorded in history. In an unfortunate phenomenon of compounded El Niño warming surface waters and a positive phase of the Southern Annular Mode (SAM), led to a strong drought with record low rainfall, which increased coastal eutrophication that resulted in two successive HAB events in the coast of southern Chile in 2016. Both blooms consisted of ichthyotoxic microalgae. The first bloom containing the dictyochophyte *Pseudochattonella* c.f. *verruculosa* in February which started in the northern region of the Patagonian fjords and expanded south. The second bloom was dominated by the dinoflagellate *Alexandrium catanella* in late March, which started in the southern region of the Patagonian fjords and expanded north (Trainer et al. 2020). The convergence of these events became known as the "Godzilla-Red tide event" due to the massive size and disastrous impact on Chilean aquaculture, with estimated losses of USD$800 million, and 100,000 metric tons of Atlantic and Coho salmon and trout (Clement et al. 2016, Trainer et al. 2020).

We study phytoplankton communities with consideration of finding methods to mitigate the harmful effects of HABs without eliminating the inherent ecological benefits. Predictions of the effect of climate change on HABs are limited by the inability to predict how strains/species of phytoplankton and evolution will change current traits; as many laboratory experiments on marine species interactions are often not substantiated by ecological observations (Legrand et al. 2003). The high species diversity and multitude of abiotic factors within natural phytoplankton communities

cannot be fully replicated in conventional laboratory conditions, but persistence in unraveling the specific interactions between different members within the community would allow us to make better predictions for the ecosystem in the future. Now that we appreciate the critical role of phytoplankton communities in the biosphere, and the value of how studying this system has inherent global benefits, we shift our focus to the investigation of different interactions between phytoplankton community members. Specifically, the role of chemical cues for internal regulation of bloom dynamics and species succession, with the potential for discovery of biotic or biochemical means of mitigating HABs.

1.D.   Chemical communication in phytoplankton communities

Understanding ecological interactions between microbes of the pelagic marine environment is notoriously difficult due to the nature of the interactions. In an environment dominated by primitive single celled organisms lacking audible or visual cues, these species must develop a way to sense the changes in the environment for their own fitness and survival. The open water marine environment is very diluted and constantly changing due to physical turbulence, but the physical properties of water make it an ideal medium for chemical signal interactions (Pohnert et al. 2007). The detection and emission of chemical cues thus makes a reasonable avenue for communication and the search for vital nutrients. Chemical ecology is the study of the origin, function and significance of the chemicals produced that mediate interactions within and between organisms in an ecosystem (Harborne 2001). These cues, known as secondary metabolites (natural products not directly involved in primary metabolism) can serve as protection against enemies, or for feeding and reproduction (Ianora et al. 2006). These compounds are believed to be the basis of ecological specialization, affecting species' distribution patterns and organization, influencing feeding patterns and biodiversity through resource and habitat partitioning (McClintock and Baker 2001). Studies have shown that secondary metabolites in phytoplankton communities also regulate and control algal biology, species succession, competition and communication (Legrand et al. 2003). In these complex microbial communities, the production of secondary metabolites may have high energetic costs but may be compensated for by defensive benefits to the producing organism. It has been postulated that these natural products evolved under the pressure of natural selection to bind to specific receptors and show ecological responses of organisms to their environment (Williams et al. 1989), and developed both physical and chemical defenses for survival (Hamm et al. 2003). Various chemical cues serve different purposes depending on the emitters and receivers, for both interspecific and intraspecific

communication. Intraspecific communication allows for internal regulation within a species, such as the concentration dependence of quorum sensing in bacterial populations, as well as the identification of mates through pheromones for phytoplankton and grazer populations. Interspecific communication involves the recognition of producing organisms to elicit a response and may include chemical defense in predator-prey interactions, allelopathic competition, facilitation and mutualism in the context of algal-bacterial interactions, algicidal bacterial interactions, as well as parasitism. These various interactions between members of this phytoplankton microbial food web are represented in the scheme in Figure 4. We will discuss the importance of each of these chemically mediated interactions, but our main studies will focus on allelopathic competition of a model toxic phytoplankter with ecologically relevant partners, and the characterization of algicidal mode and hosts of an algicidal marine bacterium.

It is important to note that in this environment, the isolation and characterization of chemical cues is particularly difficult, as they are normally present in low concentrations, and must be reasonably water-soluble for the dispersion of signals (Roy et al. 2013). This has caused a dearth in the identification of chemical identities and structures of many of these chemical cues and continues to be a problem in the investigation of phytoplankton chemical ecology. Many reviews have suggested the integration of multidisciplinary fields, in addition to analytical chemistry techniques and omics platforms to characterize the signals and ecological relationship of such signals (Heuschele and Selander 2014, Roy et al. 2013). Even without structural identification, the impact of these compounds was evident in the behavior of their target organisms and provides insight into the biochemical regulation of phytoplankton communities.
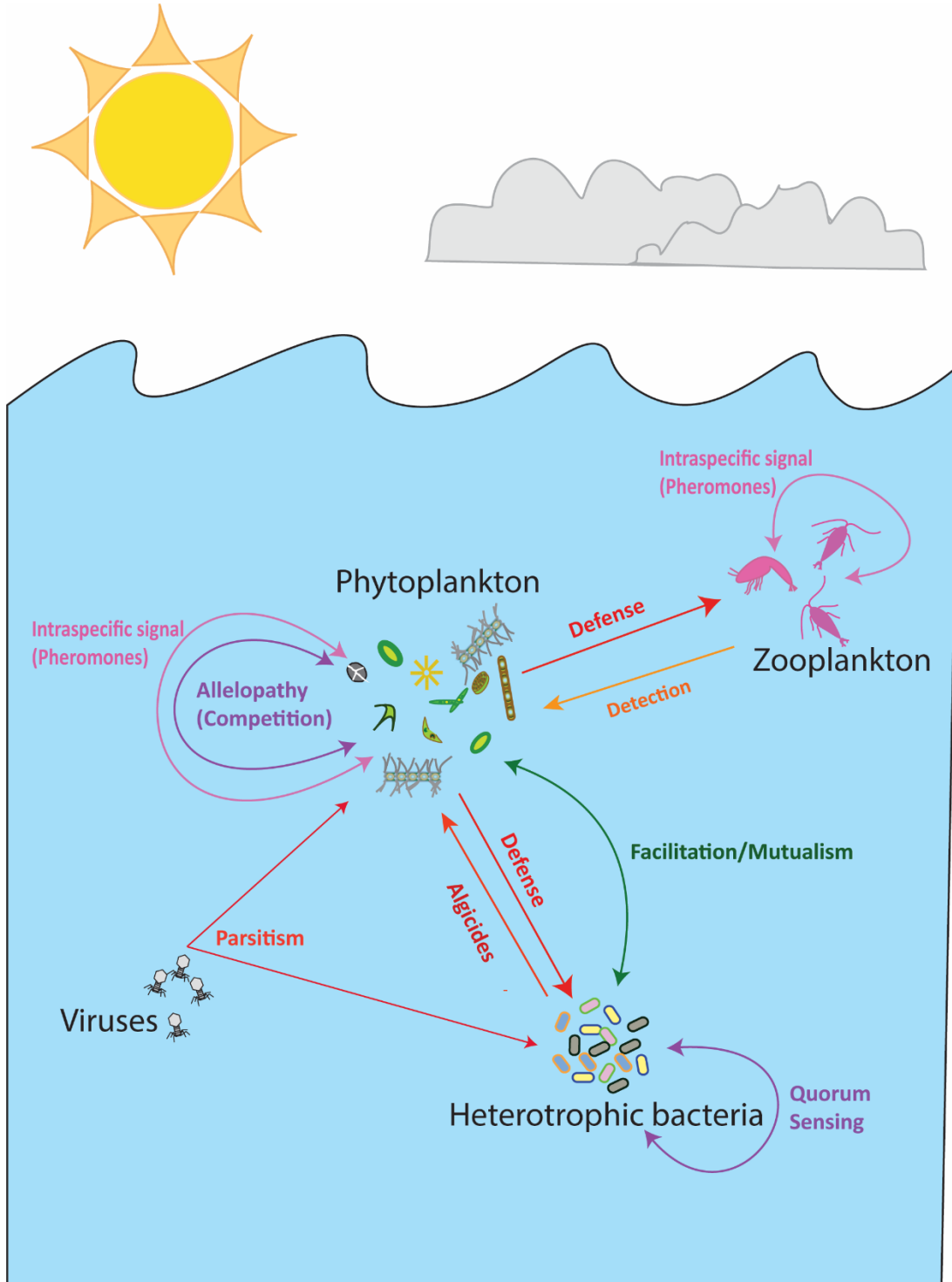
Figure 4. Varieties of inter- and intra- specific signaling in microbial phytoplankton food web

1.E.   Chemical defenses and predator-prey interactions

Marine phytoplankton are famous producers of secondary metabolites with predicted defense activity against predators (Brown et al. 2019). Defense signals may be constitutive, activated upon damage of cells, or induced by recognized chemical cues, Figure 5. The ecological purpose is the preservation of fitness for an organism, which may be achieved by the elimination or temporary immobilization of predators to allow escape. The filtrate of toxic phytoplankton *Prymnesium parvum* caused inactivity in copepod grazer *Eurytemora affinis*, which led to reduced copepod reproductive success and indicating a chemically based, constitutive anti-grazer defense in *P. parvum* (Sopanen et al. 2008). One of the most well-known wound-activated defenses in phytoplankton is the production of complex oxylipins, including polyunsaturated aldehydes (PUAs). These compounds have been shown to induce abortion or congenital malformation in copepod grazers (Ianora et al. 2006). The wounding of membrane lipids of certain microalgae activates an enzyme cascade leading to the production of volatile bioactive oxylipins from the oxidation of fatty acids (Pohnert 2000, Pohnert 2004, Pohnert 2005). Diatom derived aldehydes and metabolites with cytotoxic affect may interfere with reproduction capacity of grazers, thus reducing potential grazing stress on future generations (Miralto et al. 1999). Oxylipins also contribute to the poor food value of diatoms for copepods, and may contribute to poor reproductive outcomes due to low nutrient quality (Lauritano et al. 2012, Lauritano et al. 2015). However, when three strains of *Skeletonema marinoi* were evaluated for their effect on physiology, behavior, and reproductive success of three copepod species: *Acartia tonsa, Pseudocalanus elongatus*, and *Temora longicornis*, there were widely differing effects on copepod physiology. None of the strains of *S. marinoi* significantly altered copepod behavior and reproduction was affected differently and those changes were correlated to fatty acid and sterol content of the *S. marinoi* strains, rather than PUAs (Md Amin et al. 2011). The extent of PUA impact on copepod grazers has become debatable, and might not be as detrimental to copepod reproduction as previously thought, or species-specificity may play a stronger role in determining outcomes (Jónasdóttir et al. 2011). The swimming speed of the copepod *Temora longicornis* doubled when stimulated by a proxy for marine snow. These copepods were able to detect chemical trails released by the sinking particles of marine snow (Lombard et al. 2013).

Chemical defenses may also change with algal life cycle stage. *E. huxleyi* is chemically defended in the haploid state against ciliate predators *Strombidinopsis acuminatum*, but not in its diploid state. The ciliates showed reduced ingestion rates on *E. huxleyi* cells with prior exposure to

the predator, compared to non-exposed *E. huxleyi,* suggesting an inducible chemical defense, that was determined to be inside the cells and not secreted into the environment (Kolb and Strom 2013).

Prey organisms may detect chemical cues released by nearby predators which may trigger inducible defenses, while predators may recognize potential prey cues for feeding. This type of chemical signaling which benefits the receiver and not the host are known as kairomones and are important in driving predator-prey interactions in phytoplankton communities (Pohnert et al. 2007). Studies have shown prey response to predator kairomones by triggering inducible defenses in behavioral, morphological and physical traits (Pohnert et al. 2007).
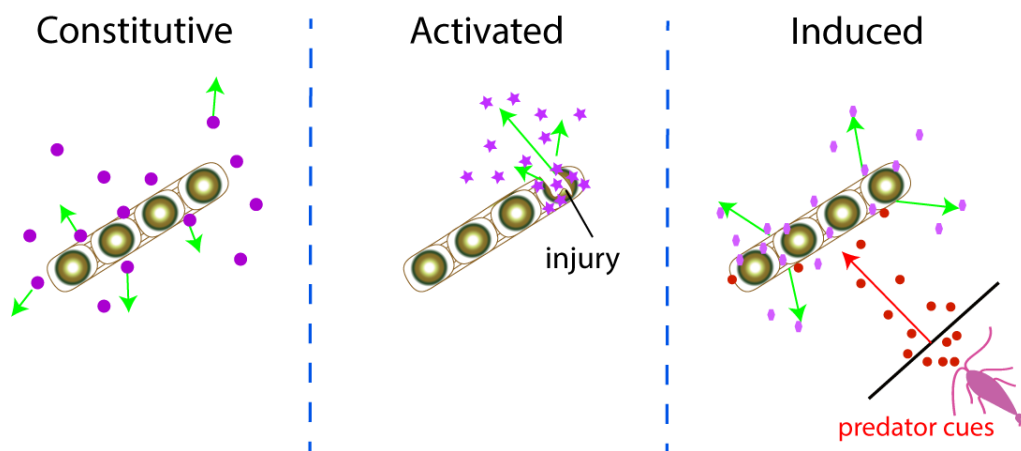
Figure 5. Constitutive, activated, and induced defenses in phytoplankton communities. Constitutive defenses are always produced. Wounding or death releases activated chemical defenses. The detection of predator cues causes the release of induced defenses.

1.F.   Prey detection and selectivity

Prey detection and capture is important to predator fitness. Chemical cues exuded or on the surface of prey help predators locate and differentiate the nutritional value of food. The mixotrophic dinoflagellates of genus *Alexandirum* have been shown to use both mucus trap and toxins in prey capture, by immobilizing prey prior to ingestion (Blossom et al. 2012). Dimethyl sulfide (DMS) the breakdown product of DMSP produced in many phytoplankton and has been known as a chemoattractant of protozoan grazers. DMS released as a result of cell lysis or excretion triggered a tail-flapping response in the copepod *Temora longicornis*, a behavioral change that altered flow patterns and predicted to assist the copepods in finding food sources (Steinke et al. 2006). DMS

might also be an infochemical for carnivores, attracting predators of planktonic grazers and reducing grazing pressure on the phytoplankton (Nevitt 2000).

Dimethylsulfide (DMS)

Dimethylsulfoniopropionate (DMSP)
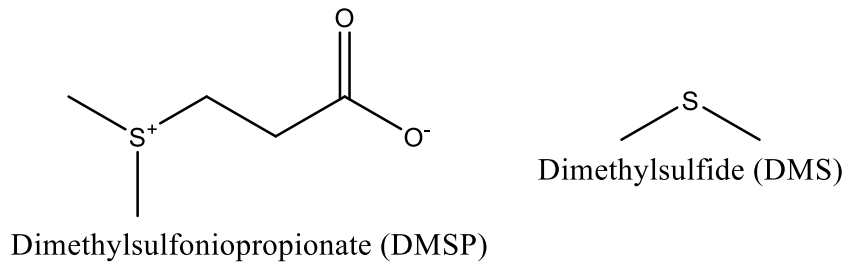
Mixotrophic and heterotrophic plankton might use chemical cues to locate and discriminate different prey types. The heterotrophic dinoflagellate *Oxyrrhis marina* displayed chemotaxis to toward synthetic DMSP while being repelled by acrylate (a degradation product of DMSP) indicating the ability of some predators to differentiate between distinct chemical cues to seek prey (Breckels et al. 2011). Two ciliates, *Chilodonella* sp. and *Tetrahymena* sp. preferentially consumed the bacteria *Pseudomonas costantinii* over *Serratia plymuthica* when presented with spatially separated biofilms of both bacterium or cell-free filtrates, suggesting the detection of exudates from the preferred prey species (Dopheide et al. 2011).

Chemoreception at cell surfaces can be utilized by protozoa to discriminate prey cells by surface carbohydrates. A calcium-dependent, mannose-binding lectin was found from surface proteins of the dinoflagellate *Oxyrrhis marina*, while mannose was detected on the surface of prey cells of this dinoflagellate. This indicated the potential for *O. marina* to use mannose-binding lectin for prey identification. Blocking the function of this lectin in live *O. marina* cells inhibited feeding by 60%, and the ability to distinguish between mannose-coated beads versus control beads was lost (Wootton et al. 2007).

1.G.   Predator avoidance

Prey avoidance mechanisms are common in the marine environment. *Alexandrium tamarense*, used chain splitting as a strategy to reduce effective size and avoided consumption by copepods, having become too small for grazing. This behavior was induced by indirect exposure to copepods, suggesting the detection of predator chemical cues by *A. tamarense* (Selander et al. 2012,

Selander et al. 2011). Additionally, when PST toxin production was measured in *A. tamarense*, exposure to grazing copepods induced production of toxin by nearly 300%, indicating the use of a combination of morphological changes and toxin production as defensive strategies post predator detection (Selander et al. 2012). Copepodamides were identified as predatory signals produced by copepods which induced toxin production in *Alexandrium minutum*, with ecologically relevant concentrations (Selander et al. 2015). Chain length reduction due to predation was also observed for the diatom *Skeletonema marinoi*, which preferred single or double chain-lengths in the presence of copepod grazers (Bergkvist et al. 2012). The opposite was shown in the gyrodinoid dinoflagellate *Cochlodinium polykrikoides*, which increased chain length in the presence of copepod grazer exudates, as a defensive strategy, by being physically too large for consumption; nutrient amendments with vitamins B1, B7, and B12 also increased chain length, also indicating the role of environmental factors in this morphological change (Jiang et al. 2010). Exposure to cells and filtrates of the ciliate predator of *Heterosigma akashiwa* caused the toxic raphidophyte to swim faster, indicative of a fleeing behavior upon detection of predator cues (Harvey and Menden-Deuer 2012). Based on these examples, this morphological, chemically induced, defensive strategy may be commonly used to avoid consumption by grazers.

Defensive strategies include the production of feeding deterrents to discourage consumption of conspecifics by grazers. Researchers determined apo-fucoxanthinoids produced by diatoms *T. pseudonana* and *Phaeodactylum tricornutum* acted as feeding deterrents to the copepod *Tigriopus californicus* at concentrations which may be ecologically relevant. Active concentrations were 1000x lower than the total concentration produced in *P. tricornutum (Shaw et al. 1995).*

The formation of biofilms may also confer defensive properties for grazers. The pathogenic bacterium *Vibrio cholerae* living on biofilms attached to chitinous zooplankton metabolizes chitin to exude ammonium which confers protection from protozoan predators. This augmentation of protective capacity of *V. cholerae* biofilms by the metabolism of chitin that produces antiprotozoal ammonium has been referred to as "metabolite-based grazing resistance" defense (Sun et al. 2015).

Surface chemistry might reduce predation for some cyanobacteria. Proteins on the surface of the cyanobacterium *Synechococcus* sp. increased susceptibility of predation. An evaluation of knockouts of glycoprotein SwmA, which has been previously implicated in the formation of the protective S layer and the large surface protein SwmB which is required for mobility, for their

protective benefits showed that SwmA and the presence of the S layer actually increased predation rates, contrary to the original hypothesis (Strom et al. 2017).

These defensive strategies rely on the accurate detection of predator cues to maximize fitness and avoid unnecessary energy expenditure. There is a high variability in species-specific grazing and consumer preference, with some species showing more resistance which may indicate evolved counter-defense or detoxification mechanisms in response (Ianora et al. 2006). Life-history parameters have been shown to influence resistance to defensive compounds. When two geographically different populations of the copepod *Acartia hudsonica* were raised on a diet of toxic dinoflagellate *Alexandrium fundyense*, the copepod with no prior exposure to *A. fundyense* blooms had lower somatic growth, maturity size, egg production and survival compared to the pre-exposed copepods (Colin and Dam 2003, Colin and Dam 2005). A metabolomics study was conducted using four different strains of *E. huxleyi* to observe the response to predation by the dinoflagellate *Oxyrrhis marina*. All strains were substantially variable in metabolic profiles in response to grazing pressure with less than 25% similarity between any two strains. None of the metabolites changed the same way in all strains. This revealed that responses to predation might not unify metabolism even within different strains of the same species. The differences in metabolic responses were more species-specific and important to understand predator-prey relationship.

1.H.   Toxins

The production of toxins by many phytoplankton species may serve many purposes, which warrants their own discussion. Toxin production by phytoplankton was traditionally considered a chemical defense, but studies indicate they may have other roles as allelopathic substances, antibacterial compounds, prey-capture, or any combination of purposes. Many toxins produced by marine microalgae, except PSTs like saxitoxins, produced highest amounts during exponential growth and experienced decreased production under nitrogen stress (Ianora et al. 2006). PSTs like saxitoxin contain nitrogen in their structures, and saxitoxin was suggested as a nitrogen store (Ianora et al. 2006). Nutrient limitation consistently caused increased concentration of toxins unless the substance contained substantial amounts of the limiting nutrient (Ianora et al. 2006). For example, domoic acid produced in diatoms of the genus *Pseudo-nitzschia* contains nitrogen, and was produced higher under phosphate limitation, but not nitrogen limitation (Pan et al. 1998, Pan et al. 1996).

Toxin effects on grazers appear to be highly species-specific, with a range of effects from severe physical incapacitation to death, or no apparent physiological effects in others (Turner and

Tester 1997). PSTs have suggested roles as anti-grazer defense. Multiple copepod species avoided consumption of PST containing prey when offered with nontoxic food. Exposure to PST of phytoplankton elicited strong physiological responses from copepods including higher heart rates, loss of motor control, decreased feeding, decreased fecundity, delayed development and direct mortality (Ianora et al. 2011). The cyanobacterium *Microcystis aeruginosa* produces toxic microcystins. When the survivorship of copepods exposed to toxic and non-toxic strains of *M. aeruginosa* was measured, the survival of copepods was inversely related to the amount of *M. aeruginosa* in the diet, regardless of microcystin production. These results indicated metabolites other than microcystins were responsible for decreased copepod survival (Ger et al. 2010). Domoic acid has neurodegenerative effects on humans, but showed no induced negative effects on plankton organisms that grazed on *Pseudo-nitzchia australis* (Wells et al. 2005). Other studies have indicated that domoic acid does not play a role in allelopathy towards competing microalgae, but did show bioaccumulation in copepod grazers (Brown et al. 2019), leaving its ecological role elusive.

saxitoxin   domoic acid
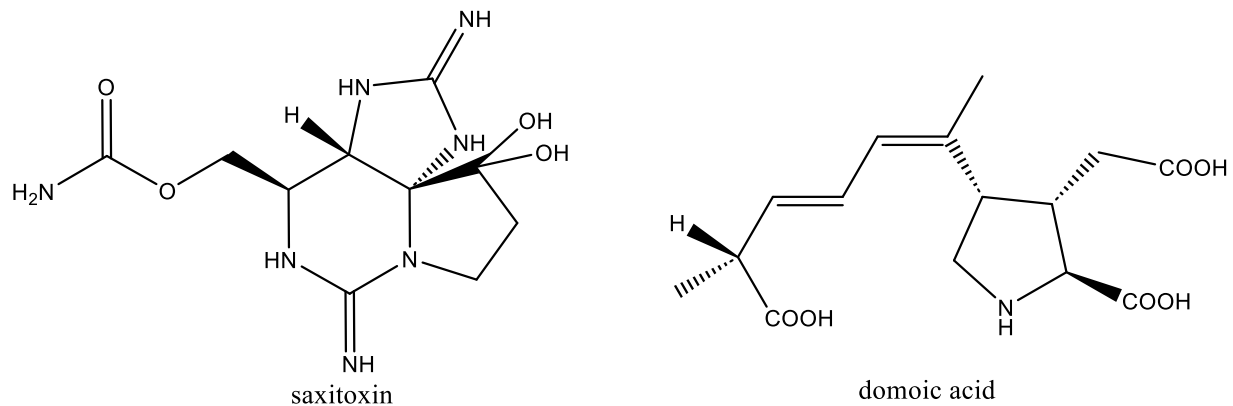
The dinoflagellate *Karenia brevia* produces polyketide brevetoxins with acute toxicity to vertebrates (Baden 1989), but was not the cause of reduced copepod egg production and survival from consumption of *K. brevis* and toxins, which was attributed to the poor quality as a food source of the dinoflagellate (Prince et al. 2006). In a later study, multiple strains of *K. brevis* with varying degrees of toxicity were fed to copepods. Those which consumed the most brevetoxin-rich strains experienced increased mortality, lower egg production and reduced feeding rates compared to consumers of less toxic strains, which indicated a potential role of brevetoxins as chemical defense of at least one sympatric copepod (Waggett et al. 2012). *K. brevis* also showed chemically mediated

grazing deterrence of rotifers, but that was not due to brevetoxins, and those signals were not identified (Kubanek et al. 2007). Karlotoxins produced by the dinoflagellate *Karlodinium veneficum* were observed to immobilize potential prey. Adding partially purified, water-born karlotoxin from *K. veneficum* reduced grazing pressure by heterotrophic dinoflagellate *Oxyrrhis marino* on non-toxic *K. veneficum* and non-toxic cryptophyte *Storeatula major*. Toxic *K. veneficum* was also grazed half as much as either non-toxic microalga prey. Karlotoxins are 90% cell associated, thus it was hypothesized that contact may be necessary (Adolf et al. 2006, Adolf et al. 2007).

The presence of predators has been shown to induce toxin production in *Alexandrium* species. A transcriptomic analysis of PST production in *Alexandrium minutum* in response to copepod grazing revealed 14 highly expressed genes in grazed *A. minutum,* and the toxin content per cell was five times higher in grazed versus un-grazed *A. minutum*. Two of those differential genes were involved in protein folding and modification (Yang et al. 2011). The toxicity of *Pseudo-nitzschia seriata* also increased when grown in the presence of water-born cues of copepod predators, suggesting toxin production may be mediated by chemical cues produced by predators (Tammilehto et al. 2015). Additionally, domoic acid production was induced when exposed to exudates of two different copepods that were actively feeding on diatom cells, measured diatoms were separated from copepods by a permeable membrane (Tammilehto et al. 2015).

The variability in toxin versus grazer effects may be dependent on the ability of predators to detoxify the chemicals, or a shared evolutionary or biogeographic history. When two geographically distant populations of the copepod *Acartia hudsonica* were reared on toxic *A. fundyense*, the copepods without prior exposure to toxic blooms of the microalga experienced lower somatic growth, maturity size, egg production and survival, compared to copepods with previous exposure, which had no effects on growth or development (Colin and Dam 2003, Colin and Dam 2005). Rotifers with shared history with *K. brevis* had a higher tolerance to *K. brevis* exposure compared to rotifers from locations with no *K. brevis* blooms (Kubanek et al. 2007).

The fate of toxins in the community and ecosystem is of high importance to many scholars due to the potential impact on humans from bioaccumulation through trophic levels, but also from the influence on local community composition and succession. The presence of other phytoplankton in the community has been shown to reduce water toxicity caused by algal exudates. Dinoflagellate and diatom species including *Skeletonema grethae* lower concentrations of brevetoxins dissolved in seawater (Myers et al. 2008, Redshaw et al. 2010, Redshaw et al. 2011). In studies with different

concentrations of *S. grethae*, the maximum removal of brevetoxin was 40% regardless of diatom concentration (Redshaw et al. 2011).

1.I.   Intraspecific Signaling

Within species communication is important for attracting mates as well as the predator defense (Schwartz et al. 2016). "Female" types of the diatom species *Seminavis robusta* produce L-diproline to attract "males" types for sexual reproduction after consecutive asexual divisions reach a minimum threshold size inciting the need for sexual reproduction. This switch to sexual reproduction produces a zygote which results in a full-sized parent cell (Gillard et al. 2013). The switch from asexual reproduction to sexual reproduction in the rotifer *Brachionus manjavacas* is regulated by the accumulation of mixis-inducing protein (MIP) in seawater (Serra et al. 2011, Snell et al. 2006). Preliminary characterizations indicate a 22 kDa protein excreted by the rotifer showed significant reduction of mixis in female progeny (Snell 2017). Chemoreception is particularly important in copepod species, where male copepods follow pheromone scent trails for female encounters, and are particularly efficient in detecting non-ovigerous females, increasing their reproductive potential (Seuront and Stanley 2014). Though these experiments were conducted in a laboratory setting, background pheromone concentrations were used in consideration of the turbulent waters of the natural habitat, and revealed sex and species-specific responses which include changes in swimming behavior (Seuront 2013, Seuront and Stanley 2014). The identity of these pheromones has yet to be determined. An untargeted metabolomics approach has been used to describe metabolites and pheromones from copepods. This revealed sex-specific differences in exudate composition and component abundance and was a proof-of-principle for this methodology. However, due to the loss of activity of attractant pheromone fractions, the structure remained unidentified (Heuschele et al. 2016, Selander et al. 2016).

L- diproline

(2E,4E)-2-4-decadienal
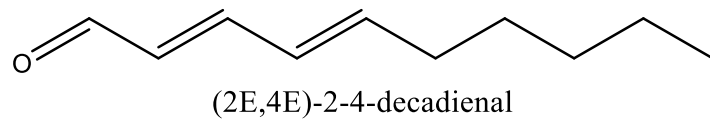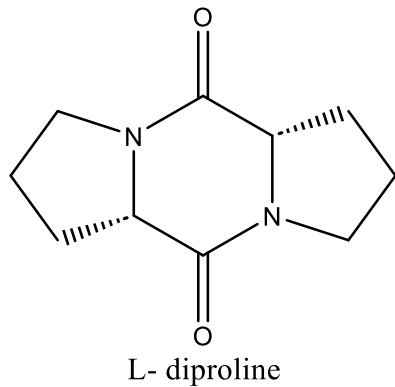
PUAs have also been implicated in communication with conspecifics in the phytoplankton community. Specifically, decadienal affects neighboring conspecifics when released by wounded diatoms. When *Thalassiosira wessfloggi* and *Phaeodactylum tricornutum* cell surfaces detected decadienal, signal cascades were initiated, leading to the buildup of intracellular calcium and nitric oxide production which led to cell death (Vardi et al. 2006). *S. marinoi* only released PUAs during late stationary phase, right before lysis was prominent. When *S. marinoi* was dosed with ecologically relevant levels of PUA during late stationary phase, the growth temporarily increased, then dramatically declined in density. The effect was significant at other stages of growth, but cultures re-exposed to PUA at late-stage growth did not induce the same response as naïve cultures in late stationary growth. It was inferred that PUAs may be sub-lethal signals with ephemeral presence in the environment, which affect diatoms during specific growth periods (Vidoudez and Pohnert 2008). Though the ecological signaling is unclear, others have postulated the detection of PUAs by diatoms in the natural environment may stimulate organized bloom termination (Casotti et al. 2005).

The haptophyte *Prymnesium parvum* produced filtrates that were toxic to less dense cultures of the same species (Olli and Trunov 2007). Cells were able to adapt when exposed to low concentration filtrates, implying that cells associated by bloom may become resistant to toxins they are emitting, with the potential to affect bloom dynamics via autotoxicity (Olli and Trunov 2007, Poulson et al. 2009).

1.J.   Quorum sensing

Quorum sensing is a term used to describe concentration based intraspecific signaling, where the buildup of autoinducers incites a change in gene expression. The best studied autoinducers in

bacteria are acyl homoserine lactone (AHL) molecules (Li and Nair 2012). Bacterial quorum sensing was previously not assumed to have a prevalent role in our oceans due to the density of bacteria in seawater usually being below the quorum sensing threshold, but they may still have impact in situations where bacteria aggregate on particles or during high density algal blooms (Brown et al. 2019). A study of bacterial communities on marine snow revealed known autoinducers, indicating the concentration of bacteria on particles can be high enough to facilitate quorum sensing (Jatt et al. 2015). Further, an examination of publicly available metagenomic sequence information from Global Ocean Sampling (GOS) revealed previously unknown autoinducer sequences, meaning quorum sensing is diverse and widespread in the marine environment (Doberva et al. 2015). It is implicated in microbial cross-talk, and social behavior in algal-bacterial symbionts (see "Facilitation and mutualism") (Zhou et al. 2016). Quorum sensing in biofilms may also confer chemical defense. Biofilms of the bacterium *Vibrio cholerae* protected themselves from predatory amoeba *Acanthamoeba casalanii* with an extracellular matrix, vibrio polysaccharide (VPS) containing proteins, nucleic acids and sugars (Yildiz et al. 2014), which is partially control by the quorum sensing regulator HapR (Sun et al. 2013).

