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Growth and mortality of microbial plankton in the Ria Formosa coastal lagoon

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

2023

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Mestrado em Sistemas Marinhos e Costeiros

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2023

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Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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(Yohann Santos)

The importance is huge, even when it is microscopic. The most fascinating and essential roles in the ocean are played by the organisms we can´t see with the naked eye.

Small is beautiful.

Acknowledgments

I feel grateful for having the opportunity to embark on this incredible journey of growth and discovery. It has been an amazing ride filled with continuous learning, where I have not only acquired new knowledge but also new skills as an aspiring researcher. However, for me, the essence of this journey goes beyond acquiring knowledge. It has been about pushing my limits. It has required perseverance, dedication, and a willingness to step out of my comfort zone. It was a journey of self-growth, intellectual pursuit, and commitment. Indeed, it was an incredible journey, and for all of that, I am grateful.

I would like to express my gratitude and appreciation to my supervisor Rita Domingues, for all the invaluable guidance and support provided during the realization of this thesis. Rita has been an exceptional supervisor, always ready to help and her constructive criticism has greatly enhanced the rigor and quality of this thesis. But especially for consistently pushing me forward to become not only a better student but also a better human being with a lifelong love for learning and a commitment to intellectual curiosity. Her guidance, mentorship, and genuine care for my academic and personal development have left a positive impact on my life.

I am also grateful to teacher Helena Galvão for believing in my abilities and for introducing me to the amazing team I had the joy to work with. It was a pleasure to be guided by someone with so much experience. From the early days in Marine Microbiology classes during my Bachelor's degree to guiding me throughout my Master's thesis, your willingness to invest time and expertise in guiding me through the challenges of this study was truly encouraging.

I would like also to express my gratitude to my lab coworkers Patrícia Nogueira and Benjamin Mosley for creating an environment that promotes growth and encourages collaboration. The opportunity to work alongside you has further enriched my experience.

I would like to extend my heartfelt appreciation to my parents and sister, for their firm support, patience, and understanding throughout this journey. Their encouragement and belief in my abilities have been a constant source of strength.

Lastly to my beloved Catarina. I want to express my deepest gratitude for her support, understanding, and love throughout this journey. Despite the demands of this academic pursuit, she has remained a pillar of support, reminding me of the importance of self-care and balance.

Abstract

Microbial plankton components interact with each other through fluxes of matter and energy, forming a complex microbial food web. It is well recognized that mortality due to viral lysis and grazing by phagotrophic protists are important processes of biomass removal of heterotrophic prokaryotes and phytoplankton. Since there are only a few studies addressing microbial mortality due to grazing in the Ria Formosa coastal lagoon and the contribution of viruses to prokaryote mortality was never addressed in this ecosystem, the main objective of this study was to evaluate growth and mortality of heterotrophic prokaryotes and phytoplankton in the Ria Formosa coastal lagoon, in order to discuss the quantitative trophic role of microzooplankton and viruses in microbial food web of this important ecosystem. The dilution technique was used to evaluate the impact of microzooplankton on microbial populations of the Ria Formosa coastal lagoon; to estimate the contribution of viruses to heterotrophic prokaryote mortality, modified dilution experiments were also performed. The dilution experiments revealed notable seasonal variations in the growth and grazing rates of heterotrophic prokaryotes and phytoplankton community, with mean grazing rates of 1.66 d^{-1} and 0.106 d^{-1} respectively, slightly higher than their mean potential instantaneous growth rate (1.57 d^{-1} and 0.080 d^{-1}, respectively). These results suggest that microzooplankton consume a significant proportion of heterotrophic prokaryotes and phytoplankton community in the Ria Formosa coastal lagoon. Regarding specific phytoplankton groups, eukaryotic picophytoplankton and cryptophytes showed higher average grazing rates, whereas eukaryotic picophytoplankton showed higher average potential instantaneous growth rates; a wide range of grazing rates among phytoplankton groups is suggestive of selectivity of grazers among taxa and highlights the need to consider and analyze the specific dynamics of each phytoplankton group separately in future studies. On the other hand, this study revealed that viral lysis is a significant source of mortality in late-summer autumn, even exceeding the grazing rates of microzooplankton. Considering the relevance of the Ria Formosa, future studies should focus on these mortality factors, as well as to predict the effects of climate change, and its associated impacts, on these important biomass removal processes.

Key words: Microbial food web, heterotrophic prokaryotes, phytoplankton, grazing, viral-lysis, dilution method, Ria Formosa

Resumo

Novas metodologias revolucionaram a compreensão dos papéis desempenhados pelo plâncton microbiano nos ecossistemas marinhos. Estes complexos microrganismos interagem entre si através de fluxos de matéria e energia, formando uma estrutura denominada teia alimentar microbiana. Os procariotas heterotróficos marinhos são os principais consumidores e transformadores de carbono orgânico (DOC), impedindo a perda de matéria orgânica dissolvida e atuando como uma via de fluxo de carbono para níveis tróficos superiores. O fitoplâncton, como o principal produtor primário na teia alimentar microbiana, suporta parte da produção do ecossistema fornecendo carbono a níveis tróficos superiores e é considerado a fonte mais importante de DOC em ambientes marinhos. A mortalidade devido à lise viral e à predação por protistas fagotróficos são importantes processos de remoção de biomassa de procariotas heterotróficos e fitoplâncton; estes processos top-down têm a capacidade de afetar significativamente os fluxos de energia e nutrientes nas teias alimentares marinhas, assim como a estrutura das comunidades microbianas. A predação e a lise viral têm diferentes efeitos no fluxo de carbono e energia ao longo da teia alimentar e, portanto, é altamente relevante quantificar as taxas de predação e lise viral em microrganismos marinhos, de forma a compreender a dinâmica dos ecossistemas marinhos. Uma vez que existem apenas alguns estudos sobre a mortalidade microbiana devida à predação na lagoa costeira da Ria Formosa e a contribuição dos vírus para a mortalidade de procariotas nunca foi abordada neste ecossistema, o principal objetivo deste estudo foi quantificar estes processos de remoção de biomassa, utilizando abordagens experimentais. Objetivos específicos consistiram em a) avaliar o crescimento e a mortalidade de procariotas heterotróficos, comunidade total de fitoplâncton e grupos específicos de fitoplâncton devido à predação de microzooplâncton; e b) avaliar a mortalidade de procariotas heterotróficos devido à lise viral. A técnica de diluição foi utilizada para avaliar o impacto dos predadores nas populações microbianas na Ria Formosa; de forma a estimar-se a contribuição da lise viral, utilizou-se a técnica de diluição modificada. A taxa de crescimento instantâneo potencial de procariotas heterotróficos foi maior durante o mês de junho ($1.99 \pm 0.11\ d^{-1}$), e a taxa de predação foi maior durante o mês de outubro ($2.17 \pm 0.26\ d^{-1}$), com a predação do microzooplâncton tendo um maior impacto durante outubro e menor impacto durante junho. Observou-se também uma variação nas taxas de predação e crescimento instantâneo potencial da comunidade de fitoplâncton ao longo das experiências realizadas