Quorum sensing is likely an important contributor to biogeochemical cycling and supply of organic matter to other phytoplankton residents (Brown et al. 2019). Bacterial degradation of particulate organic carbon (POC) may be regulated by quorum sensing. Bacteria cultured from sampled POC produced AHLs, and the addition of N-3-oxo-hexanoyl homoserine lactones to the POC caused increased activity of degradative enzymes, indicating an AHL based quorum sensing may play and important role in POC degradation (Hmelo et al. 2011). The addition of saturated long-chain AHLs to non-axenic *Trichodesmium* colonies resulted in a two-fold increase in the activity of an enzyme used to liberate phosphate from organic compounds (Van Mooy et al. 2012). Quorum sensing has also been closely associated with bacterial algicidal activity, as a mechanism for the regulation of algicide production (see "Algicidal bacteria") (Dow 2021).

1.K.   Facilitation and mutualism

Much interest is placed in the chemical cross-talk between species in phytoplankton communities. Interactions between microalgae and bacteria have become popular topics in the last years with many studies interested in how bacterial communities influence phytoplankton growth (Cirri and Pohnert 2019, Rolland et al. 2016, Venuleo et al. 2017, Zhou et al. 2016). The wide range of algal-bacterial associations include nutrient exchange, mutual support of growth factors, quorum

sensing mediation, and episodic killing of partners to obtain more resources (Cirri and Pohnert 2019). Algicidal bacterial interactions will be covered in a separate section. The dinoflagellate *Gymnodinium catenatum* requires the presence of a microbial consortium to germinate from resting cysts. In a coculture study of the dinoflagellate with two strains of associated bacteria, the bacterial free *G. catenatum* germinated at a lower rate and survived less than those grown with at least one of the two co-cultured bacterial strains (Bolch et al. 2011). Algal derived tryptophan is a substrate that can be used by bacteria to produce the plant hormone, indole-3-acetic acid, which can be a growth promoter in eukaryotic plankton (Amin et al. 2015, Segev et al. 2016). Researchers demonstrated that indole-3-acetic acid was present in field samples in concentrations similar to laboratory studies (Amin et al. 2015). Indole-3-acetic acid produced by Roseobacter *Sulfitobacter* sp. induced growth of the diatom *Pseudo-nitzschia multiseries*. *Sulfitobacter* sp grown alone or with *P. multiseries* showed detectable micromolar concentrations of indole-3-acetic acid released into the environment. Authors suggested that *P. multiseries* provided necessary organic carbon for bacterial growth promotion, which in turn benefited from ammonium derived from bacterial metabolism (Amin et al. 2015). This relationship was also reflected in *E. huxleyi* and bacterial symbiont *Phaeobacter inhibens*, where tryptophan released by *E. huxleyi* is used by the bacterium to make indole-3-acetic acid, which then stimulated the growth of *E. huxleyi* (Segev et al. 2016). Chemically mediated intraspecific facilitation may exist within some toxigenic dinoflagellate populations, where non-lytic genotypes of *Alexandrium fundyense* benefits from predatory protection by lytic genotypes of *A. fundyense* which are chemically defended from grazing by the heterotrophic dinoflagellate *Polykrikos kofoidii* (John et al. 2015). Showing the community chemical defense benefits of lytic compounds.
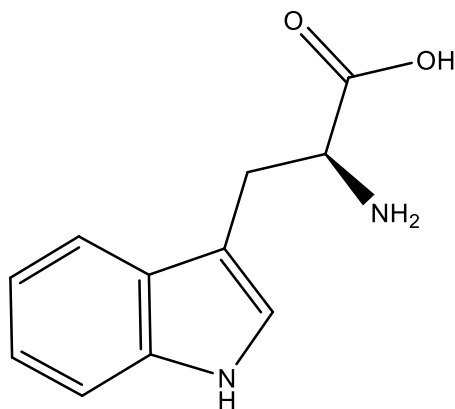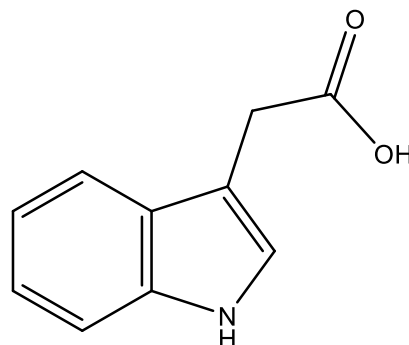
Tryptophan   indole-3-acetic acid

Other studies tracked transcriptomic or metabolomic changes in response to the presence of other species to infer chemical crosstalk between organisms and demonstrated potential chemical signal exchanges. The diatom *Thalassiosira pseudonana* regulated gene expression related to cell signaling and recognition functions in the presence of Roseobacter *Rugeria pomeroyi* (Durham et al. 2017). *Alexandrium tamarense* influenced the metabolism of *R. pomeroyi* by causing the bacteria to increase the expression of quorum sensing genes in a species-specific effect, as the same influence was not induced by the presence of *T. pseudonana* (Landa et al. 2017). The alphaproteobacterium *Dinoroseobacter shibae* influenced growth of the dinoflagellate *Prorocentrum minimum* by shifting from mutualist to pathogen during different growth phases of *P. minimum* (Wang et al. 2015). Transcriptomic analysis revealed quorum sensing, *CtrA* phosphorelay, and flagella biosynthesis genes were significantly upregulated after switching from mutualism to pathogenicity (Wang et al. 2015). These studies have shown the impact of eukaryotic phytoplankton on bacterial metabolism or physiology. The reverse is also possible. EroS protein was identified as an enzyme exuded by the marine gammaprotobacterium *Vibrio fischeri* which stimulated sexual reproduction and swarming behavior in the dinoflagellate *Salpingoeca rosetta* (Woznica et al. 2017). Resources like dissolved vitamins are also readily exchanged between members of plankton (Sanudo-Wilhelmy et al. 2014). The identification of metabolites in cross-talk has been difficult due to the rapid turnover in the phycosphere, but is likely common throughout the phytoplankton community (Brown et al. 2019).

Microbial cross-talk can be mediated by the excretion of extracellular vesicles (EVs). The production of extracellular vesicles has been seen in all domains of life. These micro-pockets of membranes can carry packages of nucleic acids, enzymes and small molecules (Schatz and Vardi 2018). EVs have been associated with functional roles in host-virus interactions, as EVs from viral infected *E. huxleyi* facilitated more rapid infection in non-infected *E. huxleyi* following internalization of these EVs (Schatz et al. 2017). The advantage of such communication is the delivery of membrane protected, high concentration signals with some target specificity, based on the lipid and protein composition of the encapsulation (Cirri and Pohnert 2019).

Chemical cross-talk has been shown to shape plankton communities which undergo annual and seasonal changes due to necessary adaptations to available niches and resources. Laboratory experiments have demonstrated bacterial colonization depends on the host and that communities can fluidly change over time (Behringer et al. 2018, Crenn et al. 2018). Diatoms established unique long-

term associations with selected bacteria to create stable communities that were likely due to chemical signaling (Behringer et al. 2018). Chemical signals are thus vital to the organization and balance of entire communities (Legrand et al. 2003, Tillmann 2004). The discovery of the phycosphere helped to make sense of facilitation of cross-kingdom communication, as gradients within this sphere guide microscale algal-bacterial interactions (Cirri and Pohnert 2019). This chemically enriched zone allows the interface of interactions between algae and bacteria, as well as other organisms mediated by exuded chemicals (Seymour et al. 2017).

1.L.   Allelopathy

Allelopathy is commonly referred to as chemically mediated competition between different phytoplankton species and may have positive (growth promotion), negative (growth inhibition), or present no effect between different parties. Models have been explored to demonstrate how environmental conditions affect competitive dynamics between two algal species in a bloom when one is allelopathic. Mandal and Banerjee predicted that environmental conditions strongly influenced which species outcompeted the other, but could not be used to account for population explosions and suggests that coexistence or dominance was largely shaped by the initial ratio of two species (Mandal and Banerjee 2013). A later model, including seasonal fluctuations in environmental conditions, where both species produced allelochemicals, predicted the co-existence of both species, due to the contribution of intraspecific competition (Mandal et al. 2014). In the situation where two species compete for a resource which could be stored, models predicted that the trade-off between the ability to compete for nutrients and the ability to produce allelochemicals or resist their effects would lead to the dominance of one species. Meaning, the allelopathic species would persist in replete nutrient conditions, whereas the better resource competitor would persist in low nutrient conditions (Grover and Wang 2013). When an additional storable nutrient was added to the model, the potential of coexistence between the two species was possible if they were able to take up one of the nutrients at a higher rate than the competitor but was less predictive in high nutrient conditions (Grover and Wang 2014). These models all show how the environment strongly influences the dynamics of two species in a bloom, but also only use general allelochemicals for prediction, as many species-specific cues are yet unidentified. They are also not programmed to account for physical characteristics of any single compound while modeling outcomes (Schwartz et al. 2016). It is important to keep in mind that models may be a good tool for hypothesis generation, but the ecological value can only be interpreted in ecologically relevant experiments, which may start with laboratory observations.
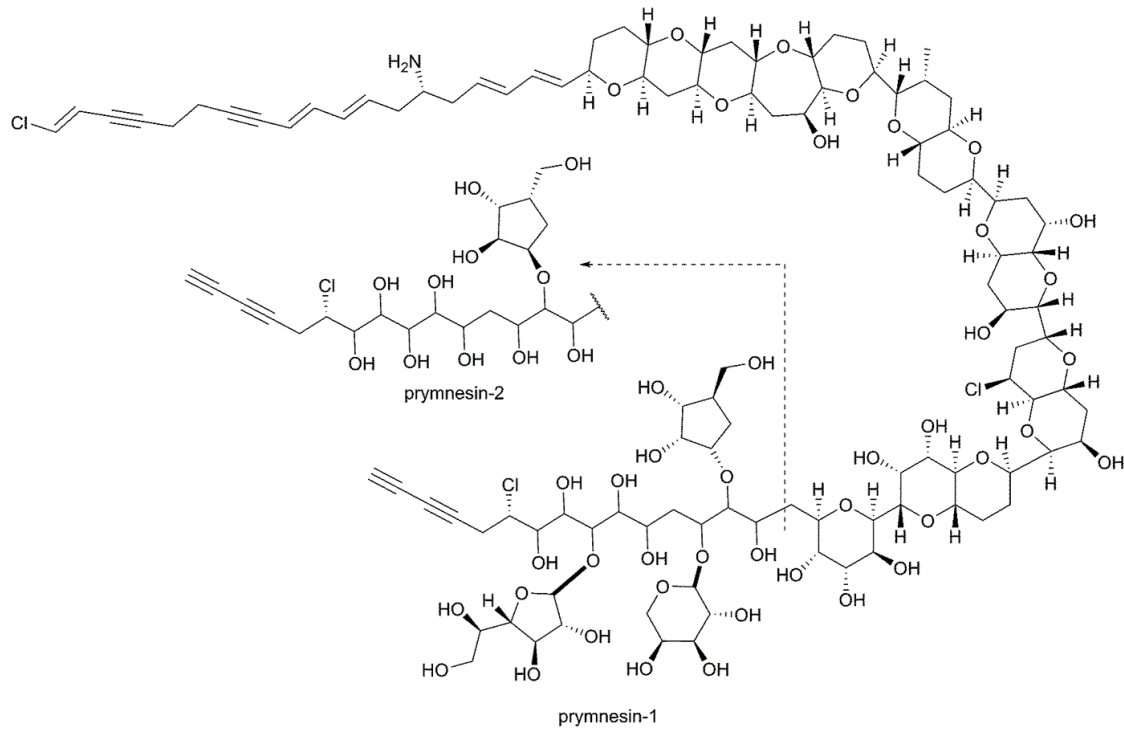
(Structures from Schwartz et al. 2016)

The many microalgae producing toxins with detrimental effects on vertebrates, are also known to be allelopathic, but the toxins are rarely responsible for competitive outcomes between phytoplankton (Poulson et al. 2009). *Prymnesium parvum* is well known for its allelopathic potential as well as toxin production. Experiments have shown that filtrates of low salinity cultures of *P. parvum* were more allelopathic towards competitor *Rhodomonas salina* compared to filtrates of high salinity cultures of *P. parvum*, but the intracellular toxicity of *P. parvum* did not change between conditions (Weissbach and Legrand 2012). There are a wealth of bioactive compounds produced by *P. parvum* in addition to the toxic polyketides prymnesin-1 and prymnesin-2, including glycolipids, galactolipids, proteolipids and lipid-carbohydrate complexes (Igarashi et al. 1996, Manning and La Claire 2010). The extent of contribution of these toxins and allelochemicals towards interactions of *P. parvum* with community members is currently unknown due to the lack of purified compounds (Ianora et al. 2011). The allelopathic effects of toxic nodularin producing cyanobacterium *Nodularia spumigena* was compared to the non-toxic *Aphanizomenon flos-aquae* towards the cryptophyte *Rhodomonas* sp.. The non-toxic *A. flos-aquae* was more inhibitory to the cryptophyte at 29% based on cell number, than the toxic *N. spumigena* (14%). Additionally, pure nodularin was added and showed no significant effect against *Rhodomonas* sp., suggesting the toxin was not responsible for allelopathic behavior in *N. spumigena (Suikkanen et al. 2006).*

Allelopathy is concentration-dependent (emitter perspective) as well as dependent on recipient cell state. For *A. fundyense* allelopathic inhibition was greater on co-occurring diatom *Thalassiosira* cf. *gravida* when *T.* cf. *gravida* cells were small. High concentrations of *A. fundyense* filtrate caused decreased growth and nutrient uptake in *T.* cf. *gravida* (Lyczkowski and Karp-Boss 2014). Experimental co-cultures have revealed that initial cell concentrations of each species, between *Chattonella antiqua* and *Akashiwo sanguinea*, ultimately determined competitive outcomes. The species with the greater initial population size inhibited the growth of the other (Qiu et al. 2011).

Species specificity also impacts the fate of allelopathic competition. Several cyanobacteria extracts stimulated or inhibited several phytoplankton species in a species-specific manner, with stimulation effects greatest when the extracted species and target species were closely related (Lopes and Vasconcelos 2011). *A. fundyenses* presented species-specific allelopathic effects in nutrient amended filtrates of multiple strains of *A. fundyenses*. Diatoms and autotrophic nanoflagellates in multiple natural assemblages reduced concentrations from exposure to *A. fundyenses* filtrates, while all strain filtrates stimulated dinoflagellate growth (Suikkanen et al. 2011).

Allelopathy is not only seen in exudates but may also depend on physical interactions between cells. Some phytoplankton may use surface mediated chemistry to lyse cells or inhibit competitor growth (Roy et al. 2013). The dinoflagellate *Heterocapsa circularisquama* lysed *Prorocentrum dentatum* cells only when directly co-cultured; filtrates of *H. circularisquama* were not allelopathic. It was speculated that surface compounds were responsible for lytic effects, but unidentified (Yamasaki et al. 2011). Some diatoms have been shown to allelopathically (and physically) slow the swimming of competing co-occurring red tide dinoflagellate *Cochlodinium polykrikoides*, reducing the access to nutrients which undermines its growth potential (Lim et al. 2014). Studies of *K. brevis* revealed different allelopathic effects on competitors depending on evolutionary history of exposure. *Asterionellopsis glacialis*, which typically co-occurred with *K. brevis* blooms showed more resistance to allelopathic effects of *K. brevis* (Poulson-Ellestad et al. 2014a, Poulson-Ellestad et al. 2014b).

The strength of allelopathy is determined by biotic and abiotic factors, with limiting nutrients having shown an effect on the production of allelochemicals and target species (Gross 2003). Under iron-replete conditions, domoic acid gives the marine diatom *Pseudo-nitzschia delicatissima* a competitive advantage over competing diatom *S. marinoi*. Though the mechanism is yet unknown, it has been hypothesized that *P. delicatissima* used domoic acid for iron uptake, reducing the

availability to competitors (Prince et al. 2013). This was based on the previously shown siderophore activity of domoic acid (Rue and Bruland 2001). An investigation of the role of bacteria in *Alexandrium* allelopathy showed no statistical difference in allelopathy for broad-spectrum antibiotic treated *Alexandrium* sp. versus non-treated controls. This suggested that these extracellular bacteria were not likely involved in the production of allelopathic compounds (Tillmann et al. 2008).

Allelopathic potency may play a role in the development of monospecific blooms and the succession of species in bloom dynamics (Granéli and Pavia 2006). Filtrate from declining cultures of *Skeletonema costatum* impacted competitors in species-specific and concentration dependent manner, but the most negatively impacted species was *S. costatum* itself. This indicated that the active compounds may also serve as bloom termination signals within this population (Vardi et al. 2006). Observations of co-blooms of the raphidophyte *Chattonella marina* var. marina and the dinoflagellate *Gymnodinium catenatum* in the Gulf of California, found that *C. marina* consistently formed two times more concentrated blooms than *G. catenatum*. Studies of the relationship found that *C. marina* caused loss of flagella, nuclear swelling, and cyst formation in *G. catenatum*, with exuded compounds accounting for about 50% of the negative outcomes on *G. catenatum*. This study showed the ability to outcompete and control a natural competitor by causing physical damage to cells through exuded allelochemicals (Fernández-Herrera et al. 2016).

While most studies of allelopathy focused on pairs of ecologically relevant species, most phytoplankton communities are highly diverse, and the presence of other competitors may have an impact on binary relationships evaluated in laboratory settings. It has been shown that allelopathic cues of one species can be undermined by the presence of a tertiary participant, or shared history may impact the resistance to allelopathic cues. Some competitors of *K. brevis* may undermine the allelopathic effects of *K. brevis,* such as *S. costatum* and *A. glacialis*. Extracellular extracts of *K. brevis* in cocultures of *S. costatum* were significantly less allelopathic than *K. brevis* monocultures. Authors predicted this may have been caused by interruptions to allelochemical biosynthesis or exudation, the metabolism of allelochemicals, or the production of counteracting compounds to *K. brevis* allelopathy (Prince et al. 2008).

Examples of allelopathy in phytoplankton communities are abundant, the identification of specific allelochemicals remains sparse. Issues of activity loss during bioassay guided fractionation and potentially low concentrations serve as persistent obstacles to the characterization of allelopathic chemical cues, but some characterization has been made. Allelopathic compounds from *A. tamarense*

showed high molecular weight (7-15 kDa) based on bioassay guided fractionation coupled with mass spectrometric characterization. Retention of lytic properties after treatment with trypsin suggested the active portion was not proteinaceous (Ma et al. 2011). *H. akashwio* releases high molecular weight (>3.5 kDa) allelopathic polysaccharide protein complexes which are active against *Thalassiosira rotula* and *Skeletonema costatum.* These compounds were also present in *H. akashiwo* bloom waters (Yamasaki et al. 2010). *K. brevis* allelopathy is very complex as allelochemicals may vary with biotic interactions and have examples of both inhibitory and growth promotive interactions with competitors. Full characterization of *K. brevis* allelochemicals is still ongoing, but have been found to be 500-1000 Da with aromatic functional groups (Prince et al. 2010).

1.M. Algicidal bacteria

There are many reviews on the ecological role and mechanisms of algicidal bacterial interactions within phytoplankton communities (Coyne et al. 2022, Mayali and Azam 2004, Meyer et al. 2017, Wang et al. 2020a, Yang et al. 2020). Algicidal bacteria play important roles in the release of organic matter from primary production of microalgae via the inhibition or lysis of algal hosts. They have been frequently associated with terminating blooms at increasing concentrations during bloom decline (Demuez et al. 2015, Skerratt et al. 2002). Algicidal bacteria may require direct contact for activity, but act by the secretion of secondary metabolites, or both (Meyer et al. 2017). Examples of algicidal bacteria using direct contact for activity include *Cytophaga spp., Saprospira spp., Pseudomonas spp.* and others (Wang et al. 2020a). These bacteria which require attachment usually lead to the degradation of surface macromolecules (Mayali and Azam 2004). An important function for these types of bacteria is cellular motility which allows for chemotaxis towards desirable hosts (Wang et al. 2016a, Wang et al. 2020a). Although there has been no phylogenetic correlation with algicidal bacterial species, there may be multiple algicidal bacterial species within a genus, with both direct and indirect modus operandi (Coyne et al. 2022). For example, *Cytophaga* sp. strain J18/M01 required direct attachment to destroy dinoflagellates (Imai and Kimura 2008), whereas *Cytophaga* sp. strain 41-DBG2 was active against the dinoflagellate *Gymnodinium breve Davis* through the release of dissolved algicidal compound(s) (Doucette 1998).

The majority of algicidal bacteria investigated act by the release of secondary metabolites (Coyne et al. 2022). Extracellular secreted compounds are mostly, agarose, amino-peptidase, lipase, glucosaminidase, alkaline phosphatase, proteins, enzymes, antibiotics, biosurfactants, bacillamide, protease, and lipid peroxidases (Wang et al. 2020a). Many algicidal chemicals produced by bacteria

may act additively or synergistically (Harvey et al. 2016, Jeong and Son 2021, Rose et al. 2021, Wang and Seyedsayamdost 2017). Additionally, they may act on physiological targets or multiple systems simultaneously (Wang and Seyedsayamdost 2017). The release of enzymes was closely associated with the algicidal lifestyle of bacteria (Li et al. 2016, Li et al. 2015, Martinez et al. 1996, Paul and Pohnert 2011, Smith et al. 1992). Hydrolase enzymes might serve dual functions, as algicides and facilitators of organic matter uptake from hosts by extracellular degradation (Meyer et al. 2017, Li et al. 2016, Li et al. 2015, Martinez et al. 1996, Paul and Pohnert 2011, Smith et al. 1992).

COOH

HO

p-coumaric acid
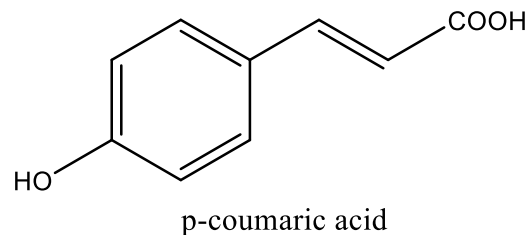
Evidence has shown that algicidal activity may be induced in bacteria based on chemical cues from phytoplankton. In the case of the relationship between marine Roseaobacter *Phaeobacter inhibens* which lives closely associated with *E. huxleyi* in mutualism; the bacteria turn algal derived DMSP into phenylacetic acid, a growth promoter in some algae, and tropodithietic acid, an antibiotic which can protect microalgae (D'Alvise et al. 2012, González et al. 1999, Thiel et al. 2010). This mutualism only exists during the growth phase of the microalga, during senescence, the microalga releases *p*-coumaric acid which triggers the bacteria to produce algicidal roseobacticides, which kill the microalga (Seyedsayamdost et al. 2011). A later study determined that two signals were needed in the switch to parasitism, one being *p*-coumaric acid and the second being a quorum sensing molecule (Wang et al. 2016b).

Algicidal effects of marine bacteria often require high concentrations, which led to the speculation that the production of algicidal compounds may be influenced by quorum sensing (Brown et al. 2019). High nutrient availability was often a prerequisite to produce algicides in laboratory settings, suggesting they were released under optimized conditions at high density (Mayali and Doucette 2002, Roth et al. 2008b). Nutrient availability might also be a cue for triggering algicidal production in algicidal bacteria, as was shown that limiting growth conditions cause production of algicides in the cyanobacterium *Oscillatoria laetevirens,* whereas optimal growth

conditions halted algicide production (Ray and Bagchi 2001). High nutrient availability reflects the conditions of declining blooms where dissolved organic matter (DOM) is released into the environment, which would benefit algicidal bacteria to release algicides to prolong optimal conditions for growth. Nutrient replete conditions may signal other species to produce algicides to prevent starvation. The adaptive strategies of algicidal bacteria to nutrient cues is likely species-specific as well.

Pronounced regulation of activity is likely required for bacteria to maintain efficient algicidal strategies within the spatial and temporal heterogeneity in the marine environment (Meyer et al. 2017). Quorum sensing regulation would thus be beneficial to this population for energy efficiency. Evidence of such regulation was indirectly shown in the isolation of the algicidal pigment, PG-L-1, from a Gammaproteobacterium with activity against various red tide phytoplankton species. The addition of a QS inhibitor halted pigment production but was recovered by the addition of AHLs (Nakashima et al. 2006). The marine bacterium *Pseudoalteromonas piscicida* was found to produce the bacterium signaling molecule 2-heptyl-4-quinolone, which acted as a QS mediator but also slowly lysed three strains of *E. huxleyi* at biologically relevant concentrations (Harvey et al. 2016). Increased algicidal proteases release, by the marine bacterium *Kordia algicida,* was induced by the addition of spent medium in a seemingly QS regulated process (Paul and Pohnert 2011).

The role of algicidal bacteria in phytoplankton bloom control would benefit from the chemical analysis of field blooms or transcriptomic monitoring of genes during natural blooms. The identification of known molecular targets would be needed for this approach but is difficult due to the dearth of fully identified algicides for monitoring. There is a lack of knowledge in algicidal mechanisms both for the emitter as well as the determinants of host susceptibility. The taxonomic selectivity of bacterial hosts can be as broad as the phylum level (Pokrzywinski et al. 2012, Wang et al. 2018), or as narrow as the strain level (Barak-Gavish et al. 2018, Roth et al. 2008a). It is especially difficult to study chemical signals from direct modes of attack as it is difficult to separate organism specific responses in such a mechanism. This has left us with a wealth of algicidal bacterial interactions on the biological level, while the identification of the chemical substances necessary for activity are mostly uncharacterized (Coyne et al. 2022, Meyer et al. 2017, Wang et al. 2020a).

1.N.   Viruses and parasitism

Evidence has shown that viruses can control phytoplankton dynamics by reducing host population or preventing higher levels of host populations. The range of viral hosts are highly

specific, but can affect algal species succession, and potentially intraspecies succession. Cellular lysis from viral infection also indirectly affects the flux of energy, nutrients and organic matter (Brussaard 2004). Annual blooms of *E. huxleyi* are terminated by coccolithoviruses (EhVs); but the mechanism remains unknown. An accumulation of viral derived glycosphingolipids in *E. huxleyi* cells correlated with increased production of caspases and metacaspases involved in regulating cell apoptosis, consistent with programmed cell death (Vardi et al. 2012). The understanding of viral mediated mortality in phytoplankton is still poorly understood and requires further investigation.

Parasitic interactions have similar roles in controlling phytoplankton to viruses. Parasites often rely on host-exuded chemical signals to locate hosts (Schwartz et al. 2016). The generalist dinoflagellate parasite *Parvilucifera sinerae* may be triggered by exposure to host exudates, using high concentrations of DMS as a cue for high densities of potential hosts. This would allow *P. sinerae* to conserve energy as dormant spores and switch to free living virulent forms, when the potential for hosts were detected (Garcés et al. 2013). Not all dinoflagellates that produce DMS were susceptible to infection, meaning other cues may be present to determine host viability.

Antiphage defenses may include aggregate formation, exopolysaccharide production, and temporary downregulation of phage receptors, as has been shown for the pathogenic marine bacterium *Vibrio anguillarum* in defense against viral infection (Brown et al. 2019). Plankton might respond to chemical cues from parasites in an analogous way to reduce risk of infection. When the dinoflagellate *A. fundyense* detected water-born cues from the parasitic dinoflagellate *Amoebophyra* sp., *A. fundyense* upregulated genes associated with energy production, signal transduction, stress, and defense, but it was inferred that molecular mechanism responsible for detection and responding to parasitic threat were different in each case (Lu et al. 2016). This indicates that defense may start with initial recognition of parasite cues, followed by further responses to physical encounter (Brown et al. 2019). Toxin-producing dinoflagellates might incur costs due to susceptibility to parasite infection (Poulson et al. 2009). The ichthyotoxic dinoflagellate *Karlodinium veneficum* are hosts to the parasitic dinoflagellate *Amoebophrya* sp. and has shown significant positive correlation between host karlotoxin concentration and susceptibility to *Amoebophrya* sp. infection (Bai et al. 2007).

Once inside hosts, parasites might manipulate hosts to their advantage. Interactions between the flavobacterium *Croceibacter atlanticus* and diatoms were investigated following attachment of the bacterium to diatom surfaces. Growth of half the tested diatoms weas inhibited following attachment, for the diatom *T. psuedonana* specifically, cellular division was halted, while individual

cells became larger, polyploid, and increased in plasmid number and chlorophyll abundance. These outcomes were predicted to create a more colonizable surface area and excretion of metabolites to aid in bacterial growth. Chemical cues from the bacteria itself did not induce any of the changes as direct contact, indicating that these bacterial cues are not constitutively released, or that the interaction is not chemically mediated. Further identification steps were not taken (van Tol et al. 2017).

1.O.   Multifaceted function of secondary metabolites

Secondary metabolites may have multiple simultaneous functions (Ferrer and Zimmer 2009, Zimmer and Ferrer 2007) due to the tendency of nature to "catch as many flies with one cup as possible (Wink 1999)." PUAs and DMSP are great examples of this multifunctionality within phytoplankton communities. The PUA decadienal is the best studied oxylipin with multiple posed functions in grazer defense, allelopathy, cell to cell signaling, antibacterial activity, and bloom termination initiation (Ianora et al. 2011). DMSP, in addition to its roles as an osmoregulatory and predator attractant, appeared to act as a chemical indicator of inferior prey. The addition of at least 20 nM of DMSP to cultures of ciliates and dinoflagellate prey caused 28-75% decrease in feeding rates (Fredrickson and Strom 2009). The role of toxins is also not yet fully understood. While there are many examples of their roles as chemical defense signals, they have also been proposed as allelopathic substances, antibacterial compounds, and prey capture, signifying the potential multifunctionality of these substances (Ianora et al. 2006).

Phytoplankton communities are highly diverse communities made up of eukaryotic and prokaryotic organisms, as well as viruses. These various players are in competition for survival and fitness, driving the evolution of chemical interactions between players. We do acknowledge that there are significant contributions from community members like grazers and viruses, which may be involved in the regulation of phytoplankton communities but take a deeper dive in the relationships of microalgae and algicidal bacteria. For this thesis, we will focus on studies involving allelopathic and algicidal bacterial interactions between microscopic phytoplankton and bacteria within the pelagic marine environment. These interactions have been implicated in the regulation of HABs and have shown ecological relevance in the natural environment (Brown et al. 2019, Coyne et al. 2022, Roy et al. 2013, Schwartz et al. 2016, Wang et al. 2020a).

2) Scope of the Thesis

The goal of this thesis was to investigate chemically mediated microbial interactions within phytoplankton communities with the potential to influence bloom succession and termination: allelopathy and algicidal bacteria. We approached it with the lens of resolving some of the challenging aspects of translating laboratory research into ecologically relevant studies.
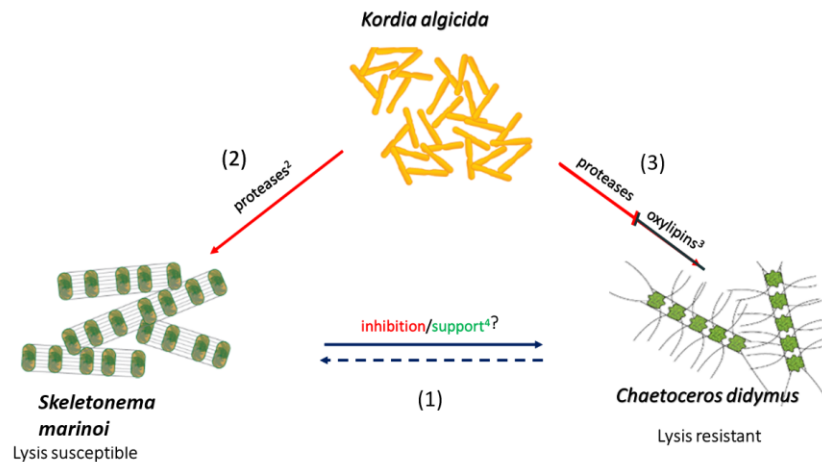
Figure 6. Scope of thesis. (1) We observed the influence of laboratory parameters on allelopathic competition between phytoplankton species. (2) We investigated the identity of algicidal proteases released by *K. algicida* which lyses phytoplankton species like *S. marinoi* (previously *S. costatum*) (Paul and Pohnert 2011). (3) We investigated the scope of target species of *K. algicida* to determine resistant and susceptible species which may further be used for insight into resistance or evasive strategies employed by phytoplankton to escape algicidal bacteria.

Our investigation of allelopathy centered around cocultivation studies of a well-known allelopathic species, *Prymnesium parvum* (Granéli and Johansson 2003, Granéli and Salomon 2010) and five ecologically relevant phytoplankton species. We observed changes in growth due to reciprocal metabolic responses and determined the plasticity of allelopathic interactions due to varied laboratory parameters and looked for ecological validation using the TaraOceans database (**Chapter 1**). One of the most challenging aspects of determining the ecological role of algicidal bacteria in controlling phytoplankton blooms is the paucity of identified algicidal targets which can be used for ecological monitoring. *K. algicida* produces algicidal proteases to lyse target phytoplankton (Paul and Pohnert 2011). We used a transcriptomics-guided approach to determine an algicidal protease encoding gene for *K. algicida* and validated the activity using molecular biology techniques

(**Chapter 2**). *C. didymus* releases proteases and oxylipins in response to exposure to *K. algicida* (Paul and Pohnert 2013). To further characterize the nature of *K. algicida* attack and potential strategies of resistance by phytoplankton, we surveyed 37 different species of phytoplankton for susceptibility to the algicidal bacteria. We looked for taxonomic trends in the target range of K. algicida, as well as trends in resistant phytoplankton species (**Chapter 3**).

3) **Chapter 1**: Systematic investigation of factors influencing phytoplankton allelopathy - a case study of *Prymnesium parvum* and ecologically relevant interaction partners

The experimental work for Chapter 1 was carried out by me with sampling assistance from Wesley Wong. Concurrently with the preparation of this thesis, the data obtained will be submitted for publication. The corresponding manuscript was written entirely by me with contributions from Dr. Remington Poulin, Dr. David A. Russo, Wesley Wong, and Prof. Dr. Georg Pohnert for corrections used in this chapter. All authors commented on the manuscript and give informed consent for the use of the data in this thesis and agree to the statement of contribution. Details of contributions are given in the following table.

Name of co-worker	Syhapanha	Poulin	Russo	Wong	Pohnert
Conception of Study	X	X			X
Experimental Planning	X	X			
Experimental set-up	X				
Data Collection	X			X	
Tara Oceans Analysis	X			X	
Metabolomics Extraction	X	X			
Data Analysis & Interpretation	X	X	X		
Manuscript Writing	X	X	X		X

3.A.   Study Introduction

Understanding the ecology of plankton communities provides insight into our ecosphere, as they play a role in all major biogeochemical cycles, as well as in the marine food web and microbial loop. As the frequency and scale of phytoplankton blooms intensified over the last decades, it is becoming increasingly important to understand the factors influencing bloom dynamics (Dai et al. 2023). Allelopathic interactions between species can facilitate competition and shift community composition, in both stimulatory and inhibitory interactions between phytoplankton and other microorganisms, indicating the importance of this chemical communication in plankton ecology (Gross 2003, Legrand et al. 2003). This dynamic process occurs on a relatively short timescale and allelopathic secondary metabolites can affect phytoplankton dynamics and influence the dominant species in blooms (Keating 1977, Legrand et al. 2003, Pratt 1966, Rojo et al. 2000). Much is still unknown about the identities of these allelochemicals and consequently, the roles they play in the natural environment. Laboratory studies have demonstrated many examples of allelopathic advantage, especially in the suppression of competing species, but few have been validated in natural communities (Legrand et al. 2003). Inconsistent methodologies make practical comparisons between studies difficult, with a common criticism being the unnaturally high concentrations of potential inhibitory allelochemicals and inadequate bioassays (Gross 2003). Most studies use cell-free filtrates or extracts of unknown composition, which made structural elucidation of allelochemicals difficult (Ianora et al. 2011). In addition to the inherent physical constraints of investigating such chemicals in aquatic environments, biotic and chemical interferences may occur in parallel in the natural community (Inderjit and Moral 1997), which adds additional challenges to the transfer of laboratory results to natural field experiments.

Allelochemical production in marine phytoplankton is often found in toxin-producing species (Granéli and Pavia 2006, Yang et al. 2022), though the toxins themselves are not usually the direct cause of interspecific allelopathic interactions, suggesting the release of additional allelochemicals as well as toxin production (Poulson et al. 2009). Laboratory studies have shown that the amount of allelochemicals released into medium are proportional the amount of donor cells (Legrand et al. 2003, Sharp et al. 1979) and the accumulation in media may be affected by pH, temperature and light (Hansen 2002, Legrand et al. 2003, Schmidt and Hansen 2001). Nutrient limitation can have a large

impact on allelopathic potency, more accentuated when target cells are nutrient deficient (Granéli and Johansson 2003).

It has been argued that phytoplankton allelochemicals' inherent value is on the effect of the target algae, as there is no self-benefit by the producing algae. Due to these complex interconnections the trade-off between costs and benefits of allelopathy remains unclear (Lewis 1986, Sliwinska-Wilczewska et al. 2021). Phytoplankton seem to not be affected by allelopathic substances released by closely related species (Granéli and Pavia 2006). An example of this is the toxin producing phytoplankter *Prymnesium parvum*, well known for its allelopathic ability to outcompete other phytoplankton. The toxins of *P. parvum* are hemolytic, ichthyotoxic, and cytotoxic but are not effective against other *Prymensium* spp. (Igarashi et al. 1996, Johansson 2000, Shilo 1981).

In addition to toxic prymnesins production, *P. parvum* is known for other secondary metabolites, which may also contribute to its bloom success in coastal environments (Granéli and Pavia 2006, Manning and La Claire 2010). The contributions of both toxins and other secondary metabolites in the success of *P. parvum* in natural blooms has yet to be discerned. Allelopathy of *P. parvum* filtrates is varies depending on abiotic factors. Filtrates of nutrient deficient *P. parvum* had a strong negative effect on the growth of other phytoplankton species, but was conversely positive when grown in replete conditions (Errera et al. 2008, Fistarol et al. 2003, Granéli and Johansson 2003, Skovgaard and Hansen 2003, Tillmann 2003). Furthermore, the action of the allelopathic compounds were in short duration (Granéli and Johansson 2003), sensitive to high light, temperature, high pH, while the production was influenced by nitrogen and phosphorus deficiency (Hansen 2002, Johansson and Granéli 1999, Shilo 1967, Skulberg et al. 1993). Though the structures of some toxins, known as prymnesins, have been identified, their allelopathic role has yet to be determined due to the difficulty of obtaining purified compounds (Ianora et al. 2011). Fistarol et al. (2003) demonstrated that *P. parvum* filtrates inhibited electron transport in Baltic Sea phytoplankton, directly depressing photosynthesis, while others suggest that *P. parvum* allelochemicals act directly on membrane surfaces, not requiring processes (Skovgaard et al. 2003). Many of the studies involving *P. parvum* were also based on filtrates or extracts of cells, and effects are density dependent (Legrand et al. 2003). It is undeniable that the presence of *P. parvum* in natural communities will have an influence on the co-existing community, but due to the varying conditions in many studies it is difficult to predict the outcome.

By conducting a systematic co-culturing survey of *P. parvum* allelopathy, against five ecologically relevant plankton competitors in non-contact cocultivation we explore the variability of interaction patterns. Two different, replete media were used to determine if media type and nutrient composition impacted allelopathic interpretation. Cell density was observed in addition to chlorophyll *a* to determine allelopathic effects on growth rate and pigmentation. By inoculating at initially low density and observing the growth over 10 days, we avoid competition situations for nutrients. Allowing the exchange of secondary metabolites through a membrane filter, we observe density dependent effects of metabolites. We searched for meaningful ecological relevance by mining the TaraOceans database for trends in natural observations relevant to our studies.

3.B.   Methods

3.B.1.   Cultures and cultivation conditions

Prymnesium parvum RCC3426 (*Pp*), *Dunaliella tertiolecta* RCC6 (*Dt*), *Emiliania huxleyi* RCC2611 (*Eh*), *Florenciella parvula* RCC446 (*Fp*), *Ochromonas triangulata* RCC21 (*Ot*), and *Pavlova gyrans* RCC1526 (*Pg*), were purchased from Roscoff Culture Collection (France) and cultivated in temperature-controlled cultivation chambers at 13 °C ± 1.5 on a 14/10 h diurnal day/night cycle with 75-90 µmol m^{-2} s^{-1} light. Artificial seawater (ASW) was prepared according to Maier and Calenberg (1994). K/2 media was prepared by adding half the normal K media nutrient concentration to 0.22 µm filtered natural seawater from Helgoland, Germany (Keller 1985, Keller et al. 1987).

3.B.2.   Cocultivation system

Non-contract cocultivation was conducted in a smaller modification of the custom cocultivation chambers described by Paul et al. (2013). The system consists of two modified glass flasks, each holding ca 200 mL, fitted together by metal rings joined by screws (SFigure1). The two chambers were membrane-separated with a 0.22 µm PVDF membrane filter (Durapore, Merk Millipore, USA). All components with direct contact to cultures were acid-washed, rinsed with MilliQ H$_2$O, autoclaved, and assembled under sterile conditions. Monoculture controls for each

cocultivation pair were established by inoculating either chamber with the same species while cocultures were established by inoculating one chamber with *P. parvum* and the other with a partner strain. Four biological replicates were inoculated for each monoculture control and coculture treatment. All cultures were randomly ordered on a single shelf within a temperature-controlled chamber.

3.B.3.   Pre-cultures and inoculation

Pre-cultures were inoculated into either ASW or K/2 and grown for $1 - 2$ weeks in the same condition and location as the cocultivation experiments. Cell density of each culture was measured via flow cytometry to ensure that both species had grown to a density of at least 1.65×10^5 cells mL^{-1} not exceeding 1.10×10^6 cells mL^{-1}. The populations were normalized to the lesser dense species by dilution with fresh media; this enabled final inoculation densities to fall between the range of $1.50 \times 10^4 - 1.00 \times 10^5$ cells mL^{-1}. 50 mL of fresh medium was dispensed into each side of a coculture chamber followed by the addition of 5 mL of normalized *P. parvum* or partner for a 1:1 inoculation ratio. When available, an extra chamber was used as a media blank with five milliliters of media added instead of plankton.

Ot was chosen to evaluate the effect of different inoculation ratios in coculture with *Pp* based on the consistent, pronounced effect in both ASW and K/2 medium. Varying ratios of *Ot*:*Pp* were observed for allelopathic influences on growth: 3:1, 1:1, 1:3.

3.B.4.   Sampling and determination of Growth Curves

Growth was observed for 10 d, including immediately after inoculation. Each half chamber was sampled daily for both flow cytometry (BD Accuri V6, BD Biosciences, NJ, USA) and chl *a* fluorescence (ex: 430 nm, em: 665 nm) (Varioskan Flash, Thermo Fisher Scientific) based density measurements. Chl *a* measurements were taken in technical triplicates and flow cytometry was validated daily using Spherotech 8 peak and 6 peak validation beads (BD Biosciences, NJ, USA).

3.B.5.   Statistical analysis and network maps

Averages and standard deviations were calculated for biological replicates (n = 4) to determine daily chl *a* and cell density measurements. Data was visualized by plotting differences

between average co-culture values from monoculture thus positive values indicate growth promotive effects and negative values indicate growth inhibitory effects for recipient microalga. Student's T-test performed on daily measurements of control versus co-cultured algal measurements determined significant deviations in daily growth rates to evaluate allelopathy. Outliers were determined via Grubbs test and excluded from further analysis.

Networks of the allelopathic interactions between pairs were visualized using Cytoscape (v 3.1.9) software with discreet mapping values for "edge", or arrows, which originated from the donor species and pointed toward the recipient and represented the allelopathic relationship. The values were directly correlated to statistical significance (Student's T-test) between control and coculture density measurements. Blue was used to represent growth enhancing effects, or positive allelopathy, and orange was used to represent growth inhibiting effects, or negative allelopathy. Grey indicated no observation of allelopathic influences on growth rates. More significant interactions were indicated by darker blue or orange color.

3.B.6.   Nutrient Analysis

After the final sampling for the growth curves, two milliliters of each sample were saved for nutrient analysis. Total nitrite and nitrate within the range $5 - 300$ µM were measured according to Zhang and Fischer (2006). Briefly, samples at room temperature and centrifuged for 2 min at 10 000 rpm to remove cells and particulates. 500 µL of the medium was added to a glass vial. 60 µL of a 2% resorcinol (Sigma-Aldrich, ACS reagent) solution was mixed in briefly, followed by 500 µL of concentrated analytical grade sulfuric acid (Supelco, 98%). The solution was vortexed and reacted for 30 min in the dark. 1.5 mL of MilliQ H_2O was added, and the reaction was allowed to cool to room temperature before absorbance at 505 nm was measured. A four-point calibration curve was created using potassium nitrate (KNO_3) (Acros Organics, ACS reagent): 10 µM, 50 µM, 100 µM, and 200 µM, and nitrate-free seawater as blanks.

Soluble reactive phosphorus within the range $0.03 - 5$ µM was determined by methods adapted from Murphy and Riley (1962) and Parsons (2013). Briefly, 1 mL of the media was allowed to react with a mixed reagent of molybdic acid, ascorbic acid and trivalent antimony to make a blue complex which can be measured at 885 nm. The mixed reagent is created by combining an 4% aqueous m/v ammonium molybdate tetrahydrate (Alfa Aesar, ACS reagent) solution, 13.5% v/v sulfuric acid solution, 5.4% m/v ascorbic acid (Sigma-Aldrich, ACS reagent) solution, and 0.136% m/v potassium

antimonyl-tartrate (Alfa Aesar, ACS reagent) solution at a ratio of 2:5:2:1. A standard was made by adding 81.6 mg of anhydrous potassium dihydrogen phosphate (Fluka BioChemika, > 99.5%) to 100 mL MilliQ H_2O + 0.1% chloroform to form a 0.0816% m/v solution, measured in triplicate. 100 μL of mixed reagent was added to each sample, standard, or blank (distilled water), and inverted to mix. The reaction was incubated for 5 min before measuring absorbance. The concentration of phosphate was determined with the following formula:

$$\mu M \text{ phosphate} = \text{corrected absorbance x F}$$

where $F = 3/(abs_s - abs_b)$, abs_s was the average absorbance of the triplicate standards and abs_b was the average absorbance of the blanks.

All samples were analyzed in randomized groups concurrently.

3.B.7.   Investigation of allelopathic trends in Tara Oceans database

Plankton diversity data within the Tara Oceans database was mined for co-occurrence trends relevant to our model system. Due to the nature of the dataset, we were only able to observe co-occurrence at the genus level for most of our target species. The information was downloaded from PANGAEA directly (https://doi.org/10.1594/PANGAEA.873277) containing the V9 rDNA taxonomic identification organized at the metabarcode level (de Vargas et al. 2015). Due to the large volume of data (>10 GB), the free database software PostgreSQL (v 15.2) was used to store and query the information. A script was written to format the header names to be digestible by the database, as well as retrieve the column titles for the database column creation. The scripts are publicly available on a GitHub repository [https://github.com/wesleyeewong/Tara_Oceans_Taxonomy_Database]. The database contained millions of rows which we queried for those containing the genera of our co-cultivations: *Prymnesium, Dunaliella, Emiliania, Florenciella, Ochromonas,* and *Pavlova.* The resulting data table was manually curated to remove taxonomic identity redundancy, and zero abundance samples were removed for non-relevant pairs. The number of samples and relative abundance of each *Dunaliella, Emiliania, Florenciella, Ochromonas,* and *Pavlova* query was compared against those samples with *Prymnesium* present or absent to evaluate potential trends of allelopathy. We evaluated the abundance

of target species in presence and absence of *Prymnesium*. We also compared relative distribution of species abundances with total abundance for each sample, based on quartile distribution.

3.C.   Results

3.C.1.   Chl *a* and cell density measurements of allelopathy in ASW medium

Phytoplankton allelopathy can impact photosynthetic functions (Gomes et al. 2017, Mao et al. 2021, Poulin et al. 2018), thus we complimented observations of chl *a* fluorescence, a proxy for photosynthetic potential, with cell density measurements for cocultivations in ASW medium. Initial observations showed consistent general trends in the allelopathic influence of *P. parvum* (*Pp*) on partners (**Figure 7**) between measurements of cell density using chl *a* and cell counts directly. *E. huxleyi* (*Eh*) and *O. triangulate* (*Ot*) growth were distinctly inhibited by the presence of *Pp* compared to monoculture controls. *Dt* and *Fp* growth were distinctly enhanced by the presence of *Pp* compared to monoculture controls. The allelopathic effect of *Pp* on *P. gyrans* (*Pg*) was measured differently between chl *a* and cell density measurements: chl *a* showed significant growth enhancement in *Pg* in the presence of *Pp,* but cell density measurements showed no indication of allelopathy received by *Pg* from *Pp* (**SFigure4**).

Pp growth was less obviously influenced by co-cultivation partners regardless of observation method, as values did not prominently deviate from baseline monoculture values (**SFigure2**). *D. tertiolecta* (*Dt*), *F. parvula* (*Fp*), and *Ot* showed some negative impacts on *Pp* growth, while *Pg* showed some growth enhancement. The impact of *Eh* on *Pp* growth varied dependent on the method of measurement. Chl *a* measurements of *Pp* in coculture with *Eh* suggested some growth enhancing effects while cell density measurements suggested no effects (no allelopathy). Allelopathic networks were created to visualize different outcomes dependent on growth observation methods for co-cultivations in ASW (**Figure 7** and **SFigure2**) as seen **in Figure 8A**. While the trends observed from chl *a* and cell density measurements were similar, several differences were noted, specifically in the distinction between significant and non-significant allelopathic effects of Eh on Pp and Pp on Pg growth. The magnitude of significance was also higher in cell density measurements of the positive effect of Pp on Dt growth than chl *a* measurements.

(A)

(B)

Dt   Eh   Fp   Ot   Pg
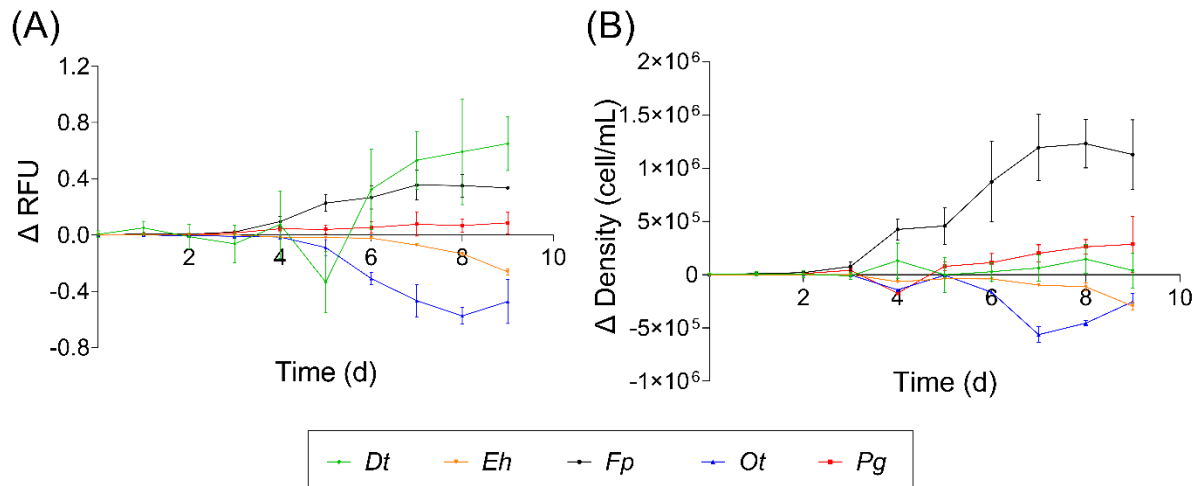
Figure 7. Differences in growth of co-cultivated algae in presence of *P. parvum* compared to monoculture controls. Measured via A) chl a (RFU) and B) cell density (cell/mL) for *D. tertiolecta* (*Dt*), *E. huxleyi* (*Eh*) , *F. parvula* (*Fp*), *O. triangulata* (*Ot*), and *P. gyrans* (*Pg*) cultured in ASW. Error bars represent standard deviation of biological replicates (n = 4).

3.C.2. Nutrient limited cocultivations in ASW

Nutrient analysis conducted at the completion of coculture exposure indicated a minor degree of nitrogen limitation (below the limit of detection) within *Pp-Pg* groups, as well as *Dt* controls for *Pp-Dt* co-cultivation, and *Pp* controls in *Pp-Eh* co-cultivation, which may influence the overall interpretation of allelopathy between pairs of phytoplankton (**STable1**). Growth curves of *Dt* control cultures in ASW, indicated stationary growth phase earlier than mixed cultured *Dt* (**SFigure 12**), suggesting nutrient limitation. The growth curves of all other groups did not indicate stationary growth phase, suggesting nutrient concentrations were still adequate for rapid cell division and not truly limited. As nutrient analysis was only carried out after final growth measurements, it was difficult to speculate at which time point the limitation occurred and if limitation had an effect on allelopathic interpretation. Phosphorus analysis revealed no limitation in any experiments (**STable2**).

Equivalent cocultivations were thus carried out using K/2 media, another replete medium to determine the influence of cultivation media on allelopathic relationships of *Pp*. For these cultivations, nutrient analysis showed no nutrient limitation in nitrogen or phosphorous (**STable1** and

STable2). The availability of nitrogen is slightly higher in ASW media and the availability of phosphorus is much higher in ASW media. The availability of nutrients in final growth measurements in K/2 together with growth curve observations (**SFigure 12** and **SFigure 13**), suggested that any limitation observed in ASW is likely only present in the final days of growth, and unlikely to have affected allelopathy.

Figure 8. Network of allelopathic interaction of *P. parvum* and partners: *P. gyrans*, *E. huxleyi*, *F. parvula*, *O. triangulata*, *D. tertiolectia* in A) ASW medium and B) K/2 medium. Allelopathic response to C) varying inoculation ratios of *P. parvum* and *O. triangulata*, *Pp*:*Ot* ratios a) 1:1, b) 3:1, and c) 1:3. Positive allelopathy indicated by shades of blue, darker blue correlating to more significantly positive interactions. Negative allelopathy indicated by shades of orange, darker orange correlating to more significantly negative interactions. Grey arrows indicate relationships where no allelopathy was observed. Black dashed arrows indicate allelopathic relationships which varied depending on measurements of chl a or cell density.

3.C.3.   Allelopathy in K/2 medium

Similar to ASW cultivations, the emitted allelopathy of *Pp* was more prominent than the received allelopathy from competitors in K/2 medium (**SFigure 2**, **SFigure 3**). *Eh* and *Fp* growth were enhanced in cocultivation with *Pp* compared to monoculture controls, while *Dt* and *Ot* growth were inhibited compared to monoculture controls. *Pg* cocultivations with *Pp* in K/2 medium showed no significant allelopathic influence on the growth of either species. *Pp* growth was inhibited in cocultivations with *Dt*, while there was no effect in *Pp* growth from *Ot*.

While the trends observed between measurements of chl *a* and cell density were similar, we saw significant differences in the response of *Pp* to *Eh* and *Fp* allelopathy between the two measurements (**SFigure 5**). There was no significant response of *Pp* to either *Eh* or *Fp* when observed via chl *a*. Cell density measurements indicated that *Pp* growth was significantly enhanced by the present of *Eh*, while *Pp* growth was significantly inhibited by the presence of *Fp*. These interactions were visualized in an allelopathic map, where differences in chl *a* and cell density measurements were indicated by black arrows in **Figure 8B**. The switch from ASW media to K/2 media also induced changes in allelopathic trends. Significant allelopathic influences of *Pg* on *Pp* growth and *Ot* on *Pp* growth in ASW were neutral in K/2 cultivations. *Pp* inhibited growth of *Eh* in ASW but supported growth in K/2 while the positive allelopathy of *Pp* on *Dt* was only observed in ASW and not K/2, where it even inhibited the competitor. It is important to note again that the controls of Dt in ASW cultures were nutrient limited and likely the cause of the perceived positive growth enhancement in ASW, while all other cultures were not nutrient limited. Changing cultivation medium thus affected the method for allelopathic measurements in addition to species-specific observed allelopathy.

3.C.4.   Influence of inoculation ratios on allelopathy of *Pp*:*Od*

We also evaluated the impact of initial starting ratios of cell counts between *Pp* and *Ot*, which maintained the most prominent and consistent interactions throughout our survey. We varied the inoculation ratios from 1:1 to 3:1 and 1:3 (*Pp*:*Ot*). The resulting allelopathic networks showed variations between chl *a* and cell density measurements as well as allelopathic intensity and direction of interaction (positive, negative, or neutral) (**SFigure 6** and **Figure 2C**). We initially observed

growth inhibition of *Ot* when inoculated at 1:1 (*Ot:Pp*). While the chl *a* of *Pp* was not affected, there was significant inhibition of *Pp* cell density at this ratio. When *Pp* was started with 3x higher concentration, *Ot* was either not affected or positively affected based on chl *a* and cell density observations respectively. The growth of *Pp* at this higher concentration was significantly enhanced by the presence of *Ot*. When *Ot* was started with 3x higher concentration, the growth of *Pp* was either positively affected or not affected by the presence of *Ot*, based on chl *a* and cell density measurements respectively. At this higher concentration, Ot growth was not significantly affected by the presence of Pp.   Co-cultures with *Pp* at 3x the starting concentration were potentially nitrogen limited at the end of observation, but this did not negatively affect the growth of *Ot*.

It should be noted that despite the observed high variability dependent on the experimental set-up, we repeatedly recorded consistent patterns if experiments were conducted in independent set-ups at different days, weeks or months (data not shown).
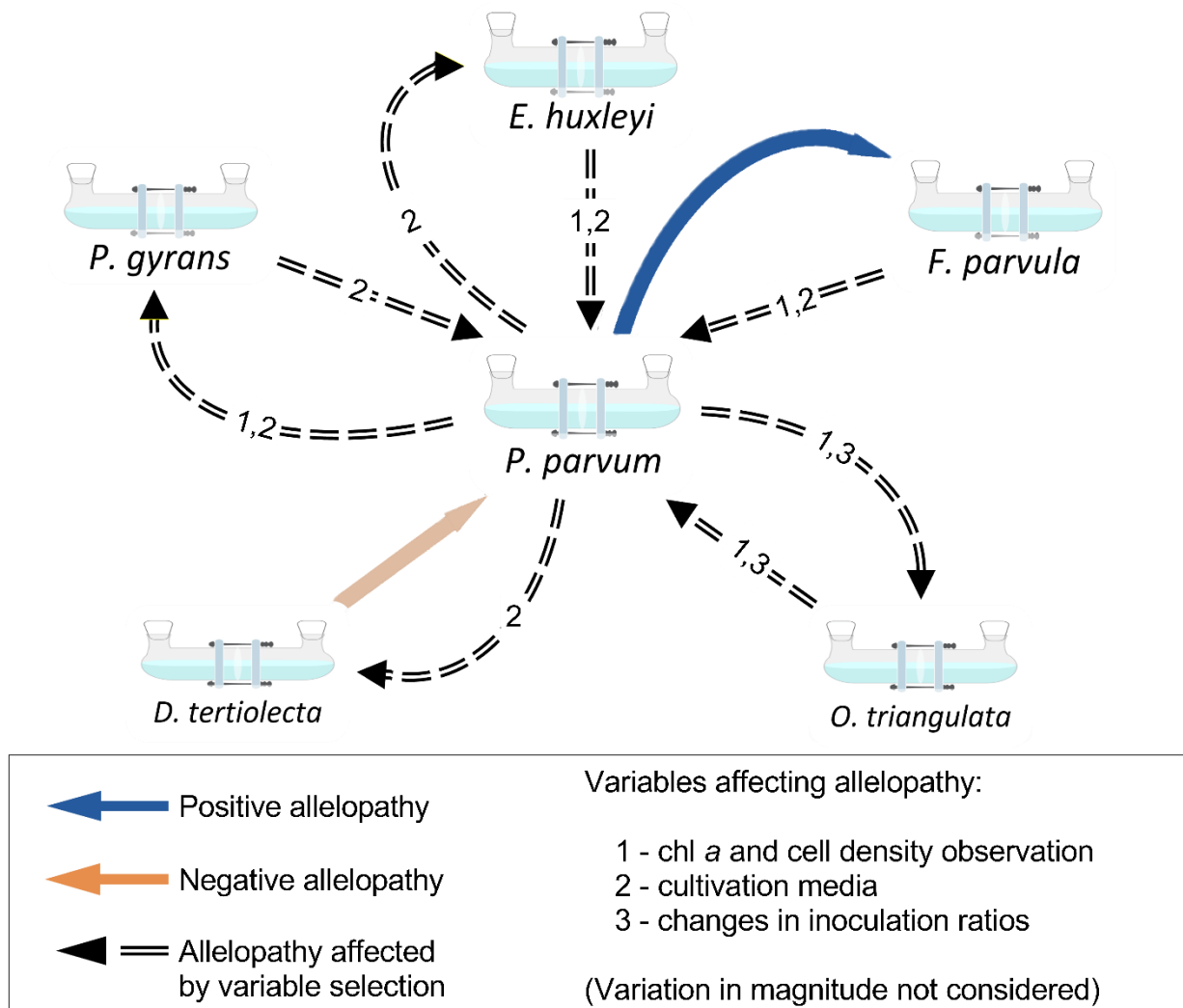
Figure 9. Summary of the variability of species-specific allelopathic interactions of *P. parvum* against *P. gyrans*, *E. huxleyi*, *F. parvula*, *O. triangulata*, *D. tertiolectia* based on changes in cultivation medium and method of observation. Positive allelopathy indicated by blue, darker blue. Negative allelopathy indicated by orange. Black dashed arrows indicate allelopathic relationships which varied depending on laboratory parameters: 1 – method of observation, 2 – cultivation media used, and 3 – inoculation ratios.

3.C.5. Allelopathic trends in natural communities characterized during the Tara Oceans expedition

To assess the ecological merit of our laboratory findings, we mined the publicly available taxonomic database of the Tara Oceans expedition. Due to the complex nature of the data, we did not focus on statistical analysis of the data, but rather on identifying species co-occurrence relevant to our system. *P. parvum* was not identified to the species level within the dataset so we focused on the *Prymnesium* genus, which included other toxin producing *Prymnesium* species such as *Prymnesium polylepis*. The same was true for the genera *Ochromonas, Florenciella,* and *Dunaliella* respectively. Full identification was available for *E. huxleyi* and *P. gyrans*. Within the Tara Oceans data, the *Florenciella* genus was most frequently found in 832 samples of the 903 total samples evaluated. This was followed by *Prymnesium* in 575 samples, *Ochromonas* in 437 samples, *Duanliella* in 369 samples, *Emiliania huxleyi* in 62 samples, and *Pavlova gyrans* at 9 sampling points. Of these samples, 580 of *Florenciella*, 329 of *Dunaliella*, 390 of *Ochromonas*, 61 of *E. huxleyi* and all 9 *P. gyrans* were detected with *Prymnesium* also present.

The relative total abundance of *Florenciella* spp. per sample in the presence of Prymnsium spp. was two orders of magnitude higher, than the total abundance of *Florenciella* spp. without Prymnesium spp. present. The majority of these cooccurring samples (69%) accounted for the highest abundances of *Florenciella* spp. throughout all samples of the TaraOceans database. This was irrespective of the total abundance of species within the database. Furthermore, in natural co-occurrences of *Prymnesium* spp. and *Ochromonas* spp., when the abundance of *Prymnesium* spp. was > 3x Ochromonas spp., the average abundance of *Prymnesium* spp. was two orders of magnitude higher than when *Prymnesium* spp. was 3x lower than *Ochromonas* spp.

Generally, for the TaraOceans data, the average abundance of *Florenciella, Duanliella, Ochromonas,* and *E. huxleyi* was higher when observed in the presence of *Pymensium* than without, and *P. gyrans* was always observed in samples with *Prymensium* present. The investigation of statistically significant trends was difficult due to the complex nature of the dataset. While we were able to observe trends for some of the most prominent allelopathic interactions in our experiments, much of the more complex allelopathic observations were left open-ended.

Table 1. Parameters influencing allelopathy in marine phytoplankton.

	Parameter	Reference
Abiotic	Nutrient availability	Johansson Granéli 1999[1], Chia et al. 2018
	Light	Hagström and Granéli 2005[1]; Barreiro Felpeto et al. 2018; Śliwińska-Wilczewska et al. 2016
	Temperature	Larsen and Bryant 1998[1]; Śliwińska-Wilczewska et al. 2016
	pH	Shilo and Aschner 1953[1]; Schmidt and Hansen 2001[1]; Hansen 2002
	Salinity	Larsen and Bryant 1998[1]; Brutemark et al 2015; Śliwińska-Wilczewska et al. 2016
Biotic	Species/phenotype	Blossom et al 2014[1]; Konarzewska et al. 2020
	Microbial community	Fistarol et al 2003[1]; Chen et al. 2020; Prince et al. 2008
	Growth stage	Shilo 1967[1]; Matsuyama et al. 2000; Suikkanen et al 2004
	Density	Johannson and Granéli 1999[1]; Legrand et al. 2003; Poulson et al. 2009
	Cell size	Schmidt and Hansen 2001[1]; Lyczkowski and Karp-Boss 2014

[1]Studies conducted involving *Prymnesium parvum*

3.D.   Discussion

Our studies highlight the pronounced plasticity in laboratory phytoplankton allelopathic co-cultivation studies. Even within highly controlled laboratory settings, changes in selected conditions may impact the outcome of allelopathy between species-specific interactions. Altering the method of observation (chl *a* or cell density), cultivation media, and inoculation ratios caused changes in the significance and direction of allelopathic relationships observed in our experimental setup (**Figure 3**). We monitored variation of allelopathic interactions qualitatively, but it is important to mention that quantitative changes were not considered, otherwise all interactions would be considered plastic. A literature survey indicates several aspects of parameter-dependent allelopathy can be identified in previous studies (**Table 1**).