em junho ($g = 0.65 \pm 0.08\ d^{-1}$; $\mu0 = 0.33 \pm 0.05\ d^{-1}$), agosto ($g = 0.55 \pm 0.16\ d^{-1}$; $\mu0 = -0.40 \pm 0.110\ d^{-1}$) e outubro ($g = 0.90 \pm 0.20\ d^{-1}$; $\mu0 = 0.31 \pm 0.12\ d^{-1}$). As taxas de crescimento e predação obtidas para procariotas heterotróficos foram significativamente superiores às da comunidade fitoplanctónica durante todas as experiências. Em relação aos diferentes grupos de fitoplâncton, o picofitoplâncton eucariótico e as criptofíceas apresentaram taxas médias de predação superiores, enquanto o picofitoplâncton eucariótico apresentou taxas médias de crescimento instantâneo potencial superiores. Isto sugere preferência por parte do microzooplâncton em se alimentar destes grupos de fitoplâncton, o que pode ser atribuído ao seu pequeno tamanho, e salienta a necessidade de se considerar a dinâmica específica de cada grupo de fitoplâncton separadamente em estudos futuros. Ao contrário do que era esperado, os resultados deste estudo revelaram que as taxas médias de crescimento dos procariotas heterotróficos e da comunidade fitoplanctónica foram inferiores às suas taxas médias de predação, sugerindo que o microzooplâncton consome uma quantidade significativa de procariotas heterotróficos e da comunidade fitoplanctónica na Ria Formosa. As altas taxas de crescimento e predação sobre procariotas heterotróficos indicaram que o "microbial loop" contribui bastante para o fluxo de carbono na lagoa costeira da Ria Formosa. Por outro lado, este foi o primeiro estudo a obter informações acerca da mortalidade induzida por vírus em procariotas heterotróficos e revelou que a lise viral foi uma forma significativa de mortalidade durante outubro, excedendo até mesmo as taxas de predação do microzooplâncton. Estudar a maioria dos componentes da teia alimentar microbiana permitiu desta forma comparar as diferentes taxas de crescimento e predação, destacando a importância de todos as componentes microbianas no funcionamento global da teia alimentar microbiana e confirma a relevância do microzooplâncton na Ria Formosa. Além disso, a lise viral interagiu com o microzooplâncton como fonte de mortalidade de procariotas heterotróficos. Tanto quanto se sabe, esta é a primeira evidência que mostra que os vírus são uma importante fonte de mortalidade de procariotas heterotróficos na Ria Formosa. Considerando a relevância destes processos e a importância do ecossistema da Ria Formosa, estudos futuros devem explorar cada um destes fatores de mortalidade individualmente, de forma a entender a dinâmica das comunidades microbianas e o seu impacto sobre a produtividade global dos ecossistemas aquáticos.

Palavras-chave: Teia alimentar microbiana, procariotas heterotróficos, fitoplâncton, predação, lise viral, método da diluição, Ria Formosa

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1. Introduction

1.1. Definition of microbial plankton

Microbial plankton are all the microorganisms that drift freely in the water column, including prokaryotes (Domains Bacteria and Archaea), fungi, protists, and viruses (Kirchman, 2018; Munn, 2011). These planktonic microorganisms may be heterotrophs, autotrophs, or mixotrophs, and can be classified according to their size into the following categories: femtoplankton (0.01-0.2 µm), which includes viruses; picoplankton (0.2-2 µm), which includes most aquatic prokaryotes (heterotrophic and autotrophic Bacteria and Archaea) and the smallest protists; nanoplankton (2-20 µm), which includes most species of flagellates, smaller diatoms, and smaller dinoflagellates and ciliates; microplankton (20-200 µm) that covers the larger-sized phytoplankton, mainly diatoms and larger species of dinoflagellates, and the larger-sized phagotrophic protists (Mostajir et al., 2015; Munn, 2011; Sherr and Sherr, 2009; Suthers et al., 2019). Microbial plankton plays important roles in many ecosystem functions, such as nutrient uptake, primary production, biomass and direct utilization of primary production in the ocean, and the basis for higher trophic levels. Furthermore, microbial plankton plays vital roles in biogeochemical cycles, climate regulation, organic matter decomposition, and water quality (Ducklow, 2008; Mostajir et al., 2015).

1.2. The importance of microbial plankton and their role in the microbial food web

Food webs include complex ecological interactions that regulate the flow of matter and energy, and thus are fundamental in understanding ecosystem functioning (Segovia et al., 2015). The importance of microbial plankton to the global metabolism of the oceans was first introduced by Vernadskii in 1926, and its importance in pelagic food webs was only seriously considered by Pomeroy in 1974 (Pomeroy, 1974; Pomeroy and Wiebe, 1988). The recognition of the role of heterotrophic microbes (heterotrophic prokaryotes, flagellates, and ciliates) to the flows of carbon and energy significantly changed the concept of food chain, which until then was considered as a simplistic and linear trophic chain (from larger phytoplankton to zooplankton to fish), where energy and carbon fluxes were based on predation (Wetzel et al., 1972). This recognition was later reinforced by the development of new techniques, like epifluorescence microscopy

(Davis and Sieburth, 1982; Haas, 1982; Hobbie et al., 1977; Zimmerman and Meyer-Reil, 1974) and the application of new radioisotopic tracer techniques (Fuhrman and Azam, 1982; Hobbie et al., 1968; Kirchman et al., 1985). These new techniques made the enumeration and determination of microorganisms abundance and activity possible, showing that heterotrophic prokaryotes and heterotrophic protists were much more abundant in the ocean than previously recognized, and were also major consumers of phytoplankton biomass (Sherr and Sherr, 2008). This new picture of energy and matter flow in the water column has been summarized conceptually by the "microbial loop", proposed by Azam et al. (1983), which describes how dissolved organic carbon (DOC) produced by all trophic levels is returned to higher trophic levels via incorporation in heterotrophic prokaryote biomass. The "microbial loop" pathway starts with released DOC being incorporated by heterotrophic prokaryotes, which are then consumed by heterotrophic protists, that contribute both to the remineralization of organic matter and link microorganisms to metazooplankton, leading to the reintroduction of energy into the food-web (Azam et al., 1983; Ducklow, 1983). During the 1980s, it was also recognized that pico- and nano-sized phytoplankton represent a large proportion of phytoplankton biomass (Waterbury et al., 1979), and that most phytoplankton production is consumed by microzooplankton rather than larger metazooplankton (Landry and Hassett, 1982) (later reinforced by Calbet and Landry (2004)). Taking all of this into consideration, Sherr and Sherr (1988) proposed that the microbial loop could not be seen independently from the rest of the food web as it is a component of a much more complex microbial food web, formed by interactions that include not only protists and heterotrophic prokaryotes, but all autotrophic and heterotrophic prokaryotic and eukaryotic unicellular microorganisms from aquatic systems, creating a model in which phytoplankton is also included. The ingestion of bacteria by mixotrophic flagellates was also incorporated (Sherr and Sherr, 1988). Viruses were also considered to be extremely abundant and they were later included in the microbial food web; viruses are known to promote biogeochemical fluxes by releasing both dissolved (DOM) and also particulate organic matter (POM) from lysed host cells (Fuhrman and Suttle, 1993; Fuhrman, 1999; Proctor and Fuhrman, 1990).