Allelochemicals can interfere with photosynthesis for which in chl *a* measurements serve as proxy (Poulin et al. 2018, Śliwińska-Wilczewska et al. 2018). *P. parvum* metabolites inhibit electron transport in Baltic Sea phytoplankton, thereby depressing photosynthesis (Fistarol et al. 2003). Cell density measurements can complement the information from chl *a* for a more robust interpretation. In general, the two measurements followed the same trends, but few examples of deviation indicate rather subtle differences in the outcome of allelopathic monitoring. Those differences that arose were not a change in the direction of allelopathy but rather in the presence or absence of statistically significant allelopathic interactions.

During cocultivation, we found that the type of media had an overall effect on the growth rates of the phytoplankton species, with ASW supporting higher density cultures. The medium also influenced the type of outcome of the co-culturing. Since the effects already manifested early in the coculturing, we can conclude that nutrient limitation plays no role, or only an additional role in the late culturing phase. Allelopathic activity is thus rather differentially expressed in the respective media. The additional influence of nutrient limitation in the late phase is likely *P. parvum* filtrates have a higher toxicity when algae were grown under nitrogen or phosphorous deficiency, while filtrates of non-deficient *P. parvum* have exhibited positive growth effects on other phytoplankton (Skovgaard and Hansen 2003, Tillmann 2003, Fistarol et al. 2003, Granéli and Johansson 2003). The nutrient limitation might explain the outcome of co-cultivation of *D. tertiolecta* response to *P. parvum*. Due to the rapid growth of *D. tertiolecta* in ASW, the controls for this phytoplankton likely became nitrogen limited earlier than mixed cultures, explaining the manifestation of different cell

counts in mixed co-cultures and control in the last days of culturing (**SFigure 12**). In K/2 media that was not depleted in nutrients at the end of the co-culturing, a negative allelopathy caused by *P. parvum* manifested already early in the experiment. For other groups, allelopathic activity was influenced by the change in ASW to K/2 media, particularly the negative allelopathy of *P. parvum* on *E. huxleyi* in ASW, was positive when in K/2 media. Positive allelopathy between *P. parvum* and *P. gyrans* in ASW, was not significant in K/2 media. While *O. triangulata* inhibited *P. paruvm* in ASW, there was no significant effect in K/2 media. Given the differences, we can assume that the media type affected the allelopathic interaction. K/2 media uses filtered and sterilized natural seawater as a base for added nutrients while ASW is completely assembled in laboratory using salts to mimic seawater conditions. The natural seawater in K/2 media likely contains secondary metabolites from organisms in the collection site. The allelopathic interactions observed are more likely to reflect the diverse metabolic environment in natural communities, than the ASW which provides more insight in controlled binary interactions.

      P. gyrans was generally resistant to harmful allelopathy of by *P. parvum*, regardless the co-culturing conditions. *F. parvula* growth benefited from *P. parvum* allelopathy. Positive allelopathy from P. parvum is typical for other *Prymnesium* species. Outside of this genus, positive allelopathy from *P. parvum* on other phytoplankton when low-density, replete filtrates of *P. parvum* were used. (Granéli and Johansson 2003). Low density filtrates supported the growth of *T. weissflogii, P. minimum,* and *R.* cf. *baltica*, while high density had a negative influence. The insensitivity of other *Prymensium* species to *P. parvum* allelopathy has been postulated to relate to inter-genus adaptations which confer protection from allelopathic substances of closely related species (Granéli and Johansson 2003).

      In our experiments, growth of competing species was monitored over an extended period of time, allowing observations from low to high cell density. During the entirety, *P. gyrans* and *F. parvula* did not display any detrimental effects in their growth from exposure to *P. parvum* allelopathy, which may indicate the ability to not only evade the deleterious allelopathic substances released by *P. parvum*, but also use them or other exudates to benefit their own growth. The ability of phytoplankton to undermine the allelopathy of a competing species has previously been reported in the case of the diatom, *Skeletonema* spp. whose presence reduces the detrimental effects of natural bloom extracts of the toxic dinoflagellate, *Karenia brevis* on competing phytoplankton (Prince et al. 2008). Whether *P. gryans* and *F. parvula* have the ability to undermine or acclimate to *P. parvum* allelopathy or have resistance mechanisms against those allelochemicals is yet to be determined but

would present an interesting research topic which could benefit aquacultures affected by *P. parvum* bloom toxicity (Moestrup 1994).

Relative starting concentrations may provide advantages in competition but may not reflect outcomes of allelopathy. When we varied the inoculation ratios of *P. parvum* and *O. triangulata,* we saw complex changes in allelopathy. When either species had a clear starting advantage, no detrimental allelopathic effects on the growth of the other species were observed. Instead, we observed mutual benefits when one species dominated the other. In highly competitive starting conditions (1:1), *P. parvum* exerted strong allelochemical pressure, dominating the coculture and suppressing *O. triangulata* growth. The significant inhibition of *P. parvum* cell density in this situation indicated that *O. triangulata* was also releasing detrimental allelochemicals in response to *P. parvum* allelochemicals.

The release of allelochemicals is likely proportional to the densities of donor cells, as more cells produce more allelochemicals (Granéli and Johansson 2003, Legrand et al. 2003) and the effect on competitors may also be influenced by changes in concentration. Another important factor which needs to be considered is the physiological state of each species (Poulson et al. 2010). The metabolism of phytoplankton is highest during exponential growth (Vidoudez and Pohnert 2012) which may allow adaptations to allelochemical cues of competitors, as growth stages have been linked to the suceptiblitly of *K. brevis* allelopathy. Only early-growth stage *Skeletonema grethae* was significantly inhibited by *K. brevis* exudates (Poulson et al. 2010). Furthermore, the diatom *Skeletonema costatum* is able to undermine the allelopathy of *K. brevis*, reducing the growth-inhibiting effects of extracellular extracts of *K. brevis* when grown in co-culture Authors predicted this strategy may be due to metabolizing the allelochemicals, the interruption of biosynthesis or exudation of allelochemicals, or the production of counteracting compounds (Poulson et al. 2009, Prince et al. 2008). The presence of competitors may stimulate complex and dynamic metabolic responses between interacting species, which may not be represented by typical filtrate-based (Suikkanen et al. 2004, Fistarol et al. 2005, Wang et al. 2013, Yang et al. 2014) or short term exposure (Tang and Gobler 2010, Yan et al. 2019) experiments of allelopathy which may limit our understanding of allelopathic interactions. The use of non-contact cocultivations for extended time periods allow for mutual responses between species (Ternon et al. 2018, Wang et al. 2020b) to be monitored and provide a more ecologically reflective allelopathic relationship.

Resistance to *P. parvum* allelopathy may provide competitors unique opportunities, as it is notoriously known for its ability to outcompete other phytoplankton, leading to monospecific blooms (Johansson 2000). Resistant phytoplankton in the natural environment may benefit not only from protection against *P. parvum* allelochemicals, but from the availability of nutrients not utilized by other species which are inhibited by *P. parvum*. Depending on the strategies of resistance, species like *F. parvula* can directly benefit from growth promotion of *P. paruvm* allelopathy. Whether the advantage is in the direct metabolism of allelochemicals by phytoplankton or associated bacteria, the suppression of production and exudation of allelochemicals, or production of remediating compounds remains uncertain.

We did not observe specific taxonomic responses to *P. parvum* allelopathy. The haptophytes *E. huxleyi* and *P. gyrans* had varying responses to *P. parvum*; itself a haptophyte. The stramenopiles *F. parvula* and *O. triangulata* also experienced opposing effects from *P. parvum* allelopathy, suggesting species-specific effects. Due to the complexity of the data, statistically significant trends were not discernible, and the more complex allelopathic interactions observed in our experiments were difficult to correlate to the TaraOceans database. However, we were able to distinguish trends for two consistent allelopathic relationships within our dataset. In our evaluation of natural plankton abundances from the Tara Oceans cruise, we saw a trend of increased abundances of *Florenciella* spp. in the presence of other *Prymnesium* spp., which was consistent with our allelopathic observations in coculture. The growth enhancement of *F. parvula* in the presence of *P. parvum* was one of the strongest and most consistent trends found in our coculture experiments. For TaraOcean samples where *Ochromonas* spp. and *Prymnesium* spp. were both present, the relative abundances between the genera were consistent with the significant allelopathic relationship observed when we skewed starting conditions in favor of *P. parvum*. In our inoculation ratio experiments between *Pp*:*Ot*, this growth enhancement of *P. parvum* in the presence of low abundance of *O. tricornutum* was a significant and constant relationship. Though the sample size where *P. gyrans* was detected is too low to make concrete assumptions, the strict co-occurrence with other *Prymnesium* is a promising observation to support the positive relationship in our experiments. Furthermore, the pronounced abundance of the *Florenciella* genus in nearly all TaraOceans samples may infer ecological implications in the positive allelopathy received by *F. parvula* in the presence of *P. parvum* in our study. In other words, the ability of *F. parvula* to thrive in the presence of *P. parvum* allelopathy may indicate mechanisms which allow it to thrive in various conditions across multiple oceans. Thus, the investigation of species-specific interactions is paramount to the understanding of the ecological

impact of natural allelopathic interactions, with specific emphasis on the reflection of natural conditions in controlled laboratory setting to find meaningful interactions. This would be extremely fitting for mesocosm studies which focus on natural phytoplankton communities and would allow dynamic monitoring of multiple competing species.

4) **Chapter 2**: Transcriptomics guided identification of algicidal protease of the marine bacterium *Kordia algicida* OT-1

The experimental work for Chapter 2 was carried out entirely by me with assistance from Dr. Yun Deng, Dr. David A. Russo, and Dr. Nils Meyer. Concurrently with the preparation of this thesis, the data obtained was submitted for publication in *MicrobiologyOpen*. The corresponding manuscript was written by me with heavy contribution from Dr. David A. Russo, and comments/corrections from Dr. Yun Deng, Dr. Remington Poulin, and Dr. Georg Pohnert included in this chapter. All authors commented on the give informed consent for the use of the data in this thesis and agree to the statement of contribution. Details of contributions are given in the following table.

Name of co-worker	Syhapanha	Russo	Deng	Poulin	Meyer	Pohnert
Conception of Study	X					X
Experimental Planning	X	X	X	X		
Transcriptomics	X					
Vector Transformation					X	
Bioassays	X	X				
PCR	X		X			
Genomic Evaluation	X	X	X			
Data Analysis & Interpretation	X	X	X	X		
Manuscript Writing	X	X	X			X

4.A.   Study Introduction

Algicidal bacteria have attracted interest for their potential to act as biotic regulators of harmful agal blooms (HABs) (Doucette et al. 1999, Mayali and Azam 2004, Meyer et al. 2017). They are commonly found associated with late-stage blooms (Imai et al. 2001, Kim et al. 1998, Skerratt et al. 2002), however the ecological role of algicidal bacteria is still yet to be clearly defined (Coyne et al. 2022, Meyer et al. 2017, Wang et al. 2020a). It has been suggested that they play a dominant role in the microbial loop and the global cycling of organic carbon in the aquatic environment (Azam et al. 1983). Additionally, recent studies have shown that algicidal bacteria may also influence the succession of natural phytoplankton communities (Bigalke et al. 2019, Onishi et al. 2021).

Algicidal bacteria act by either direct contact with algal cells or by the release of diffusible algicidal compounds (Mayali and Azam 2004, Meyer et al. 2017). The majority of reported released algicidal compounds derives from work done with cell-free filtrates (Coyne et al. 2022, Meyer et al. 2017, Wang et al. 2020a). However, due to their diverse nature, there are no standardized methods to elucidate algicidal compounds, which can range from small molecules (Sakata et al. 2011, Wu et al. 2011), to peptides (Banin et al. 2001, Hibayashi and Imamura 2003, Imamura et al. 2000) to enzymes (Kohno et al. 2007, Lee et al. 2002, Lee et al. 2000, Paul and Pohnert 2011).

Production of algicidal extracellular proteins may seem energetically costly for the producing microorganism but it is often necessary for the breakdown and uptake of essential nutrients. Identification of active enzymes can be supported by molecular techniques that also provide insights into their genetic regulation. In recent years, transcriptomics has been used to broadly identify enzymes upregulated during algicidal activity (Zhang et al. 2022a, Zhang et al. 2023). Multiple metabolic pathways were involved in the transcriptomic analysis of the algicidal mechanism of algicidal bacterium *Brevibacillus laterosporus* strain Bl-zj against the cyanobacteria *Microcystis aeruginosa* strain FACHB 095 (Zhang et al. 2022a). The co-cultured bacteria differentially expressed genes involved in amino acid, carbohydrate, and lipid metabolism, with significantly increased expression of genes in valine, leucine, isoleucine, and fatty acid degradation to obtain the energy necessary to produce algicides. The upregulation of secreted hydrolytic enzymes, antibiotics, proteases, and other secondary metabolites were hypothesized to aid in the destruction of algal cells (Zhang et al. 2022a). An integrated transcriptomic and metabolomic study was used to characterize the algicidal process of the bacterium *Enterobacter hormaechei* strain F2, also in co-cultivations with

M. aeruginosa strain FACHB-315 (Zhang et al. 2023). Analysis of differentially expressed genes during the algicidal process revealed an enrichment of energy metabolism and aromatic amino acid metabolism related pathways. Integration with metabolomic analysis revealed significant changes in peptides, co-enzymes, vitamins, and energy substances, and revealed potentially active algicides. An enrichment of chemotaxis-related genes alluded to direct algicidal mechanism used by this bacterium (Zhang et al. 2023). Exploring the genome of *Brevibacillus laterosporus* Bl-zj revealed 18 potential algicidal proteases (Zhang et al. 2021). Bioassays have led to the discovery of an increase in activity of enzymes such as β-glucosidase (Kim et al. 2009), chitinases (Li et al. 2016), and L-amino acid oxidases (Chen et al. 2010, Chen et al. 2011) during the algicidal process. Other studies predicted enzymatic algicidal activity based on the evaluation of general extracellular degradative enzymes (Mayali et al. 2008, Zhou et al. 2021). Few studies have identified algicidal proteases at the genomic level in algicidal bacteria, with corresponding *in vitro* confirmation (Kohno et al. 2007).

In our study we focus on the algicidal marine bacterium *Kordia algicida* OT-1. *K. algicida* was first reported by Sohn and colleagues (2004) as a Gram-negative marine bacterium of the *Flavobacteriaceae* family isolated from a bloom of the diatom *Skeletonema costatum* (Sohn et al. 2004). The genome was reported in 2011 and interestingly revealed gliding motility genes, although the bacterium is non-motile and non-gliding (Lee et al. 2011). The bacterium has a broad target range, with activity reported against diatoms, dinoflagellates, and raphidophytes (Sohn et al. 2004). Paul and Pohnert (2011) initially characterized the algicidal compound released by *Kordia algicida* as a serine protease which may be regulated by a quorum sensing mechanism. The diatom *Chaetoceros didymus* is resistant to *K. algicida*, producing algal proteases as part of a defensive mechanism against *K. algicida* (Paul and Pohnert 2013). In a natural community enclosure experiment, *Kordia algicida* shifted the plankton population and accelerated plankton succession by the removal of a dominant, susceptible alga (Bigalke et al. 2019).

Here, we present the identification of an algicidal protease gene *K. algicida.* The discovery of active, inactive, and subsequently inducible modes of algicidal activity in this bacterium facilitated the use of transcriptomics to identify algicidal candidates. Further analysis narrowed the candidates to a single protease, AlpA1. Other members of the *Kordia* genus showed no algicidal activity in corresponding assays though similar homologues of AlpA1 were identified with pairwise BLASTp comparisons.

4.B.   Methods

4.B.1.   Strains and growth conditions

Kordia algicida OT-1, *Kordia aestuarivivens* (accession number NBRC 114499), *Kordia periserrulae* (accession number NBRC 106077), and *Kordia* sp. (accession number NBRC 113026) were obtained from the Biological Resource Center, NITE (NBRC) from Shibuya, Japan, and stored as a cryo archive. Bacterial cultures for each experiment were initiated from cryo archives by streaking onto marine broth agar plates and incubating at 28 °C for 2 - 3 days. Single colonies were then transferred to marine broth medium (MB) and grown at 28 F with 80 - 100 rpm shaking until the desired growth phase was achieved.*Skeletonema marinoi* (accession number RCC75) was purchased from the Roscoff Culture Collection and maintained in artificial seawater media (ASW) (Maier and Calenberg 1994) at 13 °C, 14/10 day/night cycle with light intensity range 15 - 30 µmol/m^2/s.

4.B.2.   *K. algicida* growth analysis

Growth of *K. algicida* was monitored via periodic optical density measurements on a Genesys 10S UV–Vis spectrophotometer (Thermo Fisher Scientific,Waltham, MA, USA) in tandem with algicidal activity bioassays. Four biological replicates were inoculated with single colonies in 20 mL MB. Growth was monitored using optical density measurements at 550 nm starting at 12 h post inoculation, and subsequently, every three hours until 48 h. A final measurement was taken at 62 h. Algicidal activity was assessed at each timepoint.

4.B.3.   Algicidal activity bioassay

Algicidal activity was identified by the reduction of chlorophyll *a* (chl *a*) fluorescence of the diatom *S. marinoi* after 24 h incubation with *Kordia* spp. *S. marinoi* was used as a target organism in the assays due to its susceptibility to *Kordia* spp. algicidal activity (Paul and Pohnert 2011). To determine *Kordia* spp. algicidal activity, 100 - 200 µL of a culture was harvested via centrifugation for 10 min at 10 000 rpm and 13 °C. Two sample volumes of filtered ASW medium were used to wash the pellets in duplicate, to remove any residual MB that could interfere with the bioassay. The washed *Kordia* spp. was then resuspended to a working OD$_{550}$ of 0.08 in ASW. An equivalent volume of cell-free MB medium (100-200 µL) was treated similarly, as a negative control for the algicidal

activity assay, to account for potential media toxicity. 150 µL of exponentially growing *S. marinoi* was mixed with 50 µL of the resuspended *Kordia* spp. in a 96-well microplate. Activity was determined measuring the chl *a* fluorescence (ex: 430 nm, em: 665 nm) (Varioskan Flash, Thermo Fisher Scientific) of *S. marinoi* after 24 h.

For algicidal induction bioassays, inactive *K. algicida* was diluted, 1:10 into filtered ASW and incubated overnight at 28 °C and 80 rpm. The cultures were then analyzed for algicidal activity via the previously described method.

For algicidal bioassays in the presence of EDTA, *K. algicida* was grown in 10 mL MB overnight at 28 °C and shaking at 80 rpm. Cultures were diluted 1:20 in ASW and incubated overnight, same conditions. Cell-free spent supernatant was collected for EDTA treatment by pelleting cultures by centrifugation, 10 min at 13 000 rpm and 13 °C. EDTA-2Na (Alfa Aesar) aqueous stock solution (100 mM) was added to a final concentration of 5 mM and an equivalent volume of H_2O was added for control. All samples were incubated in the dark for 15 min before algicidal activity was assessed following the method delineated above.

For algicidal activity assays using recombinant proteins in ASW, cell-free spent medium of exponentially growing *K. algicida,* (20x diluted in ASW 24 h) was used as a positive control, and filtered ASW was used as a negative control. 50 uL of each treatments were added to 150 uL of *S. marinoi* cultures (n=4) and chl *a* was measured at time 0 and 24 h to observe algicidal effects.

4.B.4.   Fractionation of spent culture medium

To verify if other small molecules released by *K. algicida* contribute to the algicidal activity, we separated the spent *K. algicida* culture supernatant into a protein-rich fraction (>3kDa) and a small molecule-rich fraction (<3kDa). To this end, exponentially growing *K. algicida* in MB medium (~ 24 h) was inoculated into ASW (1:20) for overnight cultivation, 28 °C, 80 rpm and accumulation of secreted algicidal compounds. The following day, cultures were centrifuged for 30 min at 9000 rpm and 15 °C to produce cell-free spent medium. The protein-rich fraction was separated from the small molecule-rich fraction via centrifugal filtration, 30 min, 4500 x g, 4 °C, using 3 kDa Amicon centrifugal tubes (Millipore), washed with ASW medium and concentrated 10x, 30 min, 4500 x g, 4 °C. The flow through was applied to a 30 mg HLB SPE column, eluted with 3 mL MeOH (HPLC Grade), dried under N_2 and reconstituted to a 10x concentration of the original volume with ASW.

Algicidal activity was performed using the individual fractions, a recombination of the two fractions at a 1:1 ratio, as well as the unfiltered cell-free spent medium as a positive control. 50 µL of the treatments were added to 150 µL *S. marinoi* cultures and chl *a* fluorescence was measured immediately after inoculation (0 h) and again 24 h later.

4.B.5.   Preparation of bacterial samples for transcriptomic analysis

For transcriptomic analysis, *K. algicida* cultures were grown according to the section, *K. algicida* growth analysis, above. To compare active and inactive modes of *K. algicida*, 3 - 6 mL aliquots of bacteria were collected at 30 h and 75 h, based on results from growth curve analysis. Additionally, following collection of 75 h samples, the inactive cultures were subjected to the induction of algicidal activity by dilution in ASW (1:10) for 24 h. Subsequently, an additional 3-6 mL sample was collected to represent induced activity. For each sample, cells were harvested via centrifugation for 10 min at 10 000 rpm and 4 °C, flash frozen in liquid N_2, and stored at -80 °C until RNA extraction. For each sampling timepoint, bioassays were conducted concurrently to confirm algicidal activity. RNA extraction was carried out using an RNAeasy MiniKit (QIAGEN, Hilden, Germany) following lysis via bead beating using a TissueLyser II (QIAGEN, Hilden, Germany). Briefly, two cell pellet volumes of beads were added to each sample and cells were broken for 3 min at 60 Hz and then cooled in liquid N_2 (3 - 5 rounds). Lysed samples were then stored on ice and RNA extraction was carried out according to the manufacture's recommendations. Extracted RNA was stored at -80 °C and triplicate RNA samples of active and inactive *K. algicida* cultures were submitted to Novogene (Cambridge, UK) for comparative transcriptomic analysis. RNA aliquots from active, inactive, and induced cultures were kept for RT-qPCR analysis.

4.B.6.   RT-qPCR

Aliquots of RNA samples sent for transcriptomic analysis were saved for in-house analysis, with the RNA extracted from the additional induced timepoint. RNA concentrations of active, inactive, and induced samples were measured using Quibit reader for Broad RNA detection and adjusted to the same starting concentration (18 ng/µL) using nuclease free water. cDNA was generated with a SuperScript IV VILO Master Mix with ezDNase enzyme digestion (Thermo Fisher Scientific, Carlsbad, CA, USA) following manufacturer's recommendations. Briefly, to remove contaminating gDNA, RNA samples were incubated at 37 °C with ezDNase in ezDNase buffer for 2

min, then stored on ice. RNA samples were split for reverse transcription to cDNA. Super Script IV VILO Master Mix was added to one sample and SuperScript IV VILO Master Mix No-RT control was added to the other. Samples were then incubated at 25 °C, for 10 min to anneal primers, at 50 °C for 10 min to reverse transcribe RNA, and finally at 85 °C for 5 min to inactivate the enzymes.

For qPCR reactions, all primers were purchased from biomers.net GmbH (Ulm, Germany). *KAOT1_RS10890* was amplified with forward primer ATCTATGCGCAAAGCTCGTG and reverse primer TGACTTCGGAGCTGACATTC. *KAOT1_RS09515* was amplified with forward primer AGGAATTGCGCCACATTCAG and reverse primer GTACGCTACACCGATAACAC. The *K. algicida* 16s rRNA gene was used as a housekeeping gene and amplified with forward primer GGTACTGTTGGATTGCATGATTC and reverse primer TCAGAGTTGCCTCCATTGTC. qPCR was performed by combining 0.5 µL of the cDNA template generated above with 0.5 µL of both forward and reverse primers, 5 µL SYBR Green Master Mix, and 3.5 µL H_2O. Amplification was performed on a C1000 Touch Thermal Cycler CFX96 Real-Time System (Bio-Rad Laboratories, Feldkirchen, Germany) with the following program: 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 1 min, then 60 °C for 31 s followed by 60 °C for 5 s ramped to 95 °C for 0.5 °C/cycle and 0.5 °C/s for 70x cycles. Results were viewed using Bio-Rad CFX Maestro software.

4.B.7.   Heterologous expression of AlpA1

For activity testing, AlpA1 was expressed in *E. coli*. First, genomic DNA was extracted from *K. algicida* using DNeasy Blood & Tissue Kit (Quiagen, Venlo, NL). The *KAOT1_RS09515* gene, hereby known as *alpA1,* was amplified using forward primer KaP1 (GAATTGGCCATAACGGACAGTATTACATCTTCTGATGAAG) and reverse primer KaP2 (GTAACTCGAGTTTTTTAGCATTTGGAGCTGTAAATCCG), designed to cover the complete sequence of the ORF except the first 60 bp. Amplification was achieved using Herculase II Fusion DNA Polymerase (Agilent Technologies, Santa Clara, CA, USA) with 20 ng gDNA, 0.25 µM of each primer, 250 µM dNTPs and 4% (v/v) DMSO with the following program: 2 min initial denaturation at 95 °C; 35 cycles of 20 s denaturation at 95 °C, 20 s annealing at 55 °C and 45 s elongation at 72 °C; 3 min final

elongation at 72 °C. The PCR product was digested with 8 000 U *Xho*I (New England Biolabs, Ipswich, MA, USA) and 10 000 U *Msc*I (New England Biolabs) in CutSmart buffer (New England

Biolabs) for 1 h at 37 °C. Vector pET-26b (Novagen, Merck Millipore, Darmstadt, Germany) was digested using the same restriction enzymes. PCR product and open vector were purified innuPREP Gel Extraction Kit (Analytik Jena, Jena, DE) and ligated using T4 Ligase (New England Biolabs) overnight at 4 °C. The ligation product was transformed into electro-competent *E. coli* Top10 (Invitrogen, Carlsbad, CA, USA). The transformed cells were cultivated in LB medium for 1 h at 37 °C and were then plated on LB agar plates with kanamycin for transformant selection. Plasmids were verified by restriction digest with *Xmn*I (New England Biolabs) and Sanger sequencing (Eurofins Genomics GmbH, Ebersberg, DE). The plasmid was then transformed into chemically competent *E. coli* Rosetta2(DE3) (Novagen, Merck, Darmstadt, DE). Transformants were verified by colony PCR. The resulting strain was called *E. coli* P390.

4.B.8.   Expression and isolation of recombinant protein and BLASTp genera comparison

The expression and purification of AlpA1 was performed following a similar method as described in (Faezi et al. 2017). A single colony of *E. coli* P390 was grown overnight in 10 mL LB medium supplemented with kanamycin (50 µg/mL) and then diluted into fresh 50 mL LB medium containing kanamycin to an OD_{600} of 0.05. When the culture grew to logarithmic growth phase (~OD_{600} 0.6), IPTG was added (final concentration 500 µM) and expression was induced for four hours, 25 °C and 180 rpm. A 30 mL aliquot was harvested at 4 000 g for 20 min at 4 °C and re-suspended in 2 mL PBS buffer (pH 7.4) for cell lysis. The resuspended cells were lysed via ultrasonic probe with bursts of 10 s (100% power) followed by intervals of 30 s on ice. Cell debris was removed by centrifugation for 30 min at 9 000 rpm and 4 °C. The supernatant was loaded onto a pre-equilibrated HisPur Ni-NTA spin column (Thermo Fisher Scientific, Carlsbad, CA, USA) and purification under native conditions followed manufacturer's instructions.

Following elution, a buffer exchange with ASW was performed with Amicon 3 kDa MWCO 3 kDa Amicon centrifugal filters. The presence of the recombinant protein was confirmed via Western Blot. The recombinant protein was stored in -80 °C for further bioassay.

The NCBI protein sequence of AlpA1 was used in a BLASTp query against all available Kordia genomes (taxID: 221065) (Altschul et al. 1997).

4.C.   Results

4.C.1.   Loss and re-activation of algicidal activity of *K. algicida*

We performed a growth curve analysis with corresponding algicidal activity bioassays to understand the relationship between bacterial growth and algicidal activity. During the algicidal activity bioassay, *K. algicida* cultures become inactive and algicidal activity can be rescued by exposure to nutrient starvation. Algicidal activity is present from the start of the culture until the death phase (grey shaded region in **Figure 9A**). To determine if the loss of activity was governed by quorum sensing effects, the inactive cultures at 72 h were inoculated into fresh MB medium. Despite growth of the cultures, the algicidal activity was not recovered. We then rationalized that nutrient starvation may play a role in activating algicidal activity in the pressure for resource acquisition, and thus performed a subsequent incubation in nutrient limiting ASW medium (minimum ratio tested 1:10 MB:ASW). The data shows that nutrient starvation could, in fact, restore algicidal activity (**Figure 9B**).

A previous study by Paul and Pohnert (2011) reported that secreted proteases (> 30 kDa) are, at least in part, responsible for the algicidal activity of *K. algicida*. To confirm the activity was due to secreted proteases, conditioned *K. algicida* medium was fractionated to obtain a fraction enriched in proteins (>3 kDa) and a fraction enriched in small molecules (<3 kDa). This experiment extends the results from Paul and Pohnert with an investigation of the role of small molecules in algicidal activity. We thus generated separated protein enriched fractions and extracted and enriched small molecule fractions of the active spent medium of *K. algicida*. Application of these fractions in an algicidal activity assay showed that both the combined fraction and the >3 kDa fraction are algicidal, while the < 3kDa fraction is not (**Figure 9C**). Thus, confirming that the algicidal activity derives from the >3 kDa protein-rich fraction, with little to no direct contribution by small molecules. Therefore, to determine which secreted proteases could potentially be present during the algicidal phase, we proceeded with a transcriptomic analysis of active and inactive populations of *K. algicida*.
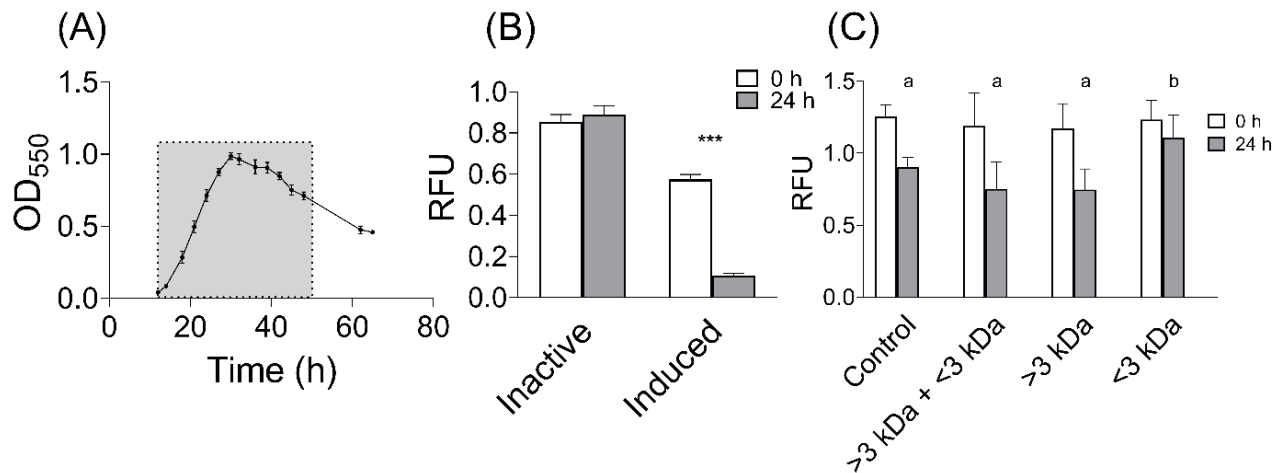
Figure 10. (A) Growth curve of *K. algicida* in marine broth medium reveals the period of algicidal activity in the grey shaded area. (n=4) (B) Inactive and induced activity of *K. algicida* measured by change in chl *a* (RFU). (n=4). Multiple paired t-test, *** p < 0.001 . (C) Activity of separated fractions of *K. algicida* spent medium: enriched small molecules fraction (< 3 kDa), mostly protein fraction (> 3 kDa) and 1:1 recombination of both (>3 kDa + < 3 kDa), with whole spent medium used as algicidal control (n=8). Measured by change in chl *a* (RFU). One-way ANOVA was performed on 24 h data, with significance indicated by letters a,b. All error bars indicate the standard deviation of biological replicates.