The microbial food web (Figure 1.1) refers to the combined interactions among various autotrophic, heterotrophic, and mixotrophic components, which include viruses, prokaryotes, phytoplankton, and heterotrophic protists (such as flagellates and ciliates). These interactions among the various components of the microbial food web are

associated with fluxes of biomass, dissolved and particulate organic matter, and inorganic nutrients (Barbosa, 2006; Herndl and Weinbauer, 2003).

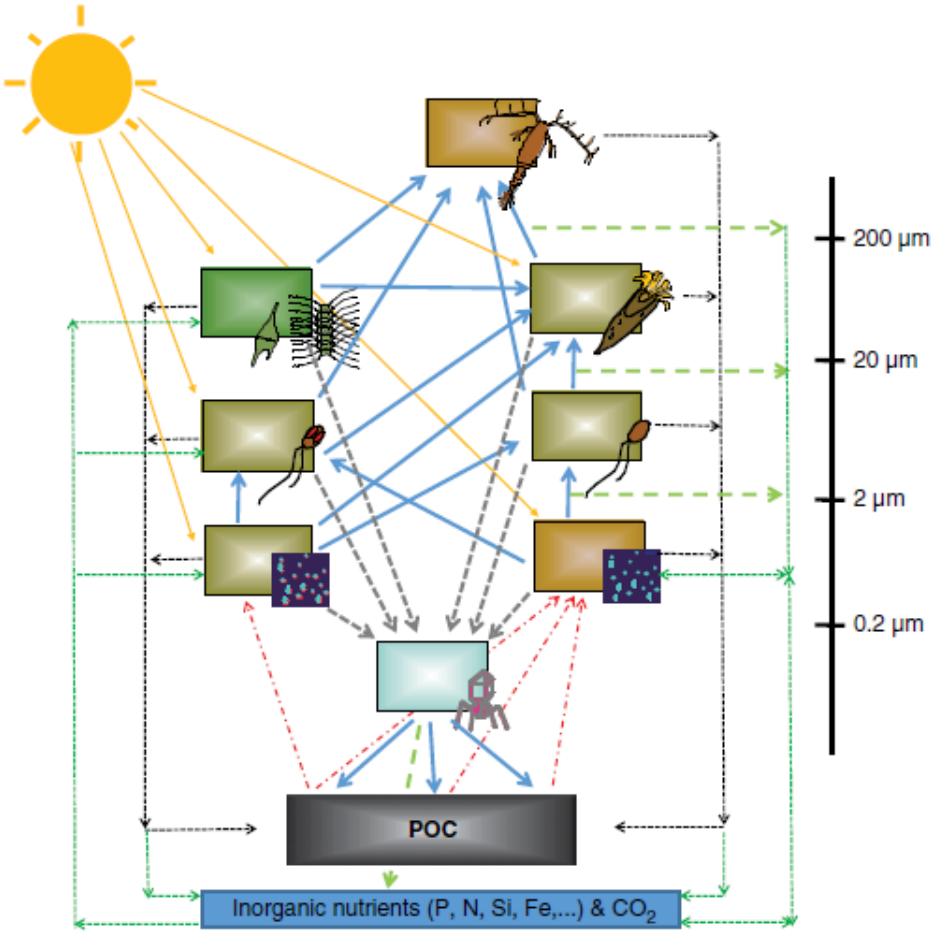

Figure 1.1: Simplified view of the microbial food web, with autotrophs in the left column and heterotrophs in the right column. Carbon fluxes are represented in blue solid lines, the use of solar light in solid yellow lines, viral lysis in grey dashed lines, flows of DOM and POM in red and black dashed lines, and the flow of inorganic nutrients in green dashed lines (in Gasol and Kirchman, 2018).

1.3. Heterotrophic prokaryotes

Heterotrophic prokaryotes represent the largest living biomass reservoir of aquatic systems (Morán et al., 2017) and are very important in processes such as nutrient uptake, carbon cycling, and remineralization, playing a crucial role in biogeochemical cycling in the global ocean (Pree et al., 2016). More specifically, heterotrophic prokaryotes are unicellular organisms characterized by having no nuclear membrane surrounding a specialized cell organelle (nucleus) housing DNA. They include two domains, Archaea and Bacteria, despite Bacteria being more abundant than Archaea in aquatic environments (Miller and Wheeler, 2012; Morán et al., 2017; Mostajir et al., 2015). These two domains

differ in many biochemical and genetic aspects, but the microscopic methods (e.g. epifluorescence microscopy) used to enumerate heterotrophic prokaryotes in aquatic systems do not allow the distinction between Bacteria and Archaea, due to their morphological similarities, and for this reason, the general term heterotrophic prokaryotes is preferred (Fuhrman and Caron, 2016; Sherr and Sherr, 2009). Heterotrophic prokaryotes derive their energy and carbon by degrading nonliving organic matter or by assimilating dissolved organic compounds, having access to the vast pool of DOM in the ocean that is mostly unavailable for larger organisms (Kaiser et al., 2011; Sherr and Sherr, 2009). In fact, the principal role of heterotrophic prokaryotes is metabolizing and transforming particulate and dissolved organic matter (DOM) in the microbial food web (Williams and Ducklow, 2019).

Half or more of the total flux of matter and energy in the marine food web is thought to pass through heterotrophic prokaryotes that are the major consumers and transformers of dissolved organic carbon (DOC), which represents the biggest reservoir of organic carbon in aquatic systems (Barbosa et al., 2001; Barbosa, 2006; Fuhrman, 1999; Herndl and Weinbauer, 2003). All organisms (heterotrophic protists, phytoplankton, and viruses) release DOM through several physiological processes, and additional DOM is also released when zooplankton fecal pellets and other forms of organic detritus dissolve and decay (Ducklow, 2001; Miller and Wheeler, 2012). In addition to using DOM, heterotrophic prokaryotes can also metabolize POM which must be first decomposed by extracellular enzymes, to small molecules that can be transported into the cell (Fuhrman and Caron, 2016; Hoppe, 1983). DOC consumed by heterotrophic prokaryotes can be either remineralized back to CO_2 (i.e., sink of organic C) or transformed into particulate organic carbon (POC), through the production of biomass and channeled to other trophic levels via ingestion by phagotrophic protists (i.e., source of organic C) (Baltar et al., 2015; Barbosa, 2006). The assimilation of organic nutrients (nitrogen and phosphate) by heterotrophic prokaryotes can also release dissolved inorganic nutrients (DIN), which are used by primary producers and can induce competition between heterotrophic prokaryotes and phytoplankton (Mostajir et al., 2015; Sherr and Sherr, 2009). The transformation of DOC into POC by heterotrophic prokaryotes represents the only way to prevent the loss of dissolved organic matter to the biota (Barbosa, 2006; Williams and Ducklow, 2019). So, the uptake of organic matter by heterotrophic prokaryotes is a major carbon-flow pathway, and its variability can change the overall patterns of carbon fluxes to higher trophic levels (Azam and Malfatti, 2007).

1.4. Phytoplankton

The term phytoplankton refers to all unicellular photosynthetic microbes that live suspended in the water column, including prokaryotes and eukaryotes (Ajani et al., 2019; Munn, 2011). Phytoplankton organisms are photoautotrophs, which means they fix inorganic carbon through the process of photosynthesis using solar radiation as an energy source; they also utilize nutrients and trace metals, and contain photosynthetic pigments such as chlorophyll *a*. The major functional groups of phytoplankton include diatoms, Cyanobacteria (blue-green algae), dinoflagellates, and other nano- and microplanktonic flagellates, such as cryptophytes (Ajani et al., 2019). Phytoplankton account for <1% of the Earth's biomass but they are the dominant primary producers in most aquatic ecosystems, contributing to nearly half of global primary production (Falkowski et al., 2004; Field et al., 1998). Furthermore, they have significant impacts on water quality and play vital roles in many ecosystem processes, such as regulating biogeochemical processes through the uptake, incorporation or transformation of numerous elements during photosynthesis and nitrogen fixation (e.g. carbon, oxygen, nitrogen), being responsible for mediating cycling, sequestration, and exportation of inorganic and organic compounds (Bidle and Falkowski, 2004; Litchman and Klausmeier, 2008).

As the main primary producer in the microbial food web, phytoplankton plays a very important role in the biological pump (Longhurst & Harrison, 1989), supports a part of ecosystem productivity by providing carbon to higher trophic levels, especially during bloom periods, and is considered the most important source of DOC in aquatic environments through exudation, losses by cell damage, or lysis (Trombetta et al., 2020), contributing with about 50% of the total organic carbon that is channeled through the microbial loop (Herndl and Weinbauer, 2003). Thus, the study of the phytoplankton community and its regulatory factors is a fundamental step in the assessment of carbon fluxes in aquatic systems, in order to increase the current predictive capacity due to changes in natural systems (Barbosa, 2006).

1.5. Microbial mortality

Since a large fraction of carbon in aquatic systems flow through microbial plankton, the knowledge of the factors controlling these microorganisms is highly relevant to the understanding of biogeochemical cycles functioning and, particularly, to the prediction of their evolution after perturbation. Two main groups of factors control microorganisms within microbial food webs. Growth of microorganisms is regulated by

"bottom-up" factors that control cell replication, such as nutrients, light, temperature, pH, salinity, and oxygen concentration. Nutrients are considered the most important factor controlling phytoplankton and often limit their growth. Biomass of microorganisms is, on the other hand, regulated by "top-down" factors, which consist in pressures leading to biomass removal like cell lyses, advection, sinking, viral lysis, and grazing (Domingues, 2010; Reynolds, 1997). It is well recognized that mortality due to viral lysis and grazing by phagotrophic protists are important processes of biomass removal of heterotrophic prokaryotes and phytoplankton, being able to dramatically affect fluxes of energy and nutrients in aquatic food webs as well as the structure of microbial communities (Barbosa and Domingues, 2008; Barbosa, 2006; Beckett and Weitz, 2018; Evans et al., 2003; Pree et al., 2016; Staniewski and Short, 2014; Suttle, 2007).

Besides contributing to primary production, many protists are mixotrophic (protists capable of both photosynthesis and phagocytosis), while other protists are strictly heterotrophic, being denominated phagotrophic protists (Kirchman, 2018; Sherr and Sherr, 2002). Phagotrophic protists are considered to be all unicellular eukaryotes that ingest (phagocytize) organic matter as part or all of their source of energy (Montagnes et al., 2008). Microzooplankton, mostly dominated by phagotrophic protists, are a group of heterotrophic and mixotrophic unicellular microorganisms ranging between 20– and 200 μm in size, that occupy a key position in marine food webs and are one of the most important groups, together with phytoplankton and heterotrophic prokaryotes, in the biogeochemical cycles of the different organic and inorganic compounds. This group includes ciliates, dinoflagellates, and foraminiferans, as well as small metazoans, such as copepod nauplii and some copepodites, and some meroplanktonic larvae. Aplastidic nanoflagellates are often included within the general category of microzooplankton, despite falling in the 2 to 20 μm size fraction (nanoplankton) that can act as a separate trophic step in the food web because they are limited to consumption of smaller microorganisms and can be preyed upon by microzooplankton (Calbet, 2008; Calbet and Alcaraz, 2009; Sherr and Sherr, 2016; Sieburth et al., 1978). Microzooplankton play an important ecological role in aquatic food webs as they are a significant source of mortality for heterotrophic prokaryotes and phytoplankton, mediate carbon transfer to higher trophic levels, remineralize nutrients and release DOM in aquatic systems (Calbet and Landry, 2004; Sherr and Sherr, 2002). Microzooplankton are the most important grazers of phytoplankton, ingesting, on average, 62.4% of phytoplankton daily production (Schmoker et al., 2013), a higher impact than that of mesozooplankton, which classically

were considered the main herbivores (Calbet, 2001; Putland and Iverson, 2007; Vargas and González, 2004). Specifically, aplastidic nanoflagellates and ciliates are the most important grazers of heterotrophic prokaryotes (Sanders et al., 1992; Sherr and Sherr, 2016, 2002; Suthers et al., 2019) being able to remove between 40 and 95% of heterotrophic prokaryotes daily production in several protected coastal systems (Barbosa, 2006). In addition, several microzooplankton organisms feed on other microzooplankton, which release some prey (Calbet et al., 2008) from their predators and therefore create trophic cascades (Calbet, 2001; Calbet and Landry, 1999).

On the other hand, viruses are the smallest and most abundant biological entities in the aquatic environment, ranging from 10^4 and 10^6 per mL, and are capable of infecting almost all organisms (Murray and Jackson, 1992; Wommack and Colwell, 2000). Temperate viruses replicate either through the lytic cycle or lysogeny. The lytic cycle involves infecting a host organism, replicating within it, and ultimately realizing viral progeny by lysing the host. Further replication requires the infection of another host (Wilcox and Fuhrman, 1994). Microbial plankton, particularly prokaryotes, are the major host of viruses since they are by far the most abundant cellular organisms (Murray and Jackson, 1992; Wommack and Colwell, 2000). As important agents of heterotrophic prokaryote biomass removal, being responsible for 10–50% of their total mortality in surface waters (Fuhrman, 1999; Suttle, 2007; Wommack and Colwell, 2000), viruses have a significant impact on the cycling of organic matter in the microbial food web (Noble et al., 1999). The large potential of viruses as agents of phytoplankton mortality is also well documented (Brussard, 2004; Fuhrman, 1999; Suttle et al., 1990). Furthermore, viral lysis of microorganisms has the strong feedback effect of preventing species dominance and enhanced species cohabitation within microbial communities, i.e., the so-called "kill the winner" hypothesis; thus, viruses also play major roles in governing microbial diversity and structuring microbial food webs (Mostajir et al., 2015).