4.C.2. Transcriptomic approach reveals algicidal candidates

For the comparative transcriptomic analysis, samples were harvested at 30 h (point of maximum cell density and pronounced algicidal activity, hereafter "active") and 75 h, well outside the previously determined algicidal window (hereafter "inactive") (**Figure. 10A**). An algicidal assay performed with an aliquot of each sample utilized for transcriptomic analysis confirmed an active and an inactive state were being compared (**SFigure 15A**). Between active and inactive states, 2589 genes displayed statistically significant differences in transcript levels (**SFigure 15B**). To search for secreted algicidal protease candidates, the list was narrowed down to genes that 1) were upregulated at the 30 h timepoint, 2) were annotated as protease or peptidase and 3) contained a signal peptide. From this search, 24 protease/peptidase candidates were identified, and the top 10 candidates, based on fold change analysis, are listed in **Table 2**.

To confirm the transcriptomic results, and further narrow down the list of candidates, the transcript levels of the two largest fold change protease/peptidases were measured by RT-qPCR. For this experiment, we used saved aliquots of RNA from the samples submitted for transcriptomic analysis of active and inactive, 30 h and 75 h respectively, *K. algicida*. Additionally, we analyzed RNA samples from induced *K. algicida*. These were generated by diluting the inactive (75 h) *K. algicida* in ASW medium for 24 h, thus inducing algicidal activity (**SFigure 15A**). We hypothesized that candidates for algicidal proteases would show a pattern of higher expression in the active phase (30 h), lower expression in the inactive phase (75 h) and an increase in the induced sample. This pattern was observed for both *KAOT1_RS10890* and *KAOT1_RS09515* (**Figure 11A**).

Table 2. Top 10 candidate protease genes determined by fold change analysis, containing a signal peptide moiety.

| Locus ID	NCBI accession	Predicted Size	Gene Description	Fold Change (Active/Inactive)
KAOT1_RS10890	WP_007094862.1	43 kDa	M57 family metalloprotease	13.396
KAOT1_RS09515	WP_007094576.1	45 kDa	S8 family serine peptidase, subtilisn-like	12.507
KAOT1_RS17795	WP_009778466.1	37 kDa	zinc metalloprotease	4.055
KAOT1_RS14060	WP_013869856.1	51 kDa	insulinase family protein (Peptidase family M16)	3.593
KAOT1_RS20965	WP_013870093.1	55 kDa	S8/S53 family peptidase	3.406
KAOT1_RS14065	WP_013549898.1	77 kDa	insulinase family protein (Peptidase family M16)	3.151
KAOT1_RS16750	WP_013750727.1	47 kDa	peptidoglycan DD-metalloendopeptidase (Peptidase family M23)	3.106
KAOT1_RS14920	WP_013869438.1	49 kDa	peptidoglycan DD-metalloendopeptidase (Peptidase family M23)	3.090
KAOT1_RS16770	WP_162014347.1	68 kDa	SprT family zinc-dependent metalloprotease	3.070
KAOT1_RS17420	WP_007096197.1	23 kDa	cysteine peptidase family C39 domain-containing protein	2.861
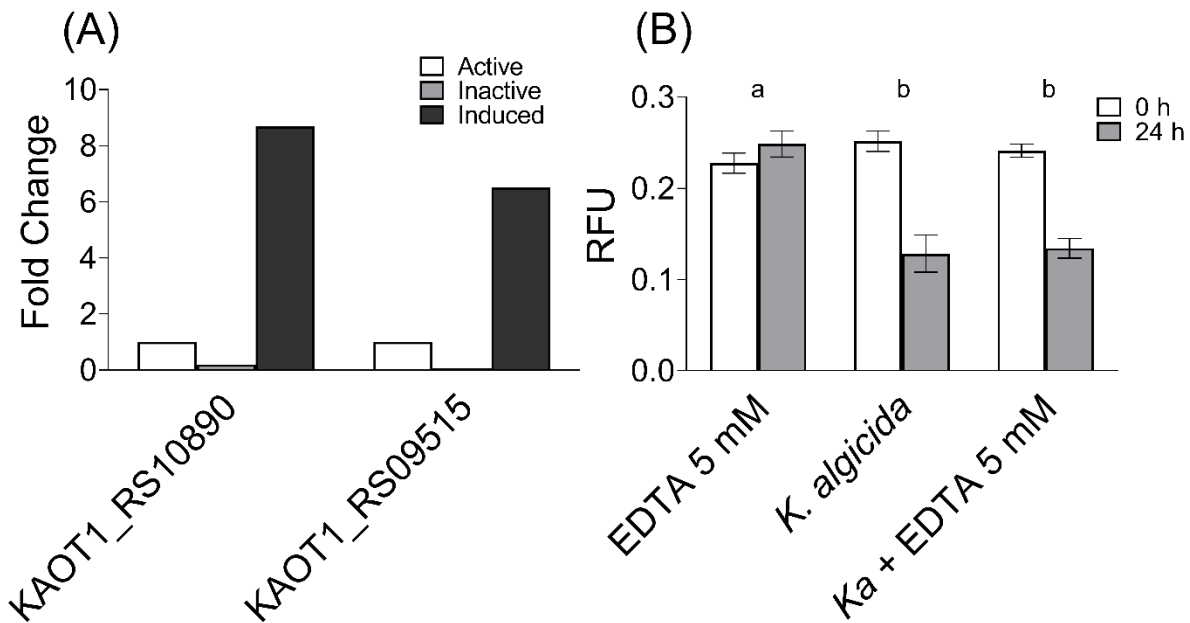
Figure 11. (A) qPCR confirmation of gene candidates for algicidal protease identification (n = 3). (B) Algicidal activity of *K. algicida* spent medium treated with EDTA (*Ka* + EDTA 5 mM), EDTA 5 mM in ASW (negative control) and spent medium of *K. algicida* (*Ka*) (positive control) (n = 4), measured by change in chl *a* (RFU). One-way ANOVA was performed on differences between time measurements among different treatments: a, b. Error bars indicate the standard deviation of biological replicates.

4.C.3.   *KAOT1_RS09515* is an algicidal protease of *K. algicida*

The transcripts of *KAOT1_RS10890* showed the highest fold change between active and inactive phases in the transcriptomic experiment and between inactive and induced phases in the RT-qPCR experiment. Based on the gene description, *KAOT1_RS10890* encodes a an M57 family metalloprotease. Therefore, we hypothesized that if this metalloprotease would be responsible for the observed algicidal activity, the addition of EDTA should show an inhibitory effect. However, this was not the case. Compared to the control, the addition of 5 mM EDTA does not significantly change the algicidal activity of the *K. algicida* cell-free supernatant (**Figure 11B**).

We, thus, focused our efforts on the S8 family serine peptidase *KAOT1_RS09515*. To test whether *KAOT1_RS09515* had algicidal activity, the gene was heterologously expressed in *E. coli* with a 6xHis tag for purification. The presence of a protein with approximately 45 kDa was observed in the elution lanes, which was consistent with the full length of the 45 kDa transcript of *KAOT1_RS09515*. Additionally, a second prominent band was present approximately 24 kDa, which likely represents post translational activation of the recombinant s serine peptidase. Following this, an aliquot of this elution fraction was buffer exchanged to ASW and applied in an algicidal assay. The fraction containing *KAOT1_RS09515* exhibited algicidal activity similar to the *K. algicida* cell-free supernatant (**Figure 12**). Therefore, we named KAOT1_RS09515 hereby named the algicidal protease encoding gene, *alpA1* (a̲l̲gicidal p̲rote̲a̲se 1).
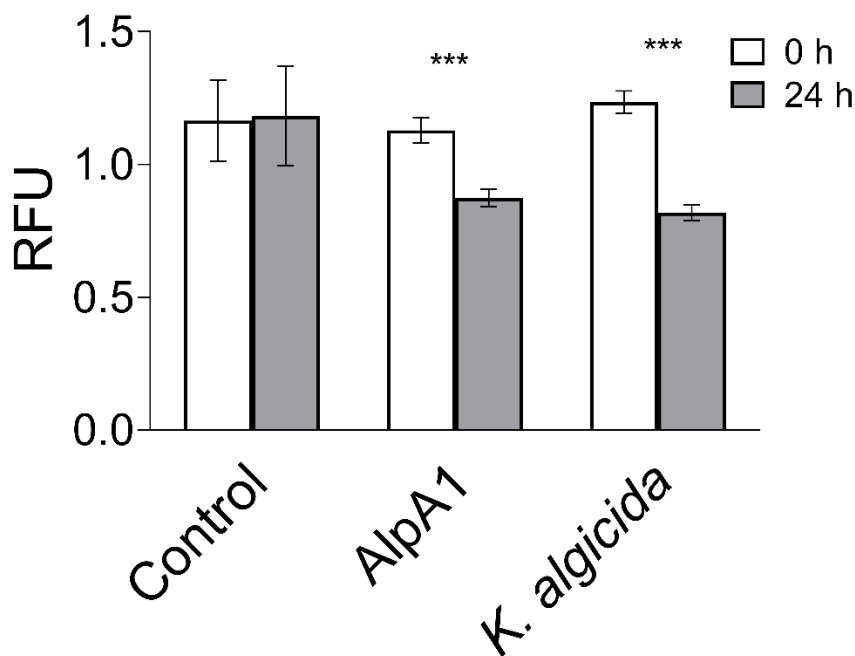

Figure 12. Algicidal activity, measured by change in chl *a* (RFU), of recombinant protein, AlpA1, from gene expressed in *E. coli* P390 cells. ASW medium (Control) and spent medium of *K. algicida* in ASW, used as controls. Error bars indicate the standard deviation of technical replicates (n = 4). Multiple paired t-tests *** $p < 0.001$.

4.C.4.   Algicidal activity is unique to *K. algicida* OT-1

K. algicida was the first member of the genus *Kordia* introduced in Sohn and colleagues in 2004. Over the last two decades many more species have been added, but algicidal activity has only been documented in *K. algicida*. To determine if such activity is prevalent within the genus, we evaluated three additional species of the genus *Kordia* for algicidal properties or inducibility as presented above. *K. aesturarivivens, K. periserrulae, and Kordia sp.* were tested for algicidal activity and algicidal inducibility in the same manner as *K. algicida*. For all three strains, initial evaluation of exponentially growing cultures in MB media showed no reduction of diatom chl *a* fluorescence (**Figure 13**, left), and remained inactive following the inducibility assays (**Figure 13,** right).
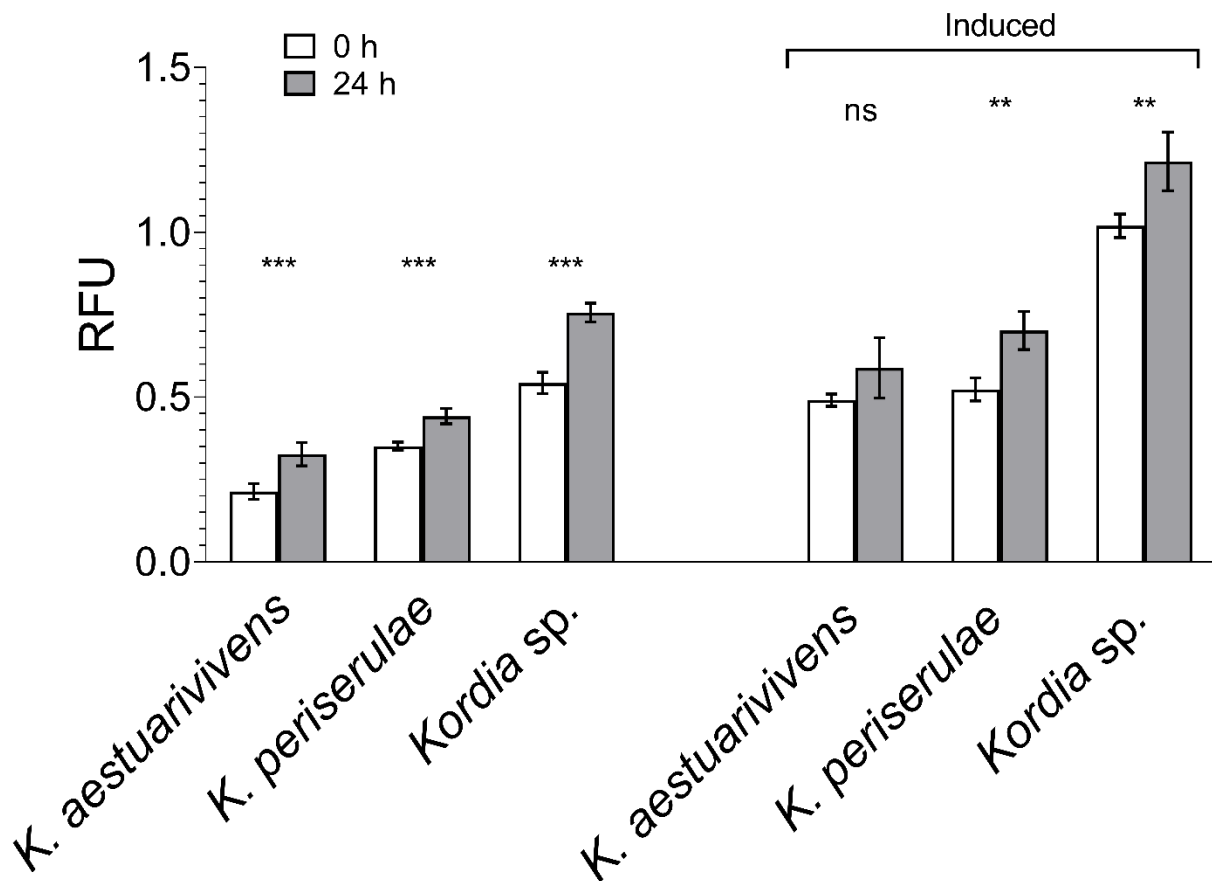

Figure 13. Algicidal activity, measured by change in chl *a* (RFU), of three additional *Kordia*. Left group grown in conditions identical to *K. algicida*. Right group was subjected to algicidal induction protocol for *K. algicida* (n = 4). Multiple paired t-tests, ** p<0.01, *** p < 0.001. Error bars indicate the standard deviation of biological replicates.

We hypothesized that the unique algicidal ability of *K. algicida* compared to other members of the genus is likely linked to specialized genes evolved in this species, which may be visible in comparisons with non-algicidal species. BLASTp identified two homologues of AlpA1 with 78% similarity in *Kordia* sp. and *Kordia aestuariivivens*, **Table 3**. However, these two species showed no algicidal activity in our bioassays. An alignment of the three proteins shows that there are several instances where amino acids are unique to AlpA1 (**SFigure19**).

Table 3. BLASTp search of AlpA1 homology in *Kordia* genera.

Scientific name	NCBI accession	Percent identity	E-value
Kordia algicida OT-1	WP_007094576.1	100.00%	0.0
Kordia sp. ALOHA_ZT_18	MCH2196544.1	78.69%	0.0
Kordia aestuariivivens YSTF-M3	WP_187560797.1	78.30%	0.0
Kordia aestuariivivens YSTF-M3	WP_187560671.1	34.31%	4e-45
Kordia aestuariivivens YSTF-M3	WP_187562711.1	30.51%	3e-37
Kordia sp. ALOHA_ZT_18	MCH2196921.1	35.42%	5.00E-14

4.D.   Discussion

We presented a transcriptomics derived, identified and verified algicidal protease gene for *Kordia algicida* for a marine algicidal bacterium in monoculture, *alpA1*. The observation of an algicidal active and inactive phase during the growth of *K. algicida* enabled a transcriptomic approach to identify potential genes encoding algicidal proteases. Evaluation of differentially expressed genes for secreted proteins, validation with RT-qPCR, and inhibition assays led to the discovery of protease gene *alpA1* (*KAOT1_RS09515*). The recombinant protein, AlpA1, generated from transformation and expression in *E. coli* showed *in vivo* algicidal activity. Our findings were further supported by the characteristic existence of this gene in *K. algicida*, compared to other non-active members of the genera.

Algicidal activity in *K. algicida* is a highly regulated process. A previous study reported a quorum sensing dependent protease activity that leads to maximum protease release in exponential growth(Paul and Pohnert 2011). Our studies show an additional dependence of algicidal activity on nutrient availability, which has been previously reported for laboratory grown strains of algicidal bacteria, favoring replete conditions for the excretion of algicides (Mayali and Doucette 2002, Roth et al. 2008b). When we grew bacteria in nutrient rich medium, activity declined when bacteria reached a declining phase but could be restored by exchanging the spent culture medium with ASW medium that contains no organic substrate. This restored activity indicates an additional nutrient-dependent metabolic regulation. This pattern might reflect the situation in the oceans where bacteria utilize algicidal protease to lyse an algal cell, thus creating a nutrient rich local environment where there is no need for further protease production. Once these resources are depleted, or the bacterium is separated from the nutrient hotspot, it re-initiates algicidal production to target the next cell. This induction of protease release in the open, nutrient limited water likely represents a strategy to save metabolic investment in algicide production.

The protease is part of the S8 family serine peptidases, which have potential multifunctionality as an algicide and facilitator of uptake by extracellular degradation of biopolymers, such as proteins (Meyer et al. 2017). The involvement of a serine protease in algicidal activity has only been documented in the algicidal effects of *Pseudoalteromonas* sp. (Lee et al. 2002, Lee et al. 2000). In this species, a metalloprotease also showed algicidal activity, albeit with 6-fold less activity than the serine protease (Kohno et al. 2007, Lee et al. 2002, Lee et al. 2000). Given the exceptionally high fold-change of the protease identified here that correlates with the activity of the bacteria, we consider it a major player in the algicidal principle, while further evaluation would be needed to confirm the extent of contribution from the other candidates. These other proteases may of course be indirectly involved in the exploitation of resources. This is not an uncommon mode of action, as it is becoming more apparent that algicidal activity can be associated with multiple components working together to make the predatory lifestyle effective. In this case it likely involves nutrient acquisition from targeted algae (Wang and Seyedsayamdos 2017; Jeong and Son 2021; Rose et al. 2021, Zhang et al. 2018, Coyne et al. 2022). It is reasonable to conclude that the full algicidal mode of *K. algicida* may involve a repertoire of other enzymes, which act together with *alpA1*. In addition, small, specialized metabolites might support algal lysis or competition with other bacteria in the phycosphere of the lysed algae.

Recent studies have favored the application of molecular techniques to describe algicidal bacterial-algal interactions, but offer descriptive characteristics of the interactions, rather than the identification of direct factors involved in attack (Hu et al. 2021, Zhang et al. 2023, Zhang et al. 2021). The transcriptomic studies were dependent on the exposure of algicidal bacteria to hosts, which introduces an additional layer of complexity to data analysis (Zhang et al. 2023, Zhang et al. 2022b). These studies identified major pathways involved in the algicidal mechanism such as energy and amino acid metabolism but did not present specific gene candidates for algicidal control (Zhang et al. 2023, Zhang et al. 2022b). In the case of *K. algicida* no host-dependent algicide production was observed (Paul and Pohnert 2011) but rather a growth state dependency of activity. For us, this regulation of *Kordia* algicidity was central to the use of transcriptomic analysis. This allowed us to analyze the transcriptional regulation without the added complexity of induced interactions from co-culturing experiments between bacteria and host algae. The candidate pool could thereby be narrowed down, reducing competing responses from the diverse chemical signaling in response to host algae and associated microbiome, and we were able to identify an algicidal gene, *alpA1,* based on monocultures of *Kordia algicida*.

Many lytic bacteria are species or genus specific, but no connection has been found between algicidal activity and phylogeny (Doucette et al. 1999). The *Kordia* genus belongs to the *Flavobacteriaceae* family and has only been discovered and explored in the last two decades. Though algicidal bacteria of the *Flavobacteriaceae* family mostly target diatoms and some dinoflagellates (Coyne et al. 2022), *Kordia algicida* exhibits an exceptionally broad host range including the raphidophyte *Heterosigma akashiwo* (Sohn et al. 2004) and the coccolithophore *Emiliania huxleyi* as observed in our lab (data not published). To explore the algicidal potential of *Kordia* spp., we sought to expand the investigation to other *Kordia* species, including *K. periserrulae*, the closest genetic relative of *K. algicida* (Lee et al. 2011). Remarkably, none of the strains tested were algicidal in our bioassay. BLASTp comparisons of AlpA1 within the *Kordia* genera showed close homology to proteins in *Kordia* sp. and *K. aestuariivivens*, with a difference of less than 30%. Interestingly, these include a serine at position 200 which is replaced by a proline in both AlpA1 homologues. If this serine belongs to the classic serine protease catalytic Ser/His/Asp triad, this could explain the difference in activity amongst the homologues (Ekici et al. 2008). Based on the lack of algicidal activity detected in these species, we infer that the 30% difference enables the algicidal effect in *K. algicida. K. algicida* has thus adapted a unique lifestyle among the members of

the *Kordia* genus. These results would reiterate the lack of correlation between phylogeny and algicidal properties observed by Wang and colleagues (2020).

Despite much research effort, the ecological role of algicidal bacteria in controlling algal blooms lacks concrete evidence, mainly based on the lack of appropriate tools for observation (Skerratt et al. 2002). Transcriptomic analysis has been postulated as a helpful tool in tracking bacterial activity, but without definitive algicidal transcripts to monitor, there is still ambiguity in the data interpretation. By elucidating a specific algicidal transcript from *K. algicida*, we introduce the possibility of real-time monitoring the activity of *K. algicida* in native blooms, as in Masan Bay where it was discovered. This could be used to clarify the ecological role of algicidal bacteria, whether they play an active role in bloom regulation or are merely opportunistic bystanders waiting for the right conditions to flourish.

5) **Chapter 3**: Survey of algicidal range and metabolic characteristics of *Kordia algicida*

The following data was collected with assistance from master students Pauline Porschitz and Hannah Lörcher as part of practical course training, and hiwi work from master student Oskar Zorc.

5.A.   Study Introduction

Algicidal bacteria play an important role in bloom termination and succession of phytoplankton communities. Aside from the direct inhibition or lysis of algal targets within the community, algicidal bacteria may indirectly affect the homeostasis of the local ecosystem (Mayali and Azam 2004). Algicidal bacteria may release algal toxins into the environment as a side effect of the lysis of susceptible toxin-producing algal targets (Wang et al. 2020a). These toxins may in turn influence the community structure depending on the sensitivity of these organisms to the released toxins (Roth et al. 2007). Brevetoxins from the *K. brevis* have not only killed clams, crabs, and fish (Prince et al. 2006) but caused harm to humans through the inhalation of aerosolized brevetoxins from lysed *K. brevis* causing conjunctival irritation, rhinorrhea, coughing, and wheezing (Fleming et al. 2007). Thus, algicidal bacteria not only have the potential to influence phytoplankton community structure but also higher trophic levels when harmful toxic algae are involved.

Algicidal bacteria may have broad or narrow targets depending on the specific bacteria. The marine bacterium, *Kordia algicida*, has demonstrated algicidal effects on a wide range of targets, from the diatom *Skeletonema costatum*¸ to the dinoflagellate *Cochlodinium polykrikoides*, and the raphidophyte *Heterosigma akashiwo* (Paul and Pohnert 2011, Sohn et al. 2004). Other algicidal bacteria have shown specificity even within the species level, such as Bacteroidetes which was active against three out of six isolates of the dinoflagellate *K. brevis* tested (Roth et al. 2008b). Understanding the range of algicidal targets would further the understanding of community structural changes influenced by the presence of algicidal bacteria and support the feasibility of using algicidal bacteria as biological controls for harmful algal blooms. An investigation of specificity may also offer a better understanding of the mode of action of the algicides and their targets (Meyer et al. 2017).

Algicidal bacteria may also be useful as biological control for specific species of harmful algal blooms (HABs) which cause approximately \$8 billion/year in damages to mariculture and marine fisheries (Brown et al. 2020). Such control could be used to minimize the impact on non-target species, but research has yet to progress beyond controlled laboratory settings (Brown et al. 2020). Other industrial interest in these bacteria is in the application for algal cell lysis for the extraction of carbohydrates, lipids and other compounds from microalgal cells to be converted into biofuels and bioproducts (Wang et al. 2020a). Wang et al. (2020a) summarized the advantages of algicidal bacteria for industrial lysis in that the process is non-toxic, easy to scale up, low-cost and sustainable, and limited by the growth and maintenance of the specific bacteria.

Investigations into the target range of algicidal bacterium would therefore benefit an ecological understanding of the impact these organisms have on phytoplankton communities, as well as improve the predictability of the succession of species, where these bacteria are naturally occurring, and their potential use for bloom control. By expanding beyond ecologically relevant phytoplankton targets, we may understand more about the characteristics of algicidal attacks by evaluating trends or differences between susceptible and resistant phytoplankton. Industries with an interest in biological mechanisms for algal cell lysis may also benefit from this broad knowledge. The following chapter details studies of the range of algicidal targets of the algicidal marine bacterium *K. algicida* as well as observations of the physical impact on certain phytoplankton species.

5.B.   Methods

5.B.1.   Strains and growth conditions:

Strains were cultivated from natural plankton assemblages in Helgoland Germany (HARB45, SEACON37, SEACON16) or received from a collaborator in Naples, Italy (Na20B4), while others were purchased from culture collections: Roscoff Culture Collection (RCC); NCMA Bigelow (CCMP); Culture Collection of Algae at the University of Göttingen, Germany (SAG); Culture Collection Yerseke (CCY); Culture Collection of Algae and Protozoa (CCAP); Belgian Coordinated Collection of Microorganisms/DCG Diatoms Collection (DCG); Scandinavian Culture Collection of Algae & Protozoa (SCCAP); and University of Texas Culture Collection (UTEX). Temperature and cultivation medium are listed in **Tables 4-6**. All species were cultivated in a 14h:10h day/night cycle with light ranging from 15-30 µmol m^{-2} s^{-1}.

The algicidal bacterium, *Kordia algicida* OT-1 purchased from the Biological Resource Center, NITE (NBRC) from Shibuya, Japan, and stored as 20% glycerol cryopreservation stocks. For growth conditions see section "4.B.1 Strains and growth conditions." Phytoplankton were grown in respective conditions for 2 weeks until visibly dense in four biological replicates.

5.B.2.   Algicidal Activity

Algicidal activity was measured by monitoring changes in chl *a* in 96 well plates over two days. At least two-time points were measured to assess changes within treated populations and against controls whenever possible. Algicidal activity assays were carried out according to section "4.B.3 Algicidal activity bioassay." A culture of *Skeletonema marinoi* strain RCC75 was included in each bioassay period as positive control for *K. algicida* activity.

5.B.3.   Statistical analysis:

Each biological replicate was measured in technical triplicates, assembled as average values ± standard deviation for each sampling. Statistical significance was determined by comparing differences between two time points within biological replicates of algicidal treatment to that of corresponding controls, using the Student's T-test performed. We interpreted significant differences as susceptibility to algicidal attack if difference values were less than controls and no significant difference as resistance to algicidal attack of *K. algicida*. You could add here what is significant P-value.

5.B.4.   Microscopy and phenotypic observations

Images were taken using an inverted Leica DM IL LED microscope (Leica, Heerbrugg, Switzerland) equipped with a Nikon DS-Fi2 CCD camera (Nikon, Tokyo, Japan), enabling time-course photographies as well as fixed time-point images. For *Skeletonema marinoi* and *Chaetoceros didymus* strain Na20B4 treated with *K. algicida*, the algicidal bioassay was performed according to the methods above mentioned. Time-scaled images were captured every 30 min for a 24 h period with 10x magnification. *S. marinoi* was used as positive controls for all bioassays as they have been

previously reported as susceptible to *K. algicida* and *C. didymus* was the first reported resistant phytoplankton toward *K. algicida* attack (Paul and Pohnert 2011).

To assess the effect of algicidal bacteria on phytoplankton motility, the dinoflagellate *Alexandrium minutum* strain RCC3018 was treated with *K. algicida* according to the methods above mentioned. Swimming activity was observed under the microscope 24 h and 48 h post-inoculation. 10 µL of cells were then transferred to a fresh medium (200 µL) to determine whether *A. minutum* could recover from the effects of *K. algicida* upon dilution in the replete medium. After 24 h incubation, the diluted cultures were examined with microscopy, and final observation were conducted after 11 days of incubation.

To test the effect of *K. algicida* on the large bloom-forming diatom *Coscinodiscus*,the bioassay was upscaled to 24 well plates, using biological duplicates for each treatment and control. The incubation was conducted concurrently with the classic algicidal activity assay in 96 well plates and we evaluated the changes in chl *a* (ex: 430 nm, em: 665 nm) using a plate reader (Varioskan Flash, Thermo Fisher Scientific). Cells were evaluated and the phenotype was observed with microscopy with a 10x magnification after 72 h incubation.

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5.C.   Results

5.C.1.   Kordia changes the physiological characteristics of susceptible phytoplankton. *S. marinoi* was susceptible while *C. didymus* was resistant against *K. algicida* attack, which was reflected in the measurements of chl *a*. The detection of this lytic activity was originally documented after 7 h incubation (Paul and Pohnert 2011). We monitored the physical effects of *K. algicida* on *S. marinoi* and *C. didymus* via microscopy (Figure 14). Changes in the cell wall structure of *S. marinoi* were noticed at 6 h post incubation with *K. algicida*. After 12 h, most of the cell walls were non-apparent and completely missing by 24 h. For *C. didymus*, there were no visible changes in cell structure through the course of 24 h treatment, which was consistent with its resistance property.

The dinoflagellate *A. minutum* was also observed with microscopy following incubation with *K. algicida*. The bacteria *K. algicida* immobilized the majority of *A. minutum* cells after 24 h, with

minor exceptions.   Control cultures without bacterium could still swim vigorously after the incubation. After 48 h, *K. algicida*-infected cell cultures remained non-motile, and the presence of hollow shells of dinoflagellates was present. *A. minutum* that was transferred to a new medium to dilute *K. algicida* effects displayed higher motility at 24 h post transfer. After 11 days, there were no live *A. minutum* cells in half of the biological replicates. Cell densities remained lower than controls and contained a mixture of motile and non-motile cells.

We observed no physiological effects of *K. algicida* when treating three different species of the large diatom *Coscinodiscus* (5 isolates), which was consistent with the observed resistance to *K. algicida* based on changes in chl *a* (**Figure 15**).
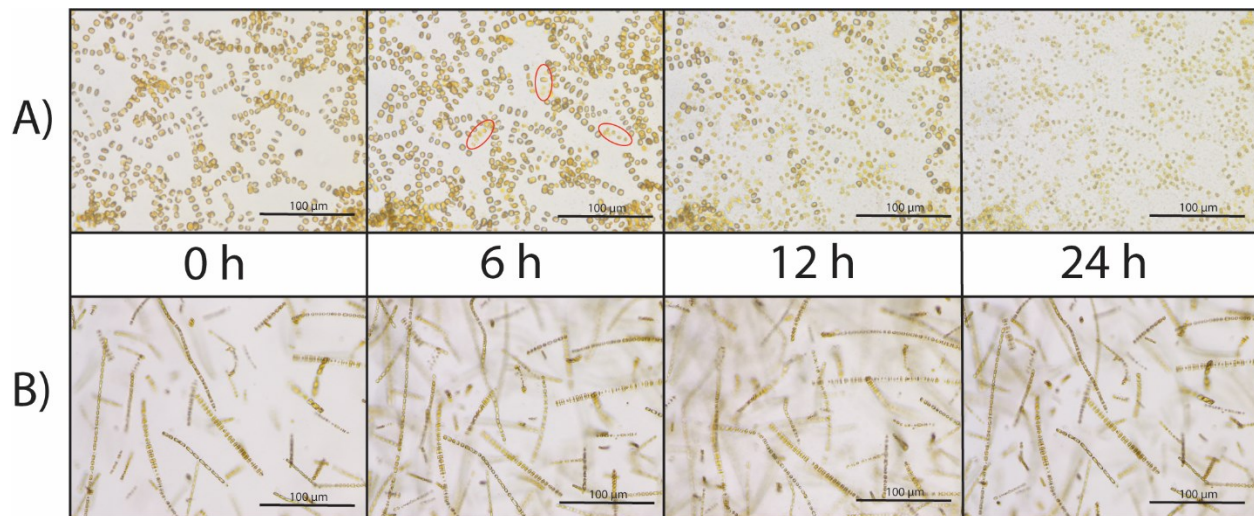

Figure 14. Timescale depiction of the morphological changes in cell physiology of K. algicida induced in (A) the susceptible *S. marinoi* and (B) the resistant *C. didymus*. The microscopy images correspond to the time (h) post inoculation with *K. algicida* starting from 0 h until 24 h. Initial changes in cell structure in *S. marinoi* are circled in red. Single biological replicates were monitored over time and exhibited similar phenotype.