Along with this, grazing and viral lysis are believed to have different effects on the flow of carbon and energy through the food web. Grazing leads to the transfer of carbon and nutrients to higher trophic levels (Tjidens et al., 2008), while viral lysis leads to the regeneration of nutrients and recycling of carbon, reducing the transfer of carbon and energy to higher trophic levels. This occurs because when viruses infect and lyse their hosts, there is a release of the host's biomass into the pool of dissolved organic matter (DOM) in a process termed the "viral shunt"; this DOM can be readily taken up by heterotrophic prokaryotes, stimulating their production (Fuhrman, 1999; Pasulka et al.,

2015; Steward et al., 2007; Tjidens et al., 2008). Furthermore, grazers and viruses also affect the structure of microbial communities in very different ways. Grazing modifies biomass and size structure through prey size preference, while the viral infection is thought to influence microbial community composition, due to the host-specific and density-dependent nature of viral infection (Mojica and Brussaard, 2020; Pasulka et al., 2015; Tjidens et al., 2008). So, whether cells are grazed or lysed has different ecological and biogeochemical consequences, since there are implications for the flow of matter and energy through the microbial food web (Evans et al., 2003). Therefore, it is highly relevant to quantify the rates of grazing and viral lysis on microbial plankton in order to understand the dynamics of aquatic ecosystems (Beckett and Weitz, 2018).

1.6. Estimation of microbial mortality

Several methods have been used to assess microbial mortality due to grazing (reviewed in Bamstedt et al. (2000)). Given its simplicity, the dilution method is a popular and prevailing technique used to estimate the growth and mortality of microbial populations due to grazing by microzooplankton. This technique was introduced by Landry & Hassett (1982) and consists in the manipulation of the encounter rates between prey and their grazers through a series of different dilutions, which are prepared using particle-free water from the same source. This creates a gradient of grazer abundances and thus, grazing rates along the different dilutions. The diluent is obtained by filtering water through a 0.2 µm filter and changes in the abundance of prey are closely monitored during a 24 h incubation period, allowing the prey's apparent growth rate within an incubation bottle to be calculated. Nutrients are added to the incubation bottles to avoid nutrient limitation during incubations that can result in incorrect estimations of grazing, with the precaution of leaving one set of undiluted bottles without nutrients, which serve as controls for the natural growth rates of the phytoplankton. The apparent growth rate is then plotted against the dilution factor, and the estimation of potential and *in situ* instantaneous growth rate of prey and grazing rate exerted by grazers are based on the coefficients of the fitted regression line. The slope represents the grazing rate and the growth rate of prey is estimated as the apparent growth rate extrapolated to 100% dilution (growth in the absence of grazers), which is given by the y-axis intercept (Figure 1.2). The dilution method is based on three fundamental assumptions: (1) the growth rate of the prey is not affected by dilution, which means that the growth of a given individual phytoplankton is independent of the presence of other phytoplankton individuals and

similar for dilution treatments (2) grazing mortality is linear with respect to prey concentration, meaning that the probability of a phytoplankton cell being grazed is directly related to its encounter rate with microzooplankton, which implies that microzooplankton are not food-satiated at natural prey densities and that the number of cells ingested by a given microzooplankton organism is linearly related to prey density, thus increased sample dilution will cause a proportional reduction in microzooplankton *per capita* ingestion rates and (3) prey growth is exponential, not being affected by light or nutrients (Landry and Hassett, 1982).

When the three fundamental assumptions are met, it is expected that the grazing impact decreases progressively with increasing dilution, this is, a negative relationship between the apparent growth rate of prey and the dilution factor. However, the linearity of this relationship is sometimes compromised and non-linear relationships are frequently reported, implying the violation of the assumption that grazers impact increases linearly to prey abundance, and so it is necessary to have a cautious interpretation of these deviations from linearity (Calbet et al., 2011; Calbet and Saiz, 2013; Gallegos, 1989; Moigis, 2006; Teixeira and Figueiras, 2009). According to Dix and Hanisak (2015), there are five response types of apparent growth rate versus dilution factor that can occur: insignificant, negative linear, negative saturated (L-shaped), saturated increasing (V-shaped), and positive linear, being the last three response types that often complicate the interpretation of results. In addition to these types, Stoecker et al. (2015) reported inverted V-shaped responses. Deviations from linearity imply the violation of a basic assumption of the dilution method, namely, that grazing mortality is linear concerning prey concentration, and can occur due to the complexity of plankton communities where saturated and selective feeding occurs (Calbet, 2008; Calbet et al., 2011; Calbet and Saiz, 2013; Gallegos, 1989; Teixeira and Figueiras, 2009). These deviations have been reported by some studies and several methods have been proposed to extract rate estimates when the apparent growth rate of prey is not strictly a linear function of the dilution factor.

This method was first introduced to estimate phytoplankton mortality due to microzooplankton grazing and it was later applied to estimate grazing on heterotrophic prokaryotes (Landry et al., 1984; Tremaine and Mills, 1987) and has been used since then by several authors (e.g. Anderson and Rivkin, 2001; Pearce et al., 2010; Pree et al., 2016; Zoccarato et al., 2016). Its increasing use has brought closer scrutiny but despite some criticism mostly about microzooplankton dynamics during incubation, like the outcome being modified due to trophic cascades (Calbet et al., 2011; Calbet and Saiz, 2013), the

potential of mixotrophy to induce wrong grazing estimates based on chlorophyll (Calbet et al., 2012), non-linear responses associated with saturated grazing (Evans and Paranjape, 1992; Gallegos, 1989), among others (Dolan, and McKeon, 2005; Schmoker et al., 2013), the dilution technique is one of the most informative yet least invasive and damaging techniques available to estimate rates of phytoplankton and heterotrophic prokaryotes growth and mortality due to grazing (Pearce et al., 2010). Although this method has been used for approximately 40 years for a wide variety of ecosystems, there still exists a lack of knowledge about the growth and mortality rates of the different components of microbial communities in coastal lagoons (Pecqueur et al., 2022).

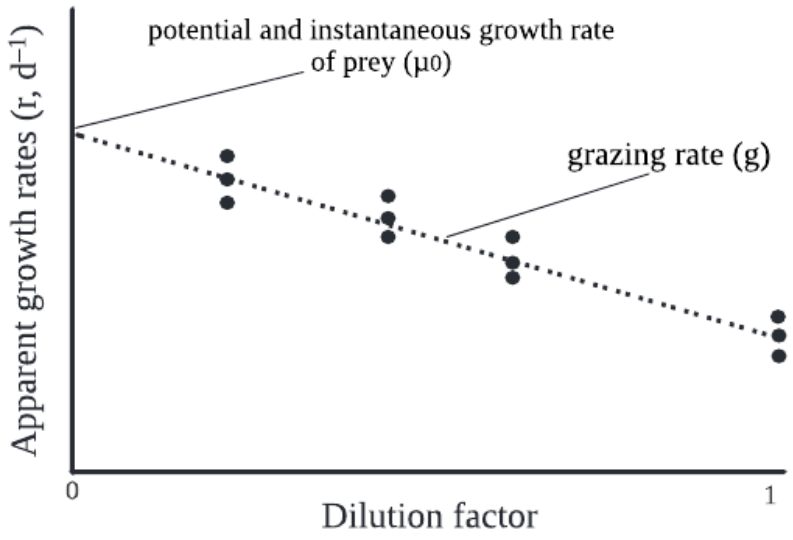

Figure 1.2: Relationship between prey apparent growth rates and dilution factor, where μ_0 is the instantaneous growth rate of prey and g is the mortality rate due to grazing.

Once viruses were recognized as ecologically important components of aquatic food webs, it also became relevant to estimate their role in microbial mortality. Several studies in coastal environments showed that levels of lytic and grazing pressure are similar (Almeida et al., 2001; Fuhrman and Noble, 1995; Steward et al., 1996), however, there was no single technique available to simultaneously estimate grazing pressure and lytic mortality until the development of the modified dilution method by Evans et al. (2003). This method is based on the original dilution method by Landry & Hassett (1982) and is used to quantify the impact of both grazing and viral lysis on microbial populations. It includes an additional dilution step obtained by combining pure seawater with grazer- and virus-free diluent in different proportions. Similar to the original dilution method,

increasing the amount of diluent in the incubation bottles decreased contact between prey and their grazers and viruses, thus reducing the impact of both agents of mortality, allowing direct measurement of grazers versus grazer and virus-induced mortality (Evans et al., 2003; Kimmance et al., 2007). The mortality of microbial plankton due to viral lysis is calculated from the subtraction of the mortality in the dilution series with grazer-free water from the mortality in the dilution series with grazer- and virus-free water. The assumptions of this method are effectively the same as the original method, although its appropriateness has not been tested (Kimmance et al., 2007). It uses two dilution series, which include several treatments with different proportions of pure seawater and diluent. The change in prey abundance is measured after approximately 24h of incubation, allowing the apparent growth rate within an incubation bottle to be calculated. This apparent growth rate is then plotted against the dilution factor, to obtain the dilution curves of both the original dilution method and the modified dilution method. The slope of the dilution curve of the original dilution method represents the grazing rate, while the slope of the modified dilution method represents the sum of both grazing and viral lysis mortality rates and, therefore, the difference between the two slopes represents the mortality rate due to viral lysis (Figure 1.3) (Beckett and Weitz, 2018; Kimmance and Brussaard, 2010; Staniewski and Short, 2014).

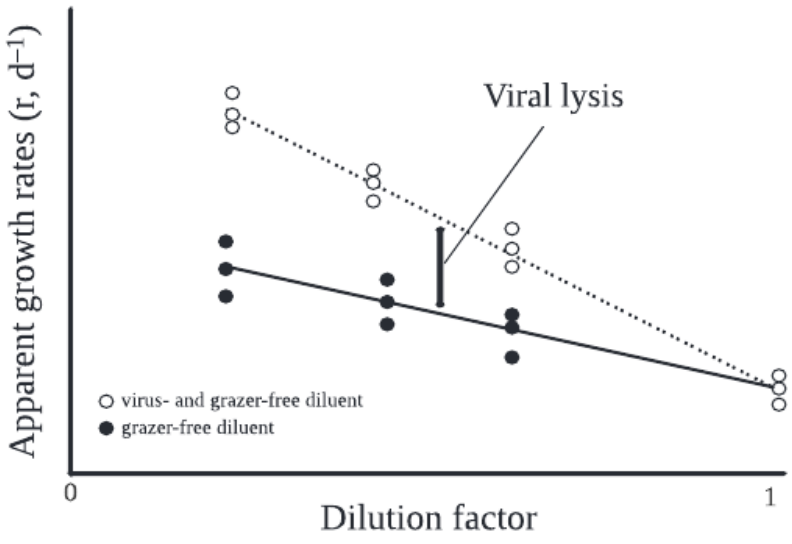

Figure 1.3: Linear regression line of prey apparent growth rates against dilution factor, in which (●) is the dilution curve of the grazer-free diluent (original dilution method) and (○) is the dilution curve of the virus- and -grazer-free diluent (modified dilution method).

Although this method has some limitations associated and some biases may arise from the interpretation of the results, this is currently the only method that can derive viral lysis rates from prey mortality directly and it is also the only single method that can, in theory, provide simultaneous estimates of grazing and virus-induced mortality, making it a promising and appealing approach to researchers (Kimmance et al., 2007; Kimmance and Brussaard, 2010; Tjidens et al., 2008). Furthermore, it requires minimal handling or disruption of the organisms being investigated and can provide a direct measurement of virus-induced mortality rates without the use of many inferred assumptions or conversion factors (Kimmance and Brussaard, 2010; Staniewski and Short, 2014). The modified dilution technique has been applied by several authors since its development in 2003, including in estimations of heterotrophic prokaryotes induced mortality by grazing and viral lysis (Cram et al., 2016; Hu et al., 2020; Jacquet et al., 2005; Taira et al., 2009; Tsai et al., 2013).

1.7. Dynamics of microbial plankton in the Ria Formosa coastal lagoon

The Ria Formosa is a very important ecosystem located on the south coast of Portugal and is one of the most important confined marine ecosystems in Portugal, from biological and social–economical perspectives (Barbosa, 2010; Newton et al., 2020). It is responsible for about 90% of the annual production of bivalves in Portugal (Chícharo and Chícharo, 2001) which is ensured by the fundamental role of microbial plankton in this ecosystem, since it is capable of using and significantly altering DOC and POC, and sustains the larval and adult stages of this particular production (Barbosa, 2006). Despite the relevance of microbial plankton in this ecosystem functions, published data about microbial plankton in the Ria Formosa coastal lagoon is unexpectedly limited, particularly for heterotrophic microorganisms (Barbosa, 2010, 2006). The importance of studying the biomass removal processes of these microorganisms has also been overlooked in this ecosystem, with only a few studies addressing microbial mortality due to grazing in Ria Formosa coastal lagoon (Barbosa, 2006; Domingues et al., 2021; Sá, 2017; Thiele-Gliesche, 1992). A recent study (Domingues et al., 2021) estimated growth and mortality rates due to microzooplankton grazing in Ria Formosa coastal lagoon using the dilution method, but the rates were estimated only for winter and the study focused more on the effects of warming on phytoplankton growth and mortality. Furthermore, no information on the contribution of viruses to prokaryote mortality is available for the lagoon. As this study aims to bridge these gaps, it shall expand what is known so far about

marine microbiology in Ria Formosa coastal lagoon, contributing to a better understanding of trophic dynamics within the microbial food web of one of the most important ecosystems in Portugal and thus, to its conservation. Furthermore, investigating the diversity and dynamics of microbial communities is of particular importance to understanding how climate changes may impact plankton communities and energy flow in food webs.