5.C.2.   Kordia changes the physiological characteristics of susceptible phytoplankton.
S. marinoi was susceptible to *K. algicida* attack and *C. didymus* was resistant, reflected in measurements of chl *a*. The detection of activity was originally documented after 7 h incubation (Paul and Pohnert 2011). We monitored the physical effects of *K. algicida* on *S. marinoi* and *C. didymus* via microscopy (**Figure 14**). Changes in cell wall structure of *S. marinoi* could be noticed 6

h post incubation with *K. algicida*. After 12 h, most of the cell walls were non-apparent and completely gone by 24 h. For *C. didymus*, there were no visible changes in cell structure through the course of 24 h treatment, which was consistent with its algicidal resistance.

The dinoflagellate *A. minutum* was also observed via microscopy following incubation with *K. algicida*. *K. algicida* immobilized the majority of *A. minutum* cells after 24 h, except for a few very small cells. Control cultures without bacterium swam vigorously. After 48 h, *K. algicida* infected cell cultures remained non-motile, and the presence of hollow shells of dinoflagellates were present. *A. minutum* transferred to new medium to dilute *K. algicida* effects displayed more motilily 24 h post transfer. After 11 days, there were no live *A. minutum* cells observed in half the biological replicates. Cell densities remained lower than controls and contained a mixture of motile and non-motile cells.

We observed no physiological effects of *K. algicida* on 3 different species of the large diatom *Coscinodiscus* (5 isolates), consistent with the observed resistance to *K. algicida* based on changes in chl *a* (**Figure 14**).
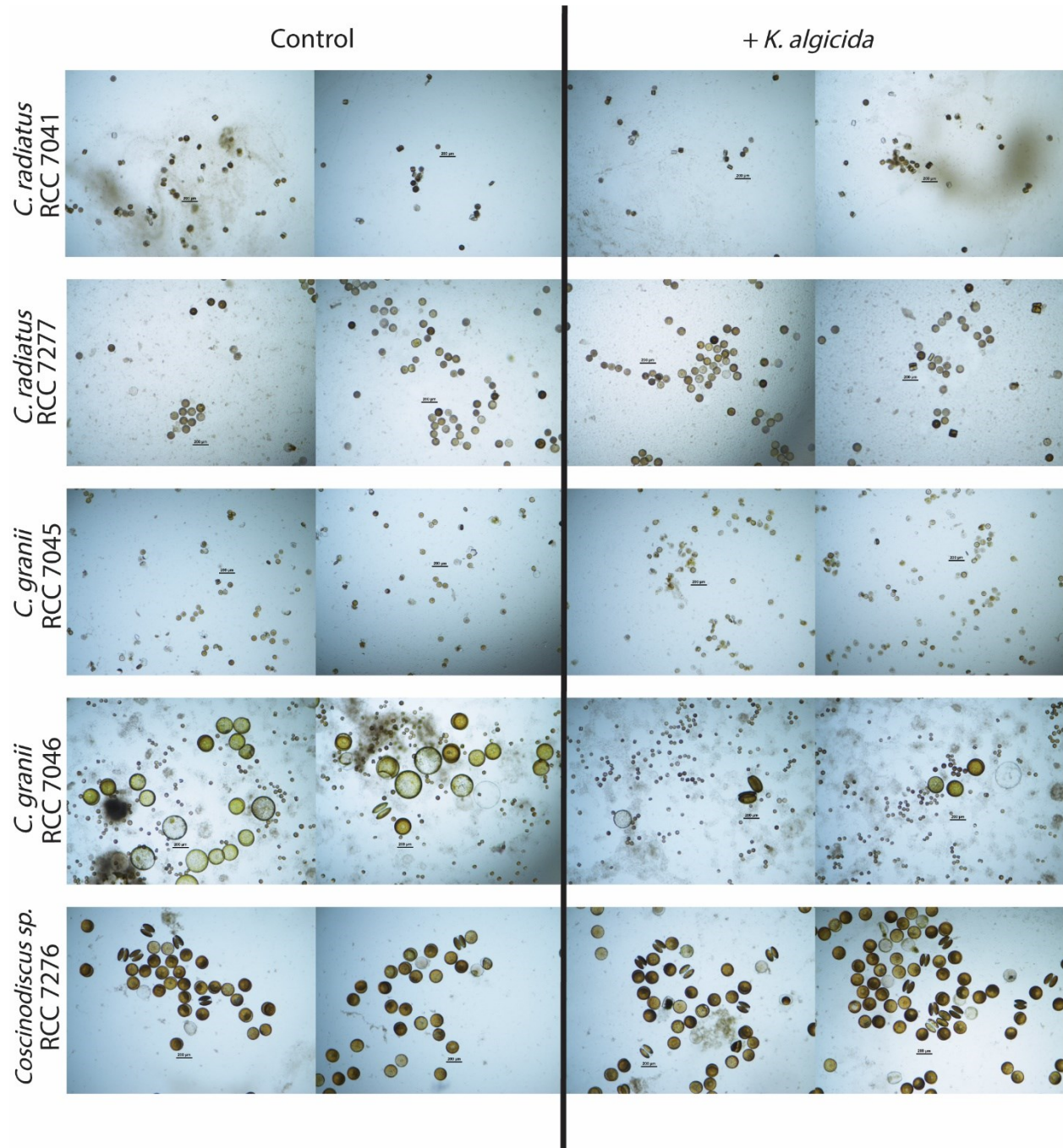
Figure 15. Depiction of cells of five strains of *Coscinodiscus* spp. at 72 h post incubation treated with *K. algicida* (right) and untreated controls (left). Species and strain ID on the left hand slide of images. Each image represents a single biological replicate.

Table 4. Species of phytoplankton susceptible to *K. algicida*

Species	Strain ID	Classification	Temp.	Media	p-value	Distribution	Toxicity
Alexandrium tamarense	RCC292	dinoflagellate	18 °C	ASW	0.0049	cosmopolitan	Toxic - red tide
Amphora sp.	CCMP129	diatom	18 °C	L1	<0.0001	cosmopolitan	no
Asterionellopsis glacialis	CCMP 137	diatom	18 °C	L1	0.0009	cosmopolitan	no
Emiliania huxleyi	RCC2611	haptophyte	18 °C	ASW	0.0003	cosmopolitan	no
Lepidodinium chlorophorum	RCC1489	dinoflagellate	18 °C	ASW	<0.0001	cosmopolitan	no - HAB
Rhodomonas lens	CCMP739	chryptophyta	18 °C	ASW	0.0076	Europe	no
Schizochlamydella capsulate	CCMP244	chlorophyta (green alga)	18 °C	ASW	<0.0001	brackish	no
Skeletonema grethae	CCMP775	diatom	13 °C	ASW	0.0002	US waters	no
Skeletonema japonicum	CCMP2506	diatom	18 °C	ASW	0.0081	cosmopolitan	no
Skeletonema marinoi	RCC75	diatom	13 °C	ASW	<0.0001	cosmopolitan	no
Synedra fragilaroides	CCMP844	diatom	13 °C	ASW	0.0319	US coast/British coast	no
Thalassiosira rotula	RCC290	diatom	18 °C	ASW	0.0002	cosmopolitan	no
Thalassiosira weissflogii	RCC76	diatom	18 °C	ASW	0.0020	cosmopolitan	no
Tisochrysis lutea	RCC1349	haptophyte	13 °C	ASW	0.0098	Niche (coast)	no

Table 5. Species of phytoplankton resistant to *K. algicida.*

Species	Strain	Classification	Temp.	Media	P-value	Distribution	Toxicity
Amphidinium carte	CCMP1314	dinoflagellate	18 °C	ASW	0.5146	cosmopolitan	toxic - HAB
Bigelowiella natans	RCC435	Chlorarachniophyte (amoeboflagellate)	18 °C	ASW	0.0203	temperate waters	no
Chaetoceros diadema	RCC1717	diatom	13 °C	ASW	0.2728	USA, South America, North Sea, Tyrrhenian Sea, Tasman Sea	no
Chaetoceros didymus	Na20B4	diatom	13 °C	ASW	0.0492	cosmopolitan	no
Coscinodiscus granii	RCC7046	diatom	13 °C	F2	0.9085	cosmopolitan	no - HAB
Coscinodiscus granii	RCC7045	diatom	13 °C	F2	0.7826	cosmopolitan	no - HAB
Coscinodiscus radiatus	RCC7041	diatom	13 °C	F2	0.0598	cosmopolitan	no - HAB
Coscinodiscus radiatus	RCC7277	diatom	13 °C	F2	0.2776	cosmopolitan	no - HAB
Coscinodiscus sp.	RCC7276	diatom	13 °C	F2	0.1378	cosmopolitan	no - HAB
Dunaliella salina	RCC3579	chlorophyte (green alga)	18 °C	ASW	0.0172	Salt marshes, Mediterranean, Atlantic	no
Dunaliella tertiolecta	RCC6	chlorophyte (green alga)	13 °C	ASW	0.3492	Coastal Atlantic	no
Florenciella parvula	RCC446	stramenopile	13 °C	ASW	0.8759	English Channel	No
Heterosigma akashiwo	RCC1502	raphidophyte	13 °C	ASW	0.0176	cosmopolitan	toxic - HAB
Heterosigma akashiwo	RCC1502	raphidophyte	18 °C	ASW	0.9058	cosmopolitan	toxic - HAB
Lepidodinium chlorophorum	RCC1489	dinoflagellate	13 °C	ASW	0.1346	cosmopolitan	no - HAB
Navicula sp.	RCC|15	diatom (benthic)	13 °C	ASW	0.0136	cosmopolitan	no
Nitzschia cf pellucida	DCG0303	diatom (benthic)	13 °C	ASW	0.9601	cosmopolitan	no
Nitzschia closterium	RCC81	diatom (benthic)	18 °C	ASW	0.6863	cosmopolitan	no
Ochromonas triangulata	RCC21	stramenopile	13 °C	ASW	0.1139	cosmopolitan (genus)	no
Odontella aurita	CCMP 1796	diatom	18 °C	ASW	0.2425	cosmopolitan	no
Oocystis	SAG257.1	chlorophyte (green	13 °C	ASW	0.0775	cosmopolitan	no

marsonii		alga)					
Pavlova gyrans	RCC1526	haptophyte	13 °C	ASW	0.0612	cosmopolitan	no
Phaeodactylum tricornutum	RCC2967	diatom	13 °C	ASW	0.4042	cosmopolitan	no
Phaeodactylum tricornutum	CCAP1055/15	diatom	13 °C	ASW	0.0524	cosmopolitan	no
Phaeodactylum tricornutum	CCMP2561	diatom	13 °C	ASW	0.0686	cosmopolitan	no
Phaeodactylum tricornutum	SCCAP K-1280	diatom	13 °C	ASW	0.0881	cosmopolitan	no
Phaeodactylum tricornutum	UTEX646	diatom	13 °C	ASW	0.2154	cosmopolitan	no
Prymnesium parvum	"unknown"	haptophyte	13 °C	ASW	0.0567	Coastal/brackish	toxic - HAB
Prymnesium parvum	RCC3426	haptophyte	13 °C	ASW	0.4275	Coastal/brackish	toxic - HAB
Prymnesium parvum	CCAP946/6	haptophyte	13 °C	ASW	0.0140	Coastal/brackish	toxic - HAB
Prymnesium parvum	UTEX2797	haptophyte	13 °C	ASW	0.9830	Coastal/brackish	toxic - HAB
Tetraselmis striata	RCC131	chlorophyte (green alga)	18 °C	ASW	0.0372	cosmopolitan	no
Tisochrysis lutea	RCC1349	haptophyte	18 °C	ASW	0.0064	Niche (coast)	no

Table 6. Species of phytoplankton with variable responses to *K. algicida* cultivated at a single temperature.

Species	Strain	Classification	Temp.	Media	p-value	Distribution	Toxicity
Alexandrium minutum	RCC3018	dinoflagellate	18 °C	ASW	0.6840 0.0061	cosmopolitan (coastal)	toxic - HAB
Cylindrotheca closterium	CCY9601	diatom, benthic	13 °C	ASW	0.1556 0.0001	cosmopolitan	no - HAB
Dunaliella tertiolecta	CCMP1320	chlorophyta (green alga)	13 °C	ASW	0.0833 0.0270	Coastal Atlantic	no
Rhodomonas baltica	RCC350	chryptophyte	18 °C	ASW	0.8140 0.0106	cosmopolitan	no

5.C.3.   Specificity of *K. algicida*'s algicidal activity toward different algal species

A total of 37 species of phytoplankton, including 48 strain isolates, were evaluated for their susceptibility or resistance against *K. algicida*. The 15 susceptible phytoplankton included the toxic and HAB forming species *A. tamarense*, and the non-toxic HAB species *L. chlorophorum* (13 °C) (**Table 4**). The 23 resistant phytoplankton (**Table 5**) included the toxic HAB-forming species: *A. carterae, H. akashiwo*, and *P. parvum* as well as non-toxic HAB species *C. radiatus, C. granii*, and *L. chlorophorum* (13 °C). The susceptible phytoplankton consisted of diatoms, dinoflagellates, chlorophyta, one chryptophyte, and one haptophyte. Four species of phytoplankton exhibited distinct results following repeated testing on the same culture (**Table 6**).

Species with multiple isolates like *P. parvum*, *P. tricornutum*, and *Coscinodiscus* spp. were resistant to *K. algicida* across all isolates. Genera with multiple species tested, such as *Skeletonema* spp. and *Thalassiosira* spp. were inhibited by *K. algicida*. Meanwhile, *Coscinodiscus* spp. and *Chaetoceros* spp. were resistant to *K. algicida*.

For the green alga *S. capsulate*, the chl *a* measurement 1 day post-treatment with *K. algicida* increased, suggesting an initial resistance to the algicidal bacterium. However, after 2 days, the cultures succumbed to algicidal attack and the chl *a* declined compared to controls (**SFigure 20**). *A. minutum, C. closterium, D. tertiolecta*, and *R. baltica* resistance and inhibition results varied after repeated testing (**Table 6**).

5.C.4.   Temperature and density

H. akashiwo, *L. chlorophorum*, and *T. lutea* were tested for *K. algicida* susceptibility at two temperatures, 13 °C and 18 °C (**SFigure 21**). *T. lutea* was susceptible at 13 °C, but not at 18 °C. In contrast, *L. chlorophorum* was susceptible to *K. algicida* at 18 °C but not at 13 °C cultures. Chl *a* values of susceptible *T. lutea* was almost three times lower than resistant cultures. Chl *a* value of susceptible *L. chlorophorum* was almost two-fold higher than for the resistant cultures. *H. akawshiwo* was the only species that expressed consistent algicidal resistance at both temperatures. Chl *a* value of 13 °C cultures of *H. akashiwo* were also almost three times higher than cultures maintained at18 °C.

Variable responses to *K. algicida* were observed for different cultures of *A. minutum*, *C. closterium, D. tertiolecta,* and *R. baltica* following the repeated bioassays at singular temperature (**Table 6**, **SFigure 22**). For *A. minutum* cultures at 18 °C, higher density (chl *a* average 1.3548) cultures were inhibited by *K. algicida*, while lower density (chl *a* average 0.3328) cultures were resistant. *C.*

closterium cultures at 13 °C were inhibited by *K. algicida* when the phytoplankton density was higher (chl *a* average 0.7572), but resistant at lower density (chl *a* average 0.2282). Lower density cultures (chl *a* average 0.0468) of *R. baltica* were inhibited by *K. algicida* while higher-density (chl *a* average 0.5225) cultures were resistant. *D. tertiolecta* CCMP1320 cultures at 13 °C were inhibited at higher density (chl *a* average 1.0235) and susceptible at lower density (chl *a* average 0.8426).

5.D.   Discussion

K. algicida produces algicides capable of damaging cell walls and inhibiting mobility of target phytoplankton. The targeting of algal cell walls is the most commonly observed effect in algicidal bacterial interactions (Coyne et al. 2022, Meyer et al. 2017). The short time-period required to see physiological changes in target organisms suggests potent algicidal mechanisms (see Chapter 2). Susceptible phytoplankton spanned a broad taxonomic range including diatoms, dinoflagellates, chlorophytes, and haptophytes. These phytoplankton had broad geographic distribution and a few niche species. *K. algicida* is also broadly distributed in the oceans and may be a factor influencing the taxonomic and geographic range of susceptible targets.

We observed high species-specificity for targets of *K. algicida*. In general, species with multiple isolates exhibited identical reactions towards incubation with *K. algicida*. Within the *Chaetoceros* spp., *C. didymus* was resistant to algicidal attack by *K. algicida*, while *C. socialis* from a natural phytoplankton bloom was susceptible (Bigalke et al. 2019, Paul and Pohnert 2011). Our study further found that *C. diadema* was also resistant, indicating complex target susceptibility within the same genus. *H. akashiwo* and *P. tricornutum* previously reported as susceptible to *K. algicida* (Paul and Pohnert 2011, Sohn et al. 2004) were resistant in our studies, which included the same isolate of *P. tricornutum* UTEX646 previously reported. Multiple species tested within the same genera also exhibited uniform effects by *K. algicida*, such as the *Skeletonema* spp., suggesting a trend within the relatedness of phytoplankton species and the susceptibility to *K. algicida* inhibition. The incongruity further emphasizes seemingly random and yet specific nature of algicidal bacterial targets (Roth et al. 2008b).

Testing multiple species at 13 °C and 18 °C revealed an influence of phytoplankton culture temperature on the success of *K. algicida* attack. The results showed no trend as *T. lutea* was resistant

when cultivated at the warmer temperature and inhibited at the colder temperature, while the reverse was true for *L. chlorophorum*. Culture density also played a role in the success of *K. algicida* attack. The effect of cell density on *K. algicida* susceptibility also appears to be species-specific as two species were inhibited at higher cell densities while one was inhibited at lower cell densities when cultivation temperature was held constant. The condition of phytoplankton influences the dynamic relationships between phytoplankton and bacteria. The effect of temperature and density may be due to physiological or metabolic factors involving cell structures and receptors relating to the life-stage and environmental condition of the phytoplankton (Meyer et al. 2017).

In a field mesocosm study, an algicidal bacteria isolate *Pseudomonas fluorescens* HYK0210-KS09 with strong algicidal effects on *Stephanodiscus* spp. in laboratory, was unable to control a natural bloom of *Stephanodiscus* in the naturally low temperatures (Kang et al. 2011). This was eventually overcome by immobilizing and packing the bacterial cells on a cellulose sponge, which provided the bacteria protection from the elements and facilitated algicidal lysis of *Stephanodiscus* (Kang et al. 2007). *K. algicida* was previously investigated in a natural mesocosm study, where it effectively accelerated the natural species succession in the environment by the removal of the presently dominant bloom species (Bigalke et al. 2019). Using *K. algicida* to control natural phytoplankton blooms or commercially for targeted cell lysis seems plausible with detailed investigations into the various surrounding factors including temperature, density, species diversity.

When we consider the dynamic relationships between phytoplankton and bacteria, species specific interactions are a common occurrence. Recent studies have shown that highly species-specific interactions depend on algal fitness, bacterial metabolism and community composition affecting the dynamics of phytoplankton composition and succession (Deng et al. 2022). Large surveys of the target range of algicidal bacteria are not typically performed, with most tests involving a select few species of the largest groups of phytoplankton ie cyanobacteria, diatoms and dinoflagellates(Coyne et al. 2022, Pierella Karlusich et al. 2020), but is essential to the predicability of the outcomes of studies involving algicidal bacteria as biological controls for phytoplankton blooms. Given the taxonomic diversity of susceptible species, the role of *K. algicida* in shaping phytoplankton communities may be broader and more prominent than initially thought. By screening a broad target range for *K. algicida*, we further the potential use for biotechnical applications beyond ecological implications. Comparative analysis may also be used to understand the resistance or evasive mechanisms employed by resistant phytoplankton species, and commonalities between

susceptible phytoplankton which would further our undersanding on the mechanisms of algicidal bacteria.

6) Conclusion and Future Perspective

Microbial communication in marine phytoplankton communities is a complex dynamic process involving diverse organisms with species-specific processes. The highly competitive environment relies on the excretion and perception of chemical cues which dictate the organization, succession, and overall fate of the community. In our studies, we focused on the evaluation of allelopathy and algicidal bacteria as they have been theorized to play major roles in determining bloom dynamics.

The use of membrane separated cocultivation chambers allowed reciprocal metabolomic response from both species for a comprehensive effect of allelopathy on respective growth. We observed plastic interactions not only from exposure to *P. parvum*, but also in the response of *P. parvum* to cocultivation partners. There is a bias against reports of allelopathy received by *P. parvum*, as it is typically used in a unidirectional approach to observe phytoplankton responses to *P. parvum* allelopathy. Our set-up allowed the observation of metabolic responses of *P. parvum* to competing species and vice-versa over a period which included exponential growth. We observed significant influences on allelopathy based on slight variations of laboratory parameters. This indicated that impact of laboratory conditions on observations of allelopathy are pronounced and likely cause the disconnect in the translation of these studies in the natural environment. To support our findings, we mined the TaraOceans database for trends of interactions relevant to our studies. We found that *Florenciella* spp. was particularly abundant in locations which contained other *Prymensium* spp.. This correlated with our study observation of growth enhancement for *F. parvula* exposed to *P. parvum*, the most significant rigid allelopathic interaction within our changing parameters.

We reported the first protease with algicidal activity from the algicidal marine bacterium *K. algicida* OT-1, AlpA1. We observed a loss of algicidal activity upon senescence which enabled the use of transcriptomic analysis to identify potential genes encoding algicidal protease. We were able to induce activity by limiting nutrient availability, a factor that helped further narrow down target genes by using RT-qPCR analysis to determine the expression of candidates based on activity profiles. We were able to validate the activity using recombinant proteins of Alp1. The identification of a specific protein and corresponding gene will allow ecological validation of the role of *K. algicida* in phytoplankton blooms. The expression of this gene can now be monitored using transcriptomic

methods over the course of blooms in locations where *K. algicida* is native. Having a recombinant protein also allows for further investigations into algicidal attack on phytoplankton targets, as proteins can be tagged with fluorescence probes and imaging tools may be used to visualize and determine mode of attack.

We conducted a survey to determine the range of algicidal targets for *K. algicida*. We discovered that targets are highly species-specific, and within similar culture conditions, isolates of the same species also responded similarly to *K. algicida*. There was a general trend for consistent responses to *K. algicida* within the same genera, with the exception of the *Chaetoceros* spp., which have both resistant and susceptible species reported previously (Bigalke et al. 2019, Paul and Pohnert 2013). We also observed some variability in the response of select species which correlated to changes in temperature and growth of the phytoplankton, suggesting an influence of algal fitness on the effect of algicidal exposure. Overall, we expanded the known algicidal range of *K. algicida* to 15 phytoplankton species across multiple taxonomic groups and reported an additional 22 resistant species. This knowledge benefits the potential use of *K. algicida* for bioengineering and biotechnological applications. The use of metabolomics for comparative analysis of groups of susceptible and resistant phytoplankton in exposure studies to *K. algicida* may provide further insight into the mechanism of algicidal attack and the resistance or evasive strategies employed by phytoplankton to avoid lysis by *K. algicida*.

A common theme underlying our investigations was the species-specificity of our bi-partite interactions, which is frequently apparent in studies of chemically mediated interactions within phytoplankton communities (Ianora et al. 2006, Pohnert et al. 2007, Poulson et al. 2009). The production of secondary metabolites responsible for allelopathic and algicidal interactions may have evolved under the pressure of natural selection to bind to specific receptors in response to organisms in their environment (Williams et al. 1989), which would explain the persistence of species-specific interactions within the marine phytoplankton environment.

We also observed plasticity in both allelopathic and algicidal interactions throughout this study influenced by changing laboratory parameters. We saw that allelopathy is not only a species-dependent trait but additionally depends on the external factors present during the interactions. This was also true for algicidal interactions, which were influenced by temperature and the condition of the phytoplankton species. These are factors which contribute to the difficulty in translating

laboratory results to field experiments as the laboratory conditions used to observe these interactions often do not reflect the natural environment of the interactions.

Biotic and abiotic factors influence chemical communication and need to be considered when performing controlled laboratory experiments to translate to meaningful connections in the natural environment. Within natural phytoplankton communities, one species may receive allelochemicals released by other phytoplankton, bacterial algicides, as well as chemical cues released during bacterial-induced lysis of other phytoplankton species. The complex overlapping effects must be considered when studying such systems (Meyer et al. 2017). We focused mainly on binary interactions, but the amalgamation of this data allows for more accurate predictions of phytoplankton dynamics in their natural environments. Resources like the TaraOceans survey can be utilized to verify laboratory observation and as preliminary hypothesis evaluations before moving to environmental experiments. Finally, laboratory experiments should take precautions to mimic the natural environment of the species interactions in question. Controlled mesocosm experiments provide the most comparable natural environments for the investigation of such complex chemical interactions. Application of metabolomics and transcriptomics studies in conjunction with mesocosm studies can provide characterization of community level interactions and will likely be a valuable resource for the evaluation of chemically mediated interactions in phytoplankton communities in the future.
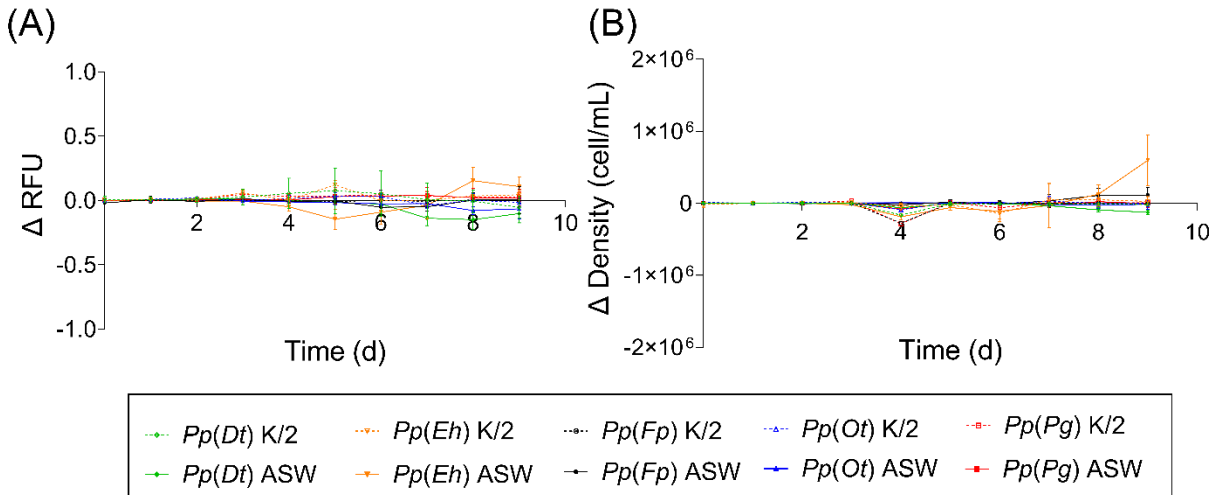
7) Supplementary
    7.A.   Supplementary Chapter 1
       7.A.1. Figures
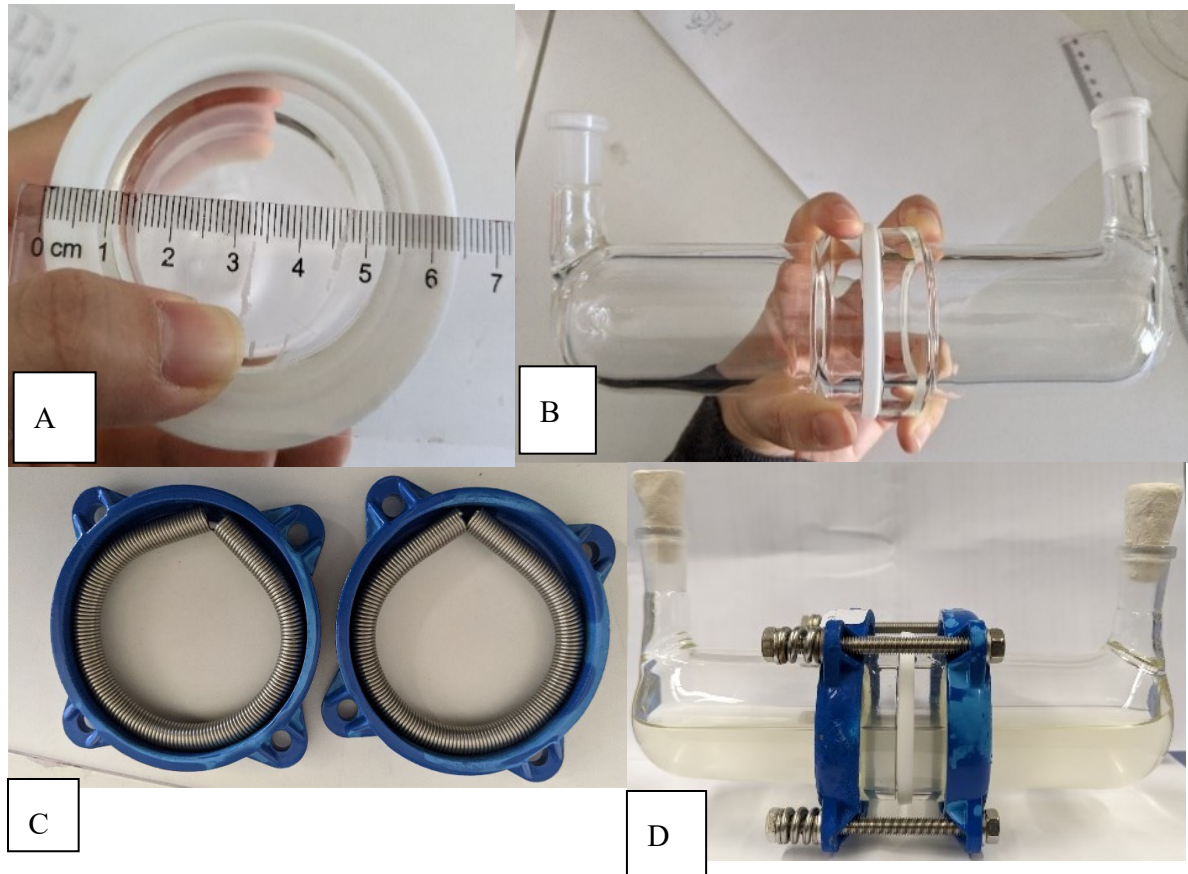
SFigure 1. Co-culture chamber. A) o-ring separating two half-chambers, B) two half-chambers joined with o-ring center, C) ring/spring assembly used to screw two half-chambers together, and D) fully assembled co-culture chamber with cotton plugs.