2. Objectives

Considering the fundamental ecological roles of phytoplankton and heterotrophic prokaryotes, these two groups of microbial plankton were specifically addressed in this study. The main goal of this study was to evaluate the growth and mortality of heterotrophic prokaryotes and phytoplankton in the Ria Formosa coastal lagoon, in order to discuss the quantitative trophic role of microzooplankton and viruses in the microbial food web of this important ecosystem. Specific objectives were a) to evaluate growth and mortality due to microzooplankton grazing of heterotrophic prokaryotes, total phytoplankton community, and specific phytoplankton groups; and b) to evaluate mortality of heterotrophic prokaryotes due to viral lysis. Based on previous studies conducted in Ria Formosa or the same type of ecosystem, it was hypothesized that 1) the mean growth rates of heterotrophic prokaryotes and total phytoplankton community are higher than their mean grazing rates (Barbosa, 2006); 2) diatoms and eukaryotic picophytoplankton display the highest growth rates, and eukaryotic picophytoplankton and plastidic nanoflagellates, particularly cryptophytes, display the higher grazing rates (Barbosa, 2006); 3) Mortality of heterotrophic prokaryotes due to viral lysis is higher than mortality due to microzooplankton grazing (Taira et al., 2009).

3. Methods

3.1. Study site

The Ria Formosa (Figure 3.1) is a coastal lagoon system located on the south coast of Portugal, separated from the Atlantic Ocean by five barrier islands and two peninsulas. It extends ~55 km (E–W) and ~6 km (N–S) at its widest point and has a total wet area of ca. 110 km^2 (Barbosa, 2010, 2006; Newton and Mudge, 2003). The lagoon is shallow, with an average depth of 2m (Andrade et al., 2004), and it is connected with the ocean through seven inlets that allow the exchange of water. The coefficient of renovation is very high and between 50 to 75% of the water in the lagoon is exchanged by tides, that are semidiurnal and mesotidal, with tidal amplitudes ranging between 1.35 m during neap tides and 3 m during spring tides. Five small rivers and fourteen streams flow into the Ria Formosa but most of these dry out completely in summer. Since the hydrodynamic circulation is dominated by the inflow and outflow of the coastal water mass throughout the tidal cycle, and because the flow of freshwater is relatively small, the salinity of the Ria Formosa water is close to the salinity of seawater (Barbosa, 2006; Newton and Mudge, 2003). Most of the rainfall occurs in the winter, between November and February, and the climate is Mediterranean, with mild wet winters and hot dry summers, with a mean air temperature in the summer of 25 ºC and 12 ºC in the winter. The water column is well mixed, with no persistent or widespread haline or thermal stratification, due to the reduced depth of the system, the absence of important sources of fresh water, and the impact of tidal currents (Newton and Mudge, 2003). The adjacent coastal region is inserted in the Gulf of Cadiz and is impacted by regular upwelling events (Barbosa, 2010; Loureiro et al., 2006), most frequent from April to October, that are associated with local westerly winds (Relvas et al., 2007; Relvas and Barton, 2002) and that may extend approx. 6 km upstream from the lagoon inlets (Cravo et al., 2014), being its natural biogeochemical cycles mainly regulated by the adjacent coastal zone (Barbosa, 2010; Cravo et al., 2019). The Ria Formosa constitutes an ecosystem of high biodiversity, serving as a breeding and development site for a large number of marine species. In addition to its ecological importance, it is also a valuable national resource for tourism, fisheries, aquaculture, and salt extraction industries (Barbosa, 2010; Duarte et al., 2007; Newton and Mudge, 2003).

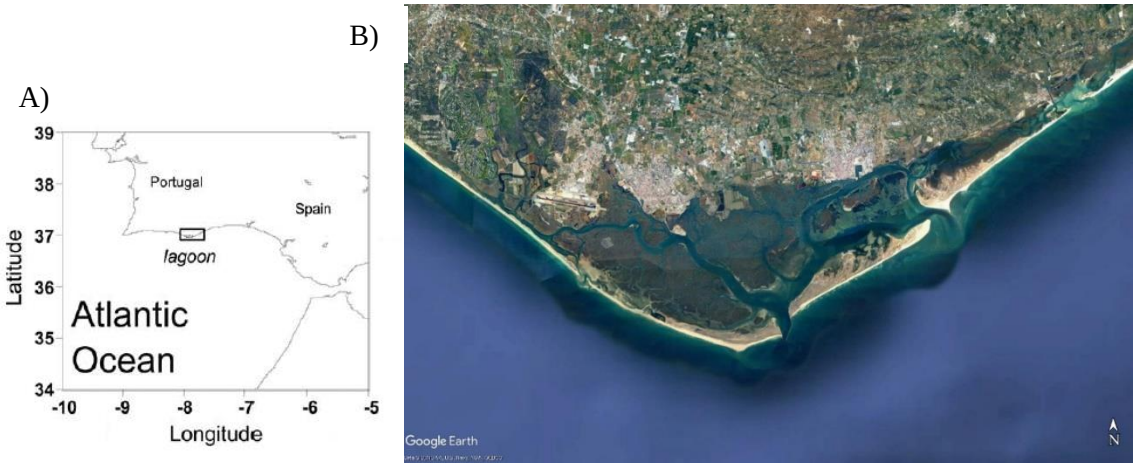

Figure 3.1: Location and satellite map of Ria Formosa coastal lagoon (A adapted from Newton and Mudge, 2003 and B from Google Earth, 2022)

3.2. Experimental approach

To estimate both heterotrophic prokaryotes and phytoplankton group-specific growth rates and mortality due to grazing by microzooplankton, mostly dominated by phagotrophic protists in the Ria Formosa coastal lagoon (Barbosa, 2006; Thiele-Gliesche, 1992), three experiments using the dilutions technique were carried out in June (early-spring), August (summer), and October (late summer-autumn) 2021, in order to evaluate seasonal variability during the productive period. To estimate the contribution of viruses to heterotrophic prokaryote mortality, two modified dilution technique experiments were performed in October (late summer-autumn) 2021 and in March (early-spring) 2022. All items used in the experiments were prepared the day before the experiment started, and they were previously acid-cleaned with HCl 10% and rigorously washed with deionized water between each use.

3.3. Sampling

For all experiments, water samples were collected at the surface (± 1m) from an established sampling location in the Ria Formosa coastal lagoon (boat pier at Praia de Faro, 37.004557 °N -7.987706 °W) using 5 L water jugs. For each dilution experiment, 65 L of water were collected at high tide. The modified dilution technique experiments required two sampling days due to time constraints, in which 10 L of surface seawater were collected on the first sampling day at low tide and 40 L were collected on the second sampling day at high tide. In each sampling expedition, water temperature and salinity were also measured using a YSI multiparametric probe, as well as the Secchi depth. All

samples were transported protected from light and arrived at the laboratory within 30 minutes of sampling.