SFigure2. Received allelopathic influence on *P. parvum* from co-culture partners: *F. parvula* (*Fp*), *P. gyrans* (*Pg*), *O. triangulata* (*Ot*), *E. huxleyi* (*Eh*) and *D. tertiolecta* (*Dt*), expressed as differences in the daily measurement of co-cultured algae and monoculture controls via (A) chl a fluorescence (RFU) and (B) cell density (cell/mL). Cultivations in both K/2 medium (dashed lines) and ASW (solid lines) are depicted. Error bars represent standard deviation of biological replicates (n = 4).
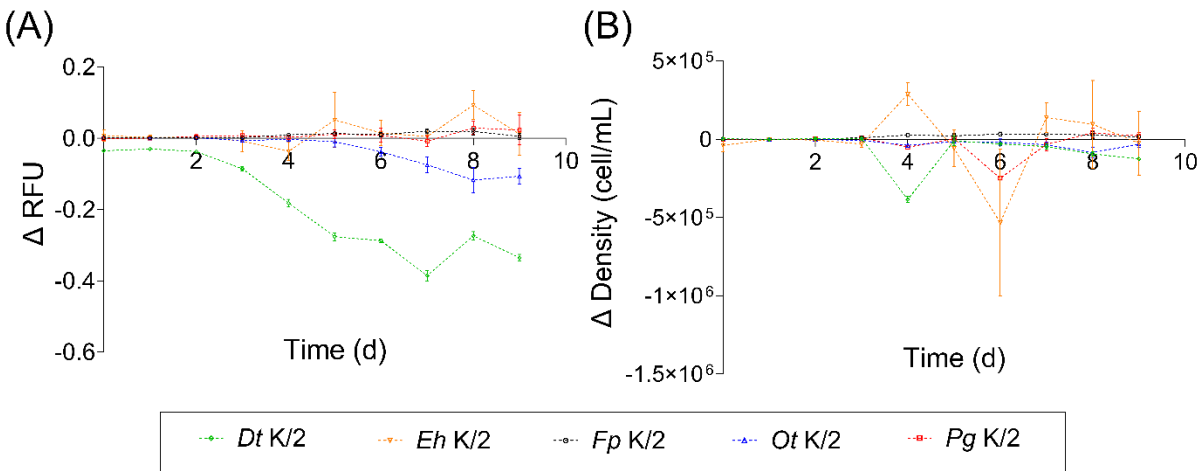
SFigure3. Emitted allelopathic influence of *P. parvum* on co-culture partners: *F. parvula* (*Fp*), *P. gyrans* (*Pg*), *O. triangulata* (*Ot*), *E. huxleyi* (*Eh*) and *D. tertiolecta* (*Dt*), expressed as differences in the daily measurement of co-cultured algae and monoculture controls via (A) chl a fluorescence (RFU) and (B) cell density (cell/mL). Cultivations in K/2 medium (dashed lines) depicted. Error bars represent standard deviation of biological replicates (n = 4).
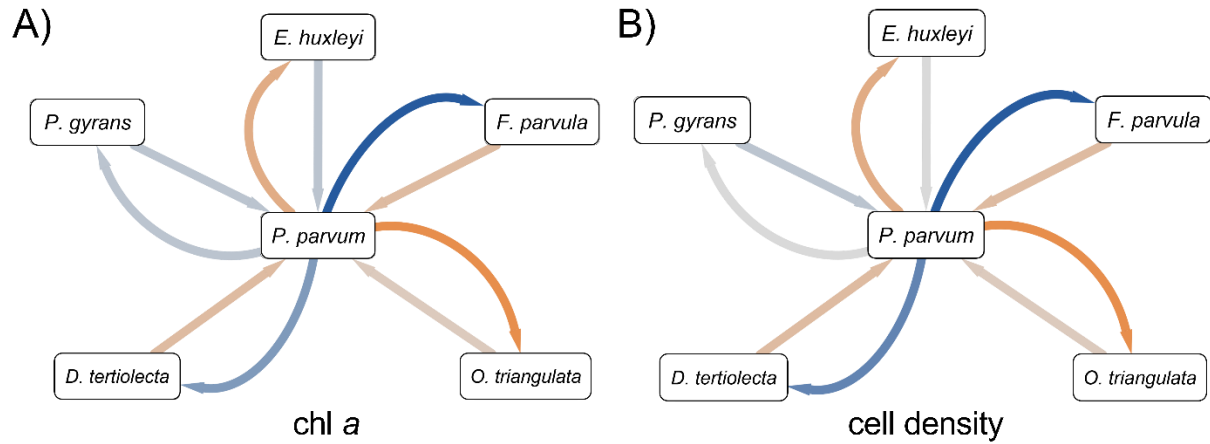
SFigure4. Network of allelopathy of ASW co-cultivations observed by measuring A) chl *a* and B) cell density.
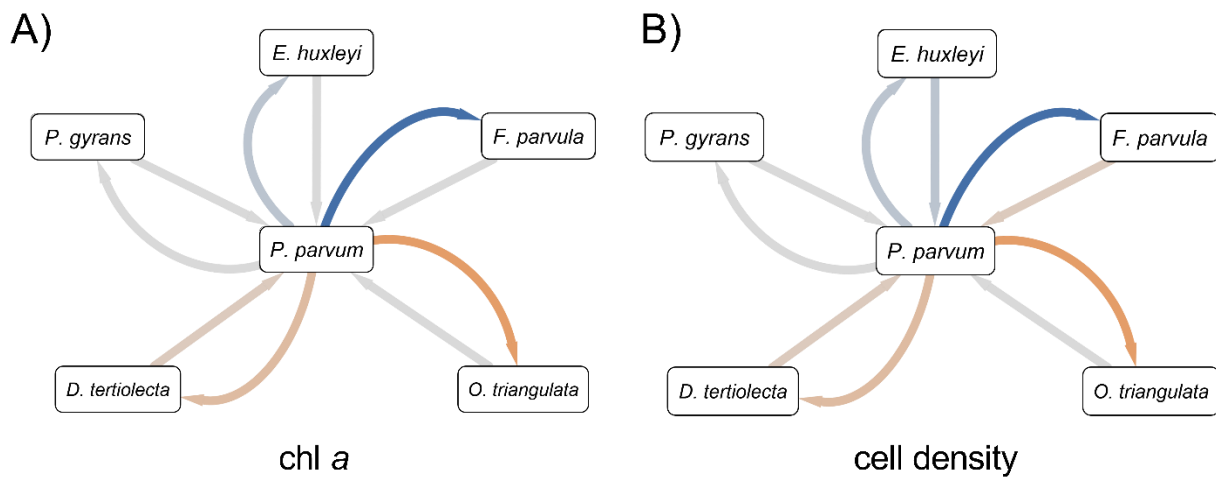

SFigure5. Network of allelopathy of K/2 co-cultivations observed by measuring A) chl *a* and B) cell density.
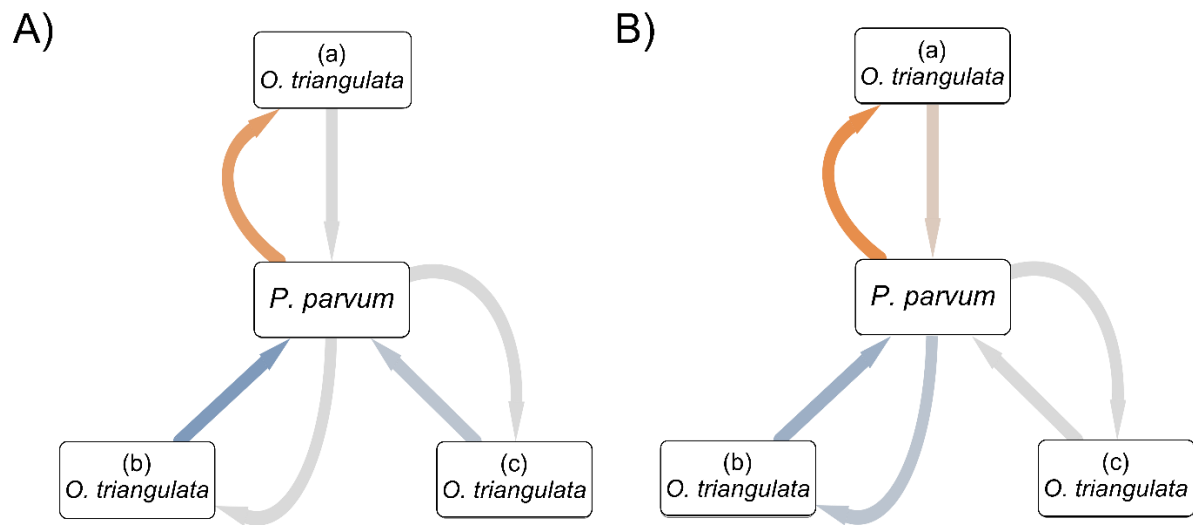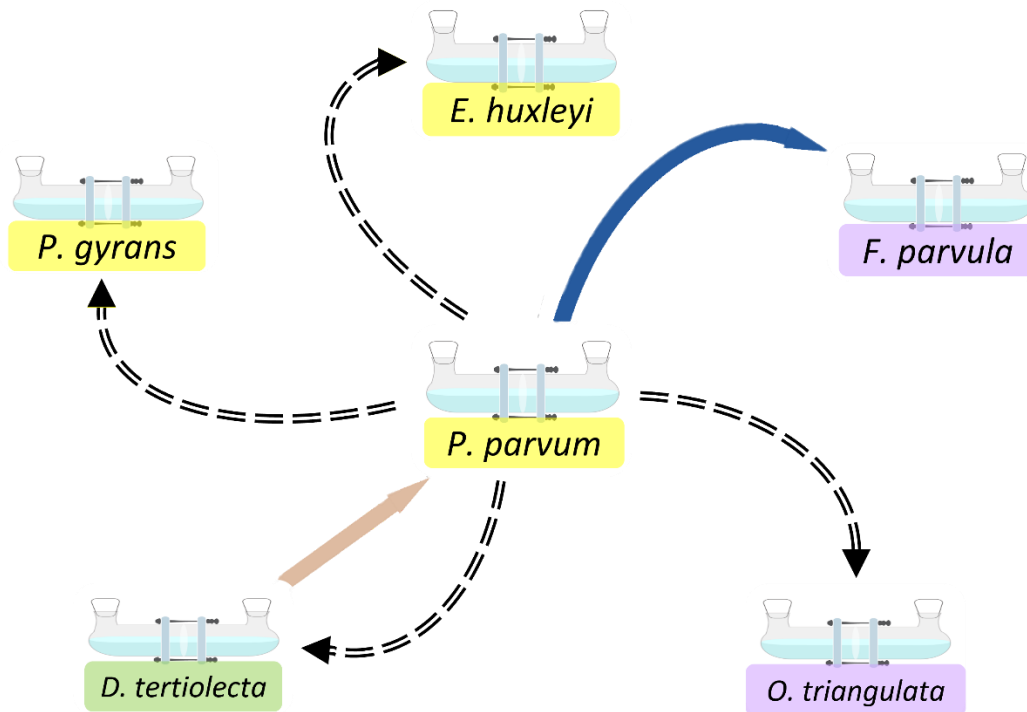
SFigure6. Network of allelopathic interaction of different inoculation ratios of *P. parvum* in co-cultivation with *O. triangulate* in ASW medium. Growth monitored via A) chl*a* and B) cell density measurements. Different starting ratios used between *Pp:Ot* (A) 1:1, (B) 3:1, and (C) 1:3. Nodes colors indicate classification haptophyte (yellow) and stramenopile (purple). Arrow colors indicate positive (green), negative (red) or no (grey) allelopathic influence, originating from emitter and pointing to receiver. Increased size and color intensity (brightness) of arrows positively correlate to significant allelopathic effect.

SFigure7. Allelopathic network from Figure 3, showing variation in allelopathic interpretation due to different laboratory parameters using black dashed arrows. This figure includes additional clade classification of the different microalgae species based on colors highlighting names: haptophyte (yellow), chlorophyte (green) and stramenopile (purple).
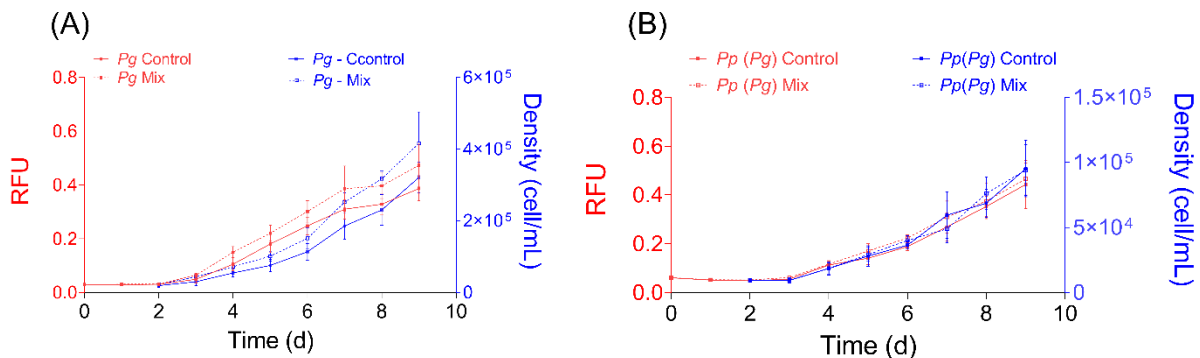
SFigure8. Comparison of growth curves of *Pp-Pg* co-culture in ASW, measured by chl *a* in RFU (left y-axis, red) and cell density (cell/mL) (right y-axis, blue). (A) Growth of *Pg* control (solid lines) and *Pg* mixed co-culture (dashed lines). (B) Growth of *Pp* control (solid lines) and *Pg* mixed co-culture (dashed lines). Error bars represent standard deviation of biological replicates (n = 4). Cell density measurements for day 0-1 missing due to file corruption.
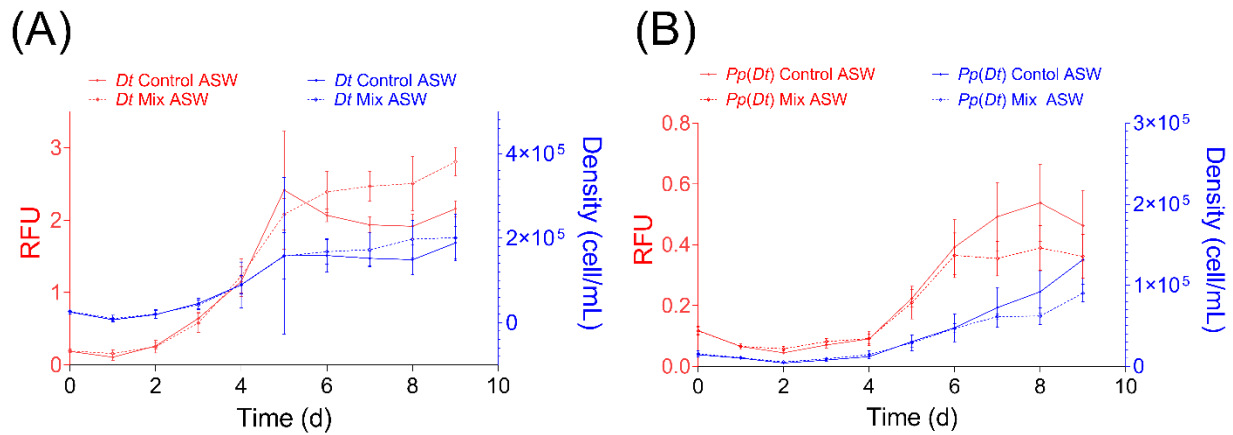
SFigure9. Comparison of growth curves of *Pp-Dt* co-culture in ASW measured via chl *a* in RFU (left y-axis, red) and cell density (cell/mL) (right y-axis, blue). (A) Growth of *Dt* control (solid lines) and *Dt* mixed co-culture (dashed lines) with *Pp*. (B) Complimentary growth of *Pp* control (solid lines) and *Pp* mixed co-culture (dashed lines) with *Dt*. Error bars represent standard deviation of biological replicates (n = 4).

SFigure10. (A) Chl a (RFU) based growth curves of P. parvum in response to O. triangulata at different ratios (Pp:Od) of starting concentrations -A (1:1), -B (3:1), and -C (1:3). (B) Complimentary growth response of O. triangulata in response to P. parvum. (C) Allelopathic effect of both P. parvum (solid lines) and O. triangulata (dashed lines), the difference of mixed co-culture versus controls. Error bars represent standard deviation of biological replicates (n = 4).
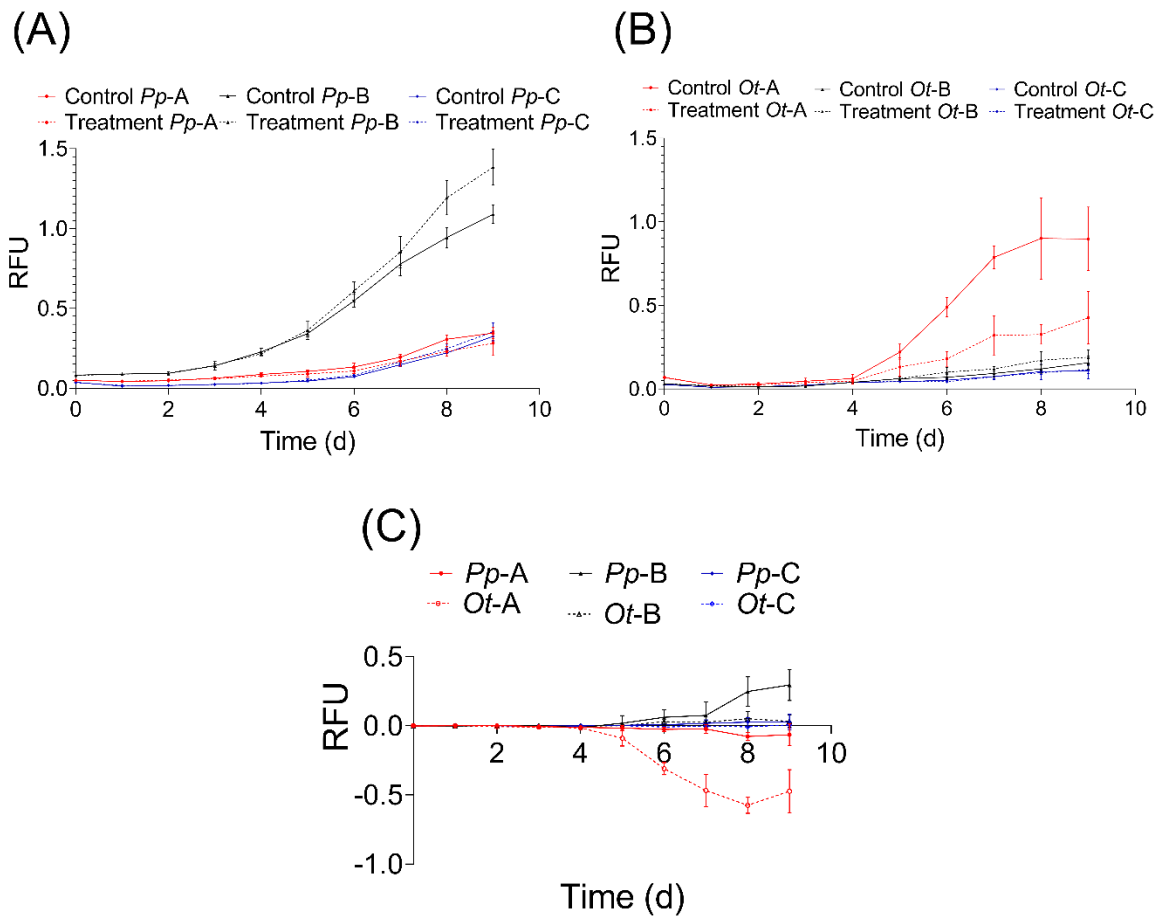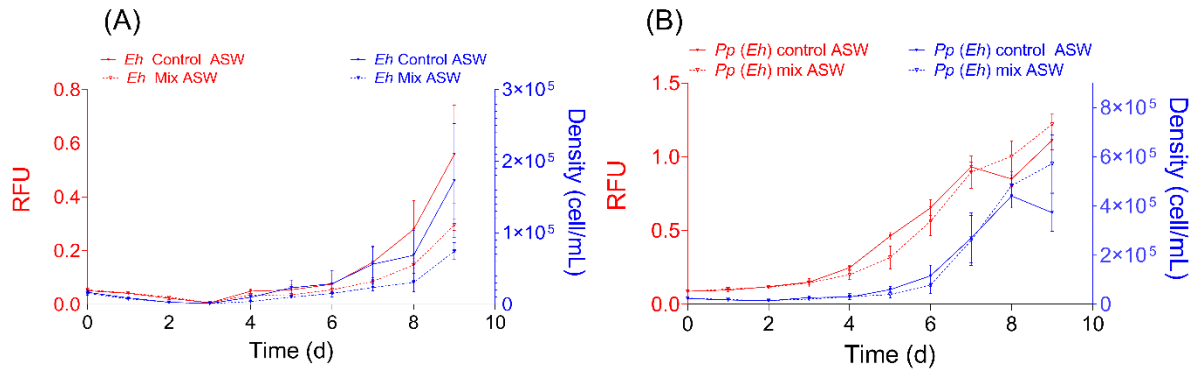
SFigure11. Comparison of growth curves of *Pp-Eh* co-culture in ASW measured via chl *a* in RFU (left y-axis, red) and cell density (cell/mL) (right y-axis, blue). (A) Growth of *Eh* control (solid lines) and *Eh* mixed co-culture (dashed lines) with *Pp*. (B) Complimentary growth of *Pp* control (solid lines) and *Pp* mixed co-culture (dashed lines) with *Eh*. Error bars represent standard deviation of biological replicates (n = 4).
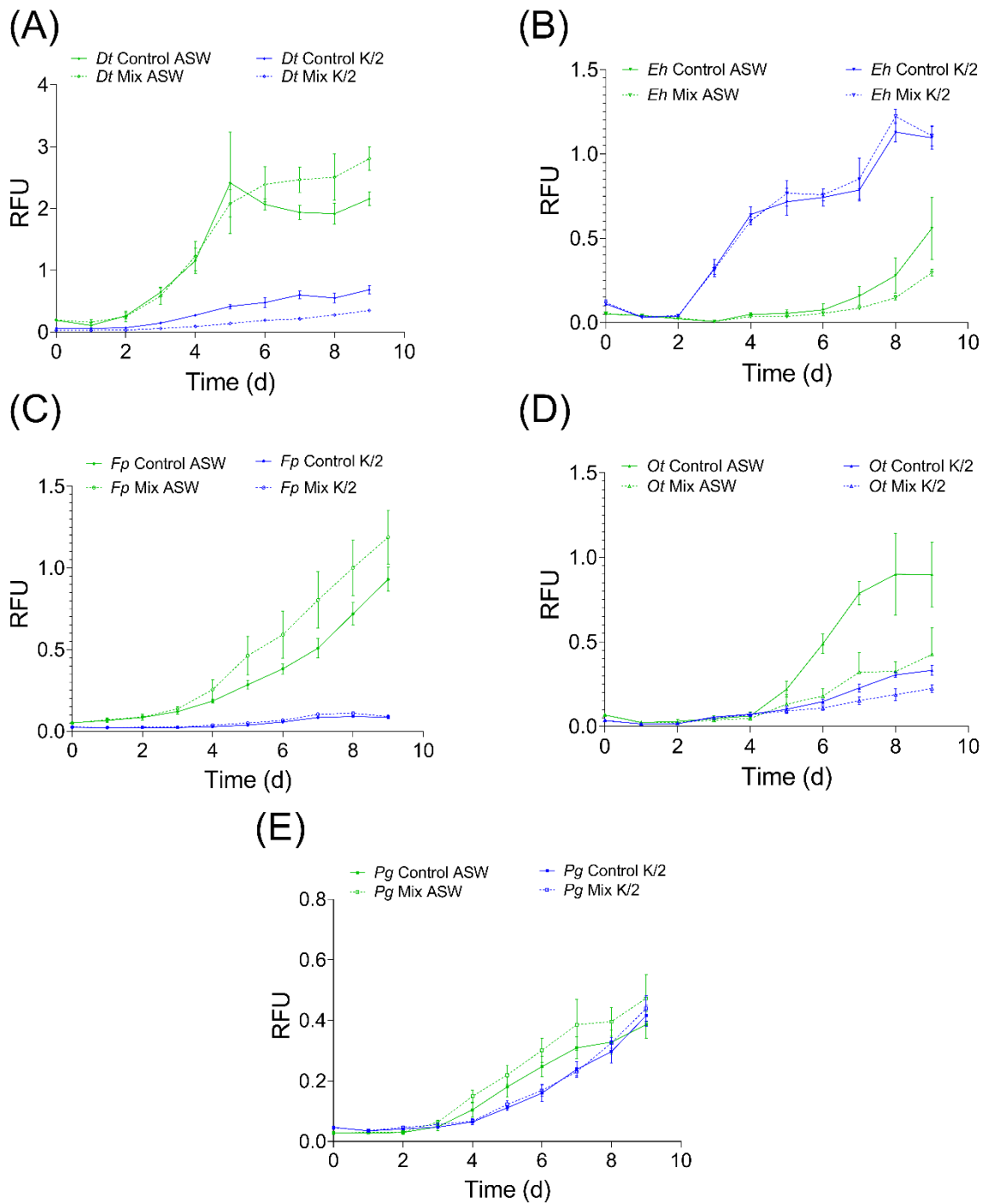
SFigure12. Growth curves of partner species of *Pp* co-cultivation: A) *Dt*, B) *Eh*, C) *Fp*, D) *Ot*, E) *Pg* in ASW media (green lines) and K/2 media (blue lines). Growth of mono-culture controls (solid lines) and co-cultivation treatments with *Pp* (dashed lines) observed by changes in chl *a* (RFU). Error bars represent standard deviation of biological replicates (n = 4).

SFigure13. Growth curves of *Pp* in response to co-cultivations: A) *Dt*, B) *Eh*, C) *Fp*, D) *Ot*, E) *Pg* in ASW media (green lines) and K/2 media (blue lines). Growth of mono-culture controls (solid lines) and co-cultivation treatments (dashed lines) observed by changes in chl *a* (RFU). Error bars represent standard deviation of biological replicates (n = 4).

7.A.2.   Tables

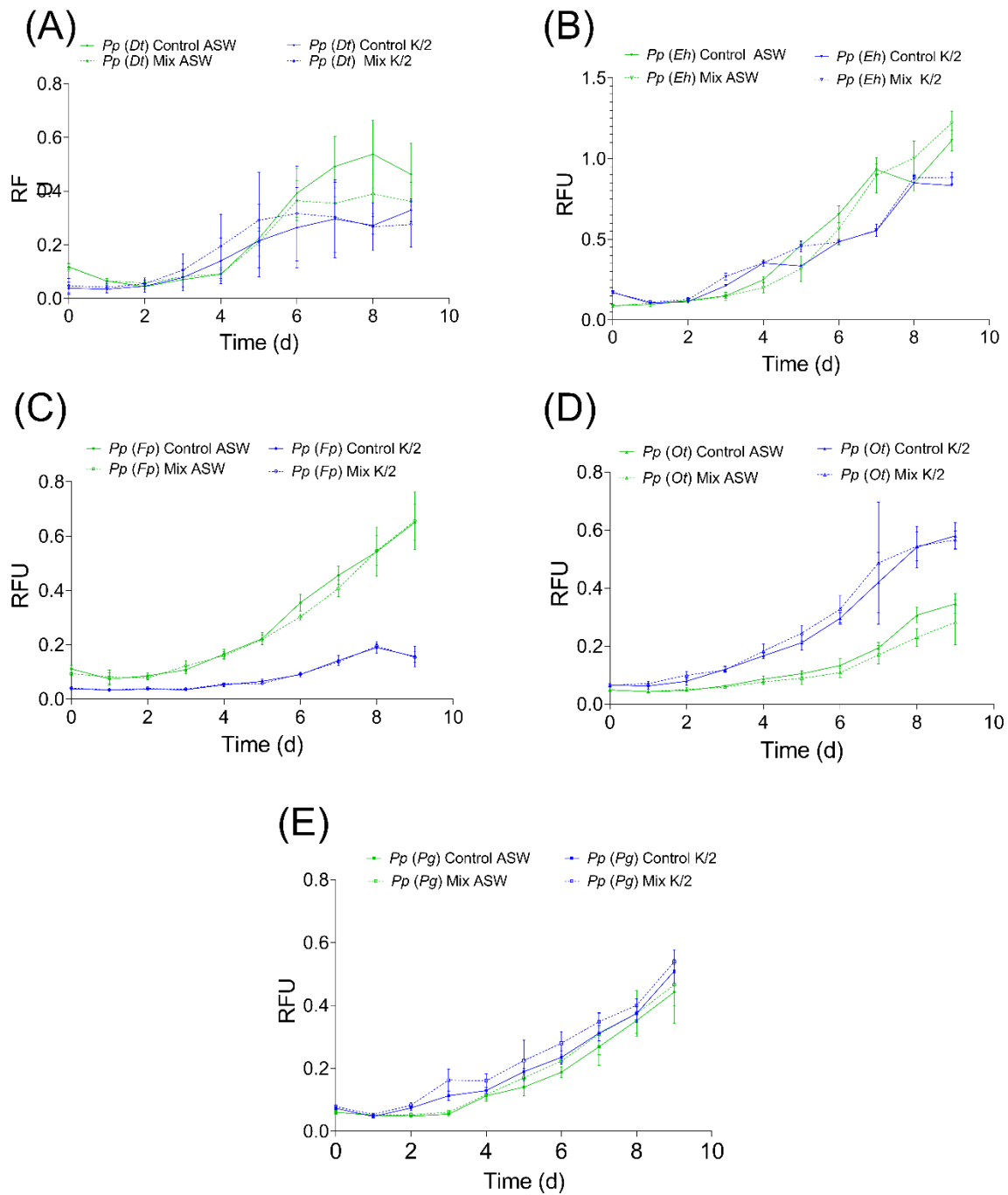**STable 1.** Inorganic nitrogen (NO_x^-) concentration (μM) from random representative samples in each co-culture. Method valid from 5-300 μM. Values given for *P. parvum* (Pp) and coculture pair (Xx) where A and B represent two halves of a single co-culture unit. Medium blanks (MB) were also measured when available.

|	Growth Medium	PpA	PpB	XxA	XxB	Pp Mix	Xx Mix	MB
Od-Pp (1:3)	ASW	<5	<5	107.31	139.62	<5	28.85	>300
Od-Pp (3:1)	ASW	>300	>300	>300	>300	172.69	>300	>300
Od-Pp	ASW	91.92	80.77	158.85	191.15	**<5**	16.92	>300
Dt-Pp	ASW	**<5**	**7.31**	**<5**	**<5**	7.31	61.15	>300
Eh-Pp	ASW	<5	<5	34.62	23.46	<5	5.00	>300
Fp-Pp	ASW	155.00	83.46	>300	232.31	29.23	6.54	-
Pg-Pp	**ASW**	**<5**	**<5**	**<5**	**<5**	**<5**	**<5**	>300
Od-Pp	K/2	223.08	167.69	218.46	244.23	158.85	253.08	>300
Dt-Pp	K/2	268.46	300.00	57.69	87.69	>300	275.00	>300
Eh-Pp	K/2	35.77	60.38	59.23	69.62	76.54	69.62	>300
Fp-Pp	K/2	262.69	174.23	168.85	225.77	264.62	233.85	160.77
Pg-Pp	K/2	181.54	194.23	101.15	128.08	165.77	105.00	168.08

ASW 558 uM NaNO3; K/2 441 uM NaNO3

STable 2. Inorganic phosphate (PO_4^{3-}) concentration (µM) from random representative samples in each co-culture. Method valid from 0.03 – 5 µM. Values given for *P. parvum* (Pp) and coculture pair (Xx) where A and B represent two halves of a single co-culture unit. Medium blanks (MB) were also measured when available.

	Growth Medium	PpA	PpB	XxA	XxB	Pp Mix	Xx Mix	MB
Od-Pp (1:3)	ASW	>5	>5	>5	>5	>5	>5	3.14
Od-Pp (3:1)	ASW	>5	>5	>5	>5	>5	>5	4.16
Od-Pp	ASW	>5	>5	>5	>5	>5	>5	3.32
Dt-Pp	ASW	>5	>5	>5	>5	>5	>5	3.80
Eh-Pp	ASW	>5	>5	>5	>5	>5	>5	4.89
Fp-Pp	ASW	>5	>5	>5	>5	>5	>5	-
Pg-Pp	ASW	>5	>5	>5	>5	>5	>5	>5
Od-Pp	K/2	2.05	2.34	1.14	0.96	1.28	2.89	0.30
Dt-Pp	K/2	1.90	0.04	1.50	0.26	1.68	0.04	1.68
Eh-Pp	K/2	2.92	2.19	4.45	1.32	2.27	2.41	1.10
Fp-Pp	K/2	3.54	3.62	4.45	4.09	4.13	3.83	2.49
Pg-Pp	K/2	2.16	1.98	3.03	2.60	2.23	4.42	0.66

ASW K2HPO3-3H20 (1 mM) + Na2-glycerophosphate-5H2O (78 uM); K/2 Na2-b-glycerophosphate-5H2O (5 uM)

7.B.   Supplementary Chapter 2
7.B.1.   Figures
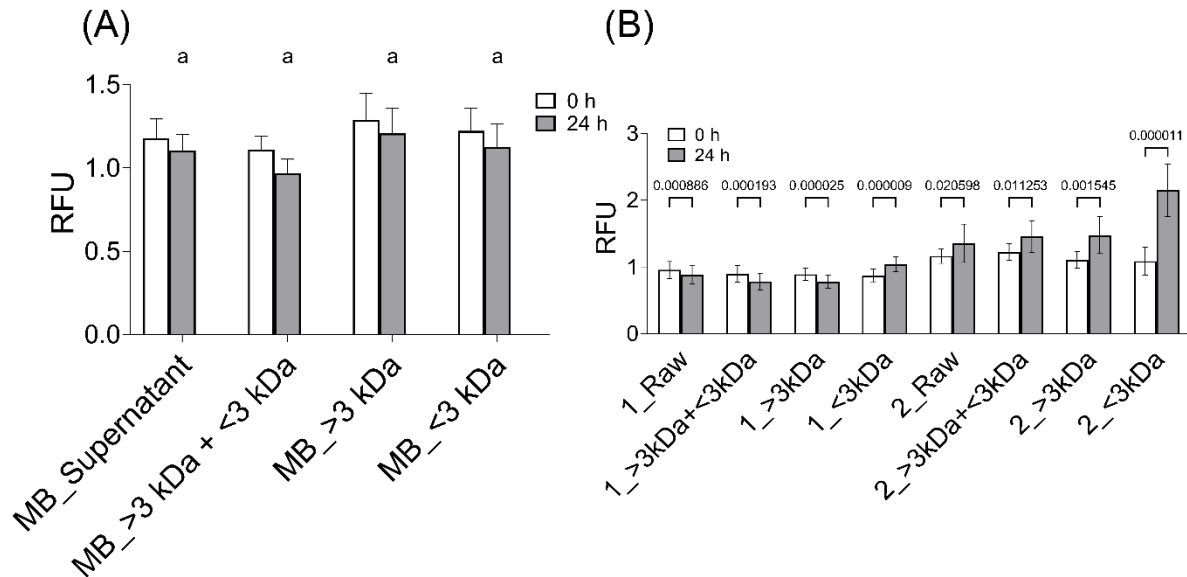
SFigure 14. Algicidal activity measured by change in chl *a* (RFU) of (A) media blank controlsfor fractionation of spent medium, no *K. algicida* added. The medium was fractioned and evaluated for algicidal activity of mostly small molecules fraction (< 3 kDa), mostly protein fraction (> 3 kDa) and the 1:1 recombination of both (>3 kDa + < 3 kDa), with whole supernatant used as algicidal control (n=8). One-way ANOVA was performed on 24 h data, with significance indicated by letter a. (B) Stability of algicidal fractions (Figure 1C) at room temperature measured over 2 days, represented by 1_ or 2_. Multiple paired t-test, ** p <0.01; *** p<0.001. All error bars indicate the standard deviation of biological replicates (n = 8).
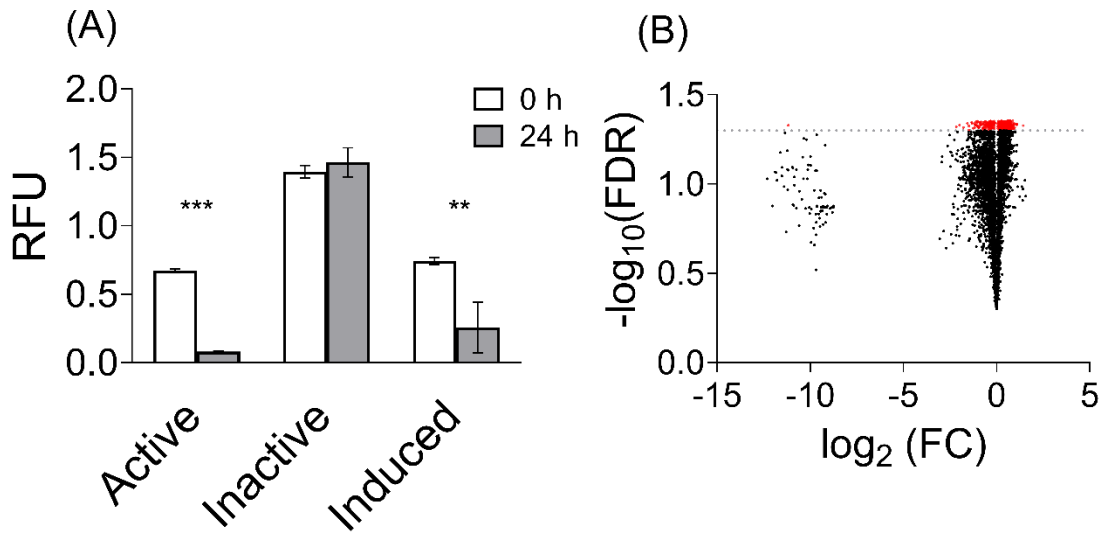
SFigure 15. (A) Activity of *K. algicida* cells prior to RNA extraction, active (30 h), inactive (75 h), and induced (10x dilution in ASW for 24 h) (n = 4), measured by change in chl *a* (RFU). Multiple paired t-tests, ** $p < 0.01$; *** $p < 0.001$. (B) Volcano plot showing fold change of genes between the two phases, active (30 h) and inactive (75 h) (n = 3). Error bars indicate the standard deviation of biological replicates.
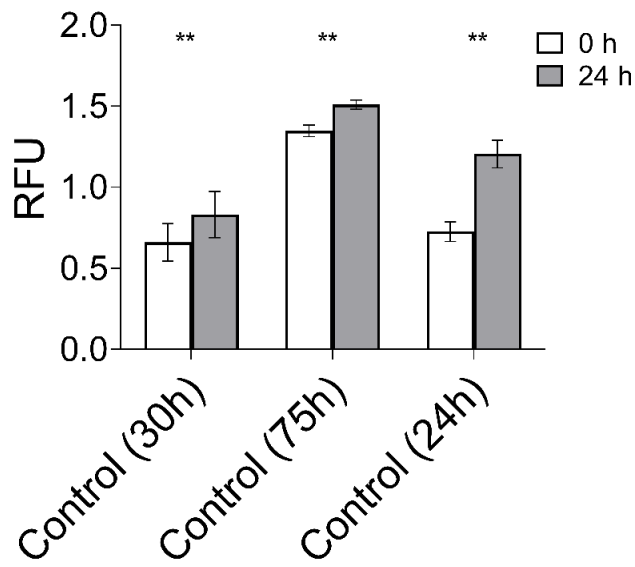
SFigure 16. Algicidal activity of medium controls, measured by change in chl *a* (RFU) for *K. algicida* samples submitted for transcriptomic analysis (n=4). Error bars indicate the standard deviation of biological replicates. Multiple paired t-test, ** $p < 0.01$.
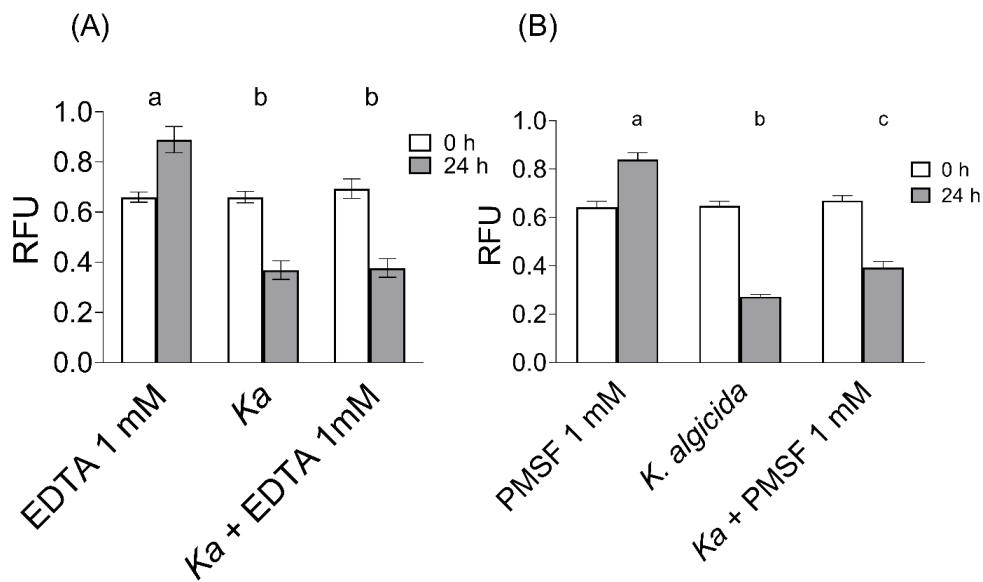
SFigure 17. (A) EDTA (1 mM) treatment to exclude metalloproteases as algicidal protease of interest (n = 4). (B) PMSF (1 mM) treatment to exclude serine proteases as algicidal protease of interest (n = 4), measured by change in chl *a* (RFU). One-way ANOVA performed to determine significant

differences between treatments: a,b,c. Error bars indicate the standard deviation of biological

replicates.

```
                  1 .........10........20........30........40........50........60
K. algicida       1 MKKNLKLIYVLLLVFVTACSTDSITSSD-----EEILLEKKGDQTEVISGEYIIIFKKGA
Kordia sp.        1 MKNNFKLIYVLLLVFITSCSTDNDETTDDLIKGKTDLLDKKLDQAEVIPGEYIIVYKKDA
K. aestuariivivens 1 MKKNFKLIYVLLLVFITSCSTDSVTTID------DDLLEKRSDQTEVIQDEYIIVYKKSA

                  61 .........70........80........90........100.......110.......120
K. algicida       56 DS-KTSYFATKRGKTPELTRIQNFYKTKALEALKLIGKGEGNLGYVYTGAIQGFHAKDLS
Kordia sp.        61 TTSKMSYTATRRGLTPELKKVQDYYKERSIATLKLIGRGEKNLGFVYTGAIQGFHAKNLS
K. aestuariivivens 55 NSGKMNYSPTKRGKTPELIKIQNYYRTKSLATLKLIGKGEDNLGFVYTGAVQGFHAKNLS

                  121 .......130.......140.......150.......160.......170.......180
K. algicida       115 SDDVKILSSIESIDYIEPNRTVFSDLPQPKNPQGVKLPTGNDIIGKSDTVLPSGDFLPWG
Kordia sp.        121 VDDVEVLLRDETIDFIEPNRRVVSNFPKPKNPQGVKLPTQSEVINKADSFLPSGDFLPWG
K. aestuariivivens 115 SEDVKILLDDESIDFIEPNRKVISDLPKPKNPQGVKLPSNNGINGKSDTTLPSGDFLPWG

                  181 .......190.......200.......210.......220.......230.......240
K. algicida       175 VDYTGRGNNAGTNRYVFVIDTGIAPHSDLTIDSGLSESFYPG--EDWVDRNGHGTHVAGT
Kordia sp.        181 VDYTGRGNNTGTNRYAFVIDTGIDQHPDLTIDIGLSASFYPG--ENWVDRNGHGTHVAGT
K. aestuariivivens 175 VDYTGRGDNTDTDRYVFVMDTGIAPHPDLTINSQYSKNFSTLNETDWIDRDGHGTHVAGT

                  241 .......250.......260.......270.......280.......290.......300
K. algicida       233 IGAKANGSGVIGVAYGSTLVAVKVLGGTQGTGSDAGILAGVDYTYNNSIAGDVFNYSVGY
Kordia sp.        239 IGAKANGFGVIGVAYGSTLVAIKVLGGTQGSGSDAGILAGVDYTYNNSIAGDVFNYSVGY
K. aestuariivivens 235 IGAKANGSGVIGVAYGSTLVAVKILG-DDGSGSLDGILAGVDYTYNTSLAGDVYNYSVGF

                  301 .......310.......320.......330.......340.......350.......360
K. algicida       293 RTRRTSTAIDNAFTTLDNKIYGALAAGNSNDNTLYYSPQRLQTSRTWMVGNLTRSITPNG
Kordia sp.        299 RTRRTNAAIDNAFTTLDNKIYGALAAGNSNDNTLYYSPQRLQTSRTWMVGNLRRNITPNG
K. aestuariivivens 294 ENRQTSTAIDNAFTTLGNKISGATAAGNSNDNTLFYSPQRLQTSRTWMVGNLRRNITPSG

                  361 .......370.......380.......390.......400.......410.......420
K. algicida       353 SSCYGASVDRWAPGTDVWSTWLNGNYNRISGTSMASPHVAGILAVRGNNSVGTQGNTTKN
Kordia sp.        359 SSNEGASVDRWAPGTDVWSTWLNGQYNRISGTSMASPHVAGILAVRGNNSVGTNGNTSKG
K. aestuariivivens 354 SSCYGASVDRWAPGTDVWSTWLNGGYNRISGTSMASPHVAGILVVRGNNSVGTNGNTSKN

                  421 .........
K. algicida       413 GFTAPNAKK
Kordia sp.        419 GYTAPNARR
K. aestuariivivens 414 GFSAPNAKR
```
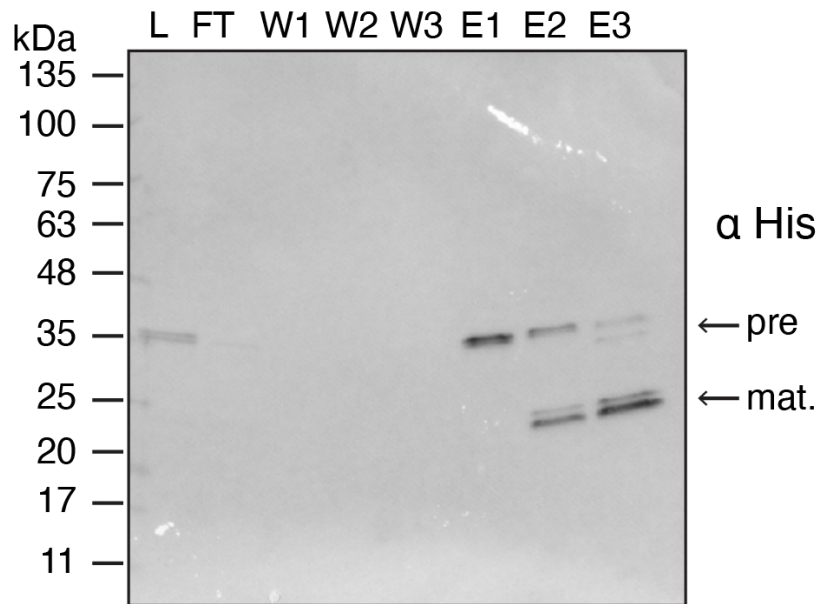
SFigure 18. An alignment of closest homologues of AlpA1 (*K. algicida)*, from *Kordia* sp. and *Kordia aestruariivivens* (Table 2). Shows several instances where amino acids are unique to AlpA1.

SFigure 19. Anti 6xHis-tag immunoblot of the purification of recombinant AlpA1. Pre- and mature protein bands are indicated with arrows. L lysate, FT flow through, W wash and E elution.

7.B.2.   Tables

STable 3. qPCR fold change analysis > Raw data (all values, Active, inactive, induced > replicates)

Fold Change Analysis (2^(-ΔΔCq))			
	Active	Inactive (Inactive-Active)	Induced (Induced - inactive)
KAOT1_RS09515	1	0.0675	6.5055
KAOT1_RS10890	1	0.1957	8.6838

7.C.   Supplementary Chapter 3
7.C.1.   Figures
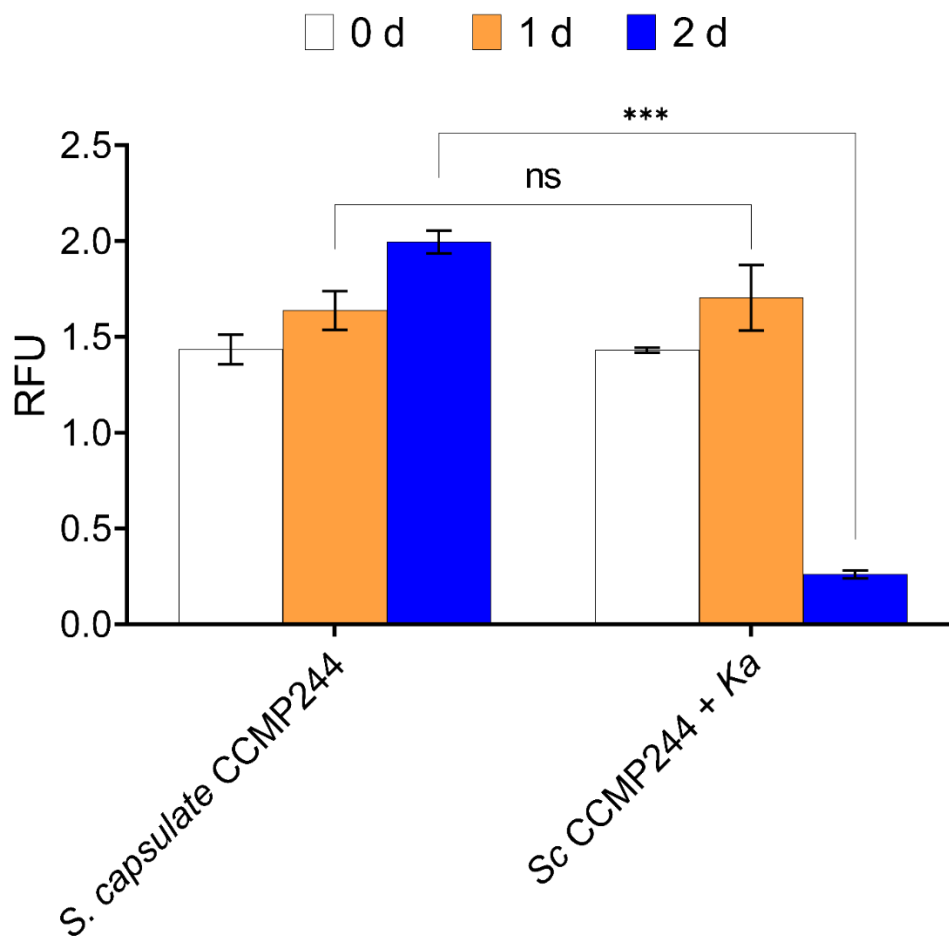
SFigure 20. Chl *a* (RFU) monitoring of *S. capsulate* CCMP244 exposed to *K. algicida*. N = 4 biological replicates of *S. capsulate*. Error bars indicate SD of replicates. *** P<0.001
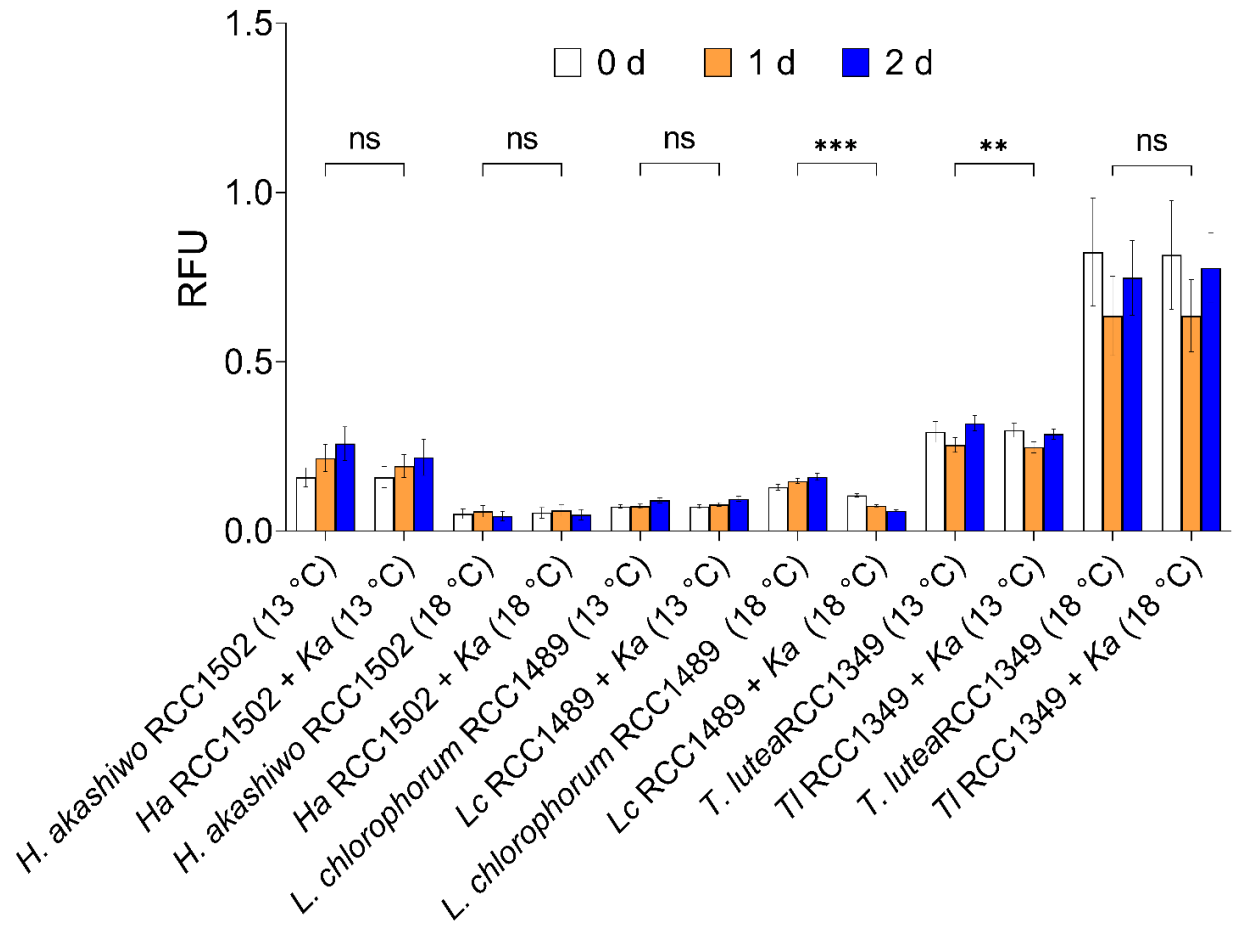
SFigure 21. Chl *a* (RFU) monitoring of 13 °C and 18 °C cultures of *H. akashiwo, L. chlorophorum,* and *T. lutea* exposed to *K. algicida*. N = 4 biological replicates of phytoplankton. Error bars indicate SD of replicates. ** p < 0.01, *** p <0.001.
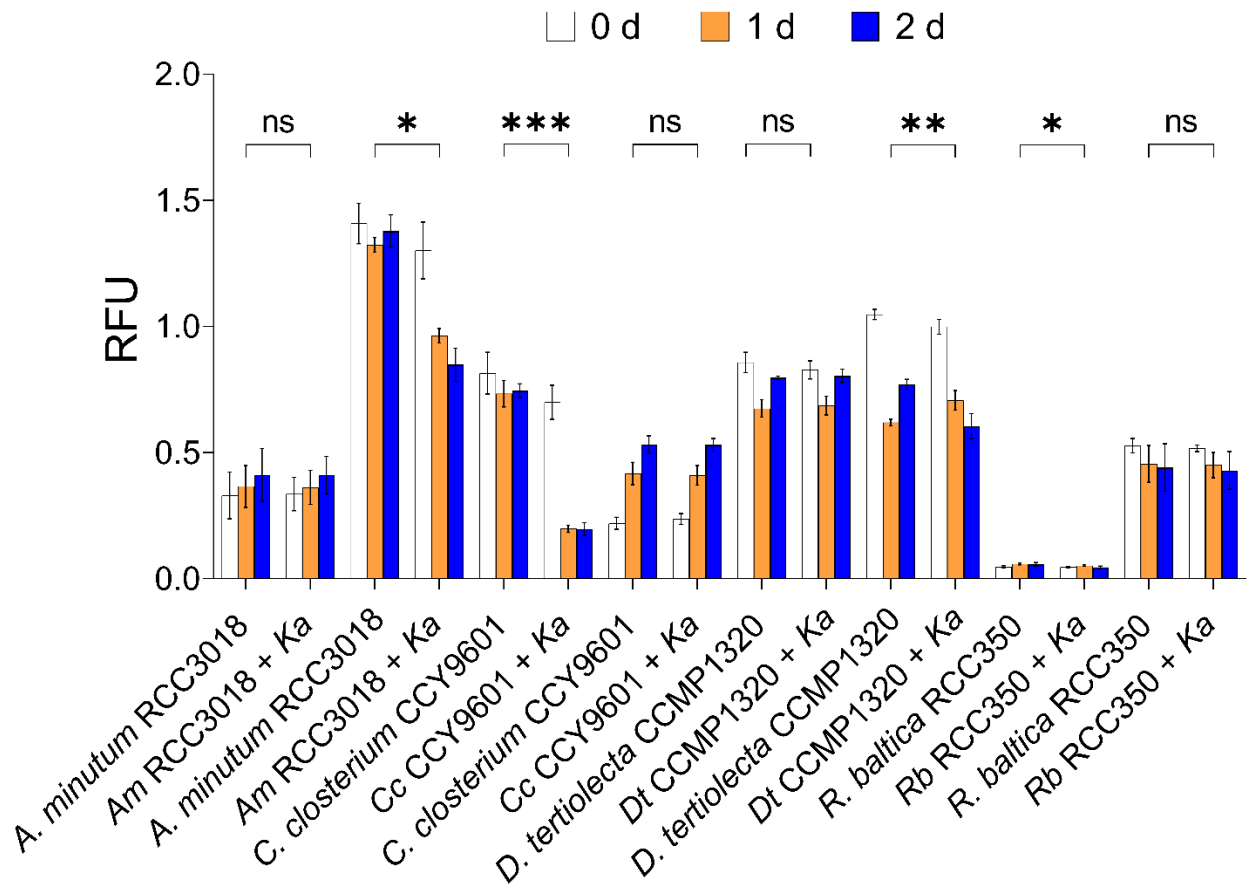
SFigure 22. Chl *a* (RFU) monitoring of cultures of *A. minutum, C. closterium, D. tertiolecta CCMP1320* and *R. baltica* exposed to *K. algicida* on two separate occations. N = 4 biological replicates phytoplankton. Error bars indicate SD of replicates.* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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9) *Curriculum Vitae* | Kristy Southysa Syhapanha

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Work Experience

July 2020 - Present	**Dr. rer. nat. (PhD) Chemical Ecology/Analytical Chemistry: Algicidal bacteria in plankton communities: resistance, lysis and heterotrophy, CRC ChemBioSys C01, Pohnert lab, Friedrich Schiller University Jena, Germany**

I analyze the algicidal properties of the marine bacterium *Kordia algicida* as a potential bioregulator for HABs. This includes a metabolomic analysis of the chemical fingerprint of *K. algicida* in the presence of resistant and susceptible diatoms using a high resolution LCMS Oribtrap to analyze endo- and exo-metabolomic fingerprints, as well as genomic investigations of candidate proteases which contribute to algicidal activity by creating *E.coli* transformants for target protein purification and the use of RT-PCR to determine the correlation to algicidal activity. I also investigate the interspecific allelopathy of different plankton using co-culturing techniques and metabolic extraction to understand the complex hierarchy of interactions within complex plankton communities.

February 2019 – May 2020	**MSc Chemical Biology: Optimization of a metabolomics investigation of algicidal bacteria within plankton communities, Pohnert Lab, Friedrich Schiller University Jena, Germany**

I optimized a method for extracting secondary metabolites of the algicidal marine bacteria, *Kordia algicida* and analysis on LCMS and GCMS Orbitraps. Hydrophilic-lipophilic balance SPE was used to extract exometabolomic compounds, while three different lysis methods were compared for the extraction of endometabolomic

compounds. The extracts were used to establish LCMS and GCMS metabolic profiles for the algicidal bacteria as a foundation for investigating algicidal metabolites.

Septempber 2018 – Jan 2019 **Intern/Consultant Georgia State University Technology Transfer Office**

In this role, I research and analyze the commercial viability and appeal of potential chemistry products developed from GSURF inventions. This includes organizing patent application documents, and the analysis of prior art that may affect the technologies currently being developed. I also help prepare small business and Georgia Research Alliance grants to access funds to support the development of inventions.

July 2016 – July 2018   **Chemist, ORISE Fellow, CDC IRAT Branch**

In this role, I used ICP-MS instrumentation to detect trace amounts of 23 different metals for contamination in patient sample collection devices. I helped establish a record keeping process for the progress of methods run, (Multielemental, Whole Blood, Serum, HgI), as per requests and maintain the inventory of supplies needed for this lab. I was also responsible for the use and upkeep of the ICP-MS instruments, Elan DRC2-O and Nexion 300D. I analyzed human blood samples for trace amounts of chromium and cobalt, using iCAP_Qa ICP-MS technology, mainly from the NHANES study and human urine samples for creatinine levels using a Roche Cobas Integra 400. I also participated in national public health studies involving the FDA (Leadcare – Magellan Instruments), PATH, NHANES and in-house studies for the CDC IRAT Branch.

May 2016 – August 2016   **Project Coordinator for ICBG Grant, Georgia Institute of Technology**

I was responsible for the maintenance of hundreds of algal samples that have been collected from Fiji and the Solomon Islands to access their pharmaceutical potential. I helped coordinate the flow of information between collaborators at Georgia Tech as well as USP and analytical companies. I also managed and performed anti-microbial bioassays

(MRSA, VREF, MDREC, WTCA, ARCA, ECO35) on extracts of samples with high potential.

May 2015 – May 2016   **Laboratory Technician, Kubanek Lab, Georgia Institute of Technology**

In this role I maintained and repaired a variety of laboratory equipment including the HPLC, LCMS, rotary evaporators, vacuum pumps, compound microscopes, and freeze dryers. I managed and organized chemical compounds/laboratory equipment by taking inventory and placing orders for necessary experiments. I also routinely performed microbial bioassays (MRSA) and wrote weekly reports analyzing results and outlining immediate goals.

August 2014 – May 2016   **Undergraduate Research: Molecular Networking of Fijian Algae, Kubanek Lab, Georgia Institute of Technology**

I conducted a comparative analysis of chemical metabolites of a variety of macro-algae and their relationship to their ecological environment. I used methanol extractions, column chromatography, LCMS and analyzed MS/MS data using Cytoscape software. In this project I also had the opportunity to mentor two other fellow undergrads on basic lab protocol, and concepts of common analytical techniques.

May 2014 – May 2016   **Plankton Culturing Technician, Kubanek Lab, Georgia Institute of Technology**

I maintained the health of 15 different plankton species, including diatoms and dinoflagellates, by using sterile transfer techniques and making seawater media. I was also responsible for ordering new species and integrating them into our stock cultures and preparing/growing cultures for further experiments in allelopathy and drug discovery.

January 2013 – August 2014 **Undergraduate Research: Chemically Mediated Predatory-Prey Interactions in the Georgia Oyster Reef, Kubanek Lab, Georgia Institute of Technology**

I investigated the chemically related predator-prey interactions in Mud Crab feeding deterrence due to Blue Crab urine. We utilize animal husbandry techniques, liquid-liquid extraction, solid-phase extraction, HPLC purification, 1H-NMR spectroscopy, LCMS and multivariate data analysis in a metabolomics style investigation.

Publications/Presentation

Syhapanha, K.S., Poulin, R.X., Russo, D.A., Wong, W., Pohnert, G., (2023) Systematic investigation of factors influencing phytoplankton allelopathy - a case study of *Prymnesium parvum* and ecologically relevant interaction partners (working title). Manuscript in Progress.

Syhapanha, K.S., Russo, D.A., Deng, Y., Meyer, N., Poulin, R.X., Pohnert, G., (2023) Transcriptomics guided identification of algicidal protease of the marine bacterium *Kordia algicida* OT-1. *MicrobiologyOpen.* 12, e1387. https://doi.org/10.1002/mbo3.1387

Syhapanha, K.S., Poulin, R.X., Pohnert, G. (2023, Mar.) Systematic evaluation of *Prymnesium parvum* allelopathy against ecologically relevant competing phytoplankton. Poster session presented at the Phycology Conference 2023, Jena, Germany.

Syhapanha, K.S., (2022, Oct.) Metabolic Marketplace: CO1 Algicidal Bacteria in Plankton Communities: Resistance, Lysis and Heterotrophy. PowerPoint slides presented at the CRC ChemBioSys retreat 2022, Masserberg, Germany.

Syhapanha, K.S. (2019, Jun.) C01: Algicidal Bacteria in Plankton Communities: Resistance, Lysis and Heterotrophy. Poster session presented at the CRC ChemBioSys retreat 2019, Masserberg, Germany.

Syhapanha, K.S. (2018, Jan.) Evaluation of trace metals lot sceening data. Poster session presented at the 2018 Winter Conference on Plasma Spectrochemistry, Amelia Island, Fl.

Ward, C. D., Williams, R., Mullenix, K., Syhapanha, K., Jones, R., and Caldwell, K. (2017) Trace Metals Screening of Devices used for the Collection, Storage, and Analysis of Biological Specimens. Atomic Spectroscopy, 39(6).

Education

May 2020 - Present	Friedrich Schiller University Jena
	Doctor rerum naturalium Chemical Ecology (Analytical Chemistry)
February 2019 – May 2020	Friedrich Schiller University Jena
	Master of Science in Chemical Biology
August 2012 – May 2016	Georgia Institute of Technology
	Bachelor of Science in Chemistry, 3.4 gpa

Skills and Training

Analytical Techniques	Spectrometry (LCMS Orbitratp, GCMS Orbitrap, ICP-MS), Electrochemistry (LeadCare II, LeadCare Ultra), Chromatography (HPLC, SPE), Spectroscopy (Electron absorption, Raman, IR, Laser-Induced Breakdown), Nuclear Magnetic Resonance (1H, 13C, 31P), Immunoassay (Roche Cobas Integra 400).
Biological Techniques	Polymerase chain reaction (PCR), vector transformation, SDS-PAGE, Thin Layer Chromatography (TLC), Flow cytometry, plankton culturing, cell culturing, microbial culturing, Gel-electrophoresis, Anti-microbial bioassays, sterile techniques, plate streaking
Training/Projects	Responsible Conduct of Research (RCR), Confocal Microscopy (ZEN software), Bloodborne Pathogens, Clinical Laboratory Improvement Amendments (CLIA) certified, FDA Magellan LeadCare project
Programming/Computer	STARLIMS, Python programming, HTML, Microsoft office

References

Prof. Dr. Georg Pohnert
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10) Selbstständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und unter Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe.

Jena, Datum   Unterschrift (Kristy Southysa Syhapanha)