3.4. Dilution technique experiments

Under low light conditions and using appropriate graduated measuring cylinders, five different dilutions were prepared in 10 L Thermo Scientific Nalgene bottles using grazer-free diluent, created by gravity filtration of collected seawater through a Pall cartridge filter (<0.1 µm) into a clean carboy utilizing a vacuum pump. The following dilutions were prepared, combining the respective diluent with full seawater: 0.125+, 0.25+, 0.50+, 0.70+, 1+ and 1-, considering that the value represents the relative volume of unfiltered seawater. To avoid damaging initial phytoplankton composition, water samples were not pre-filtered to remove larger grazers since this procedure can retain large-sized phytoplankton cells, like diatoms, and thus, increase the problems associated with the extrapolation of experimental outcomes to the natural environment (Nogueira et al., 2014). All dilutions, except 1-, were enriched with inorganic macronutrients at saturating concentrations (+40 µM of nitrate as potassium nitrate (KNO_3), +10 µM of ammonium as ammonium chloride (NH_4Cl), +4 µM of phosphorus as potassium dihydrogen phosphate (KH_2PO_4), and +50 µM of silicon as sodium hexafluorosilicate (Na_2SiF_6)) to promote constant phytoplankton growth. Nutrient enrichment intends to avoid potential nutrient limitation and thus keep growth rates similar among dilution treatments, assuring that only grazing mortality was manipulated without strongly impacting community composition. Nutrients were not added to dilution 1- in order to account for the potential effects of nutrient addition on the estimation of in situ phytoplankton instantaneous growth rates. After homogenization, water from each experimental treatment was transferred into triplicate 2 L polycarbonate Nalgene bottles. The bottles were incubated in the laboratory inside a tank filled with tap water (to maintain a constant, ambient temperature) for 24 h, under an ambient light: dark cycle set according to seasonal conditions, and exposed to photosynthetically active radiation (PAR) intensity of 90 – 120 µmol photons m^{-2} s^{-1} (Figure 3.2). Bottles were manually homogenized to avoid the settlement of non-motile cells.

Water samples for quantification of heterotrophic prokaryotes, and pico- (< 2 µm) and nanophytoplankton (2-20 µm) were collected at the beginning and end of incubation, fixed with glutaraldehyde 25% (final concentration 0.2%), and kept in a refrigerator (4ºC) until slide preparation for epifluorescence microscopy. The slide preparation was done

24h after the fixation of the sample. Water samples were also taken for identification and quantification of microphytoplankton (20-200 µm); these samples were preserved with acid Lugol´s solution (final concentration approx. 0.003%) and kept in the dark before observation by inverted microscopy. Finally, water samples were also taken before incubation to determine the initial chlorophyll *a* concentration.

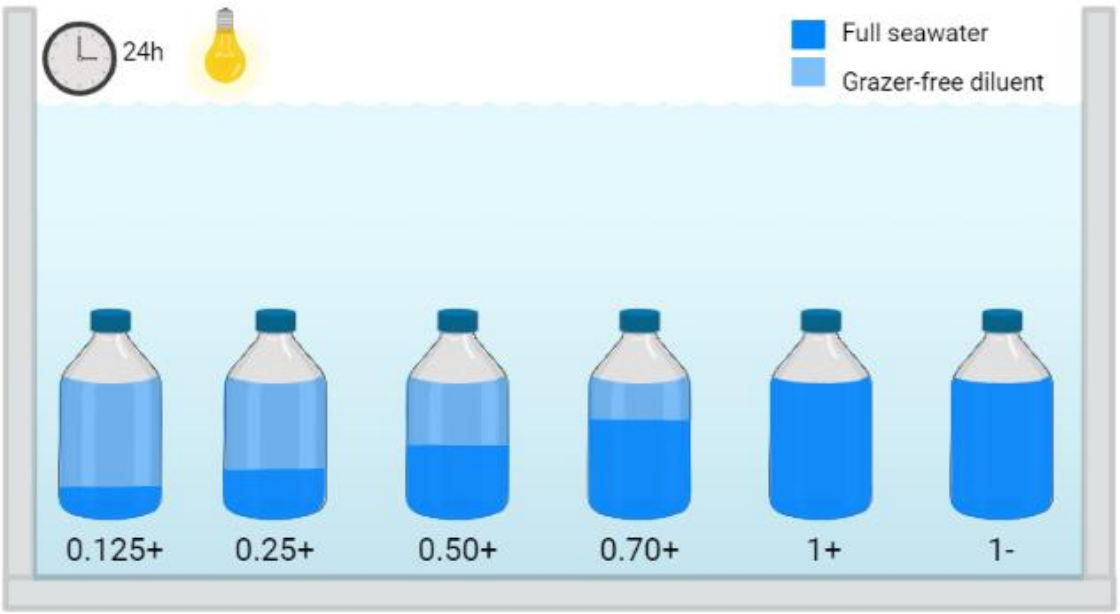

Figure 3.2: Schematic representation of experimental treatments with different proportions of diluent (light blue) and unfiltered seawater (dark blue). Note that designations include the dilution factor. Nutrient amended sample dilutions are marked with a (+) sign; (-) denotes no nutrient amendment.

3.5. Modified dilution technique experiments

In the modified dilution technique, two diluents were created using different procedures. On both sampling days, when the samples arrived at the laboratory, the collected seawater was filtered through a Pall cartridge filter (<0.1µm) to obtain a grazer-free diluent. However, on the first day, half of the grazer-free diluent was additionally transferred to several Pyrex flasks and autoclaved at 121 ºC and 1 bar for 15 min to inactivate viruses and thus obtain the grazer and virus-free diluent. The Pyrex flasks were left to cool overnight.

Two series of dilutions were prepared, one using the grazer-free diluent, and the other using the grazer and virus-free diluent (Figure 3.3). For each series, the following dilutions were prepared, combining the respective diluent with full seawater: 0.25, 0.50, 0.75, and 1 on the first experiment, and 0.125, 0.25, 0.50, 0.75 and 1 on the second

experiment, where the value represents the percentage of seawater. The dilutions were prepared in 10 L polycarbonate Nalgene bottles. After homogenization, water from each dilution was transferred in triplicate to 2 L polycarbonate Nalgene bottles, which were incubated for 24 hours in a tank filled with tap water (to maintain a constant temperature). Water samples to evaluate the abundance of heterotrophic prokaryotes were collected at the beginning and end of incubation, fixed with glutaraldehyde 25% (final concentration 0.2%), and kept in the fridge (4 ºC) until slide preparation. Slides were prepared within 24h of sample collection in order to minimize cell loss (Turley, 1993). Additionally, total counts of nanoplankton were also made using the same slide preparations as for heterotrophic prokaryotes in both experiments.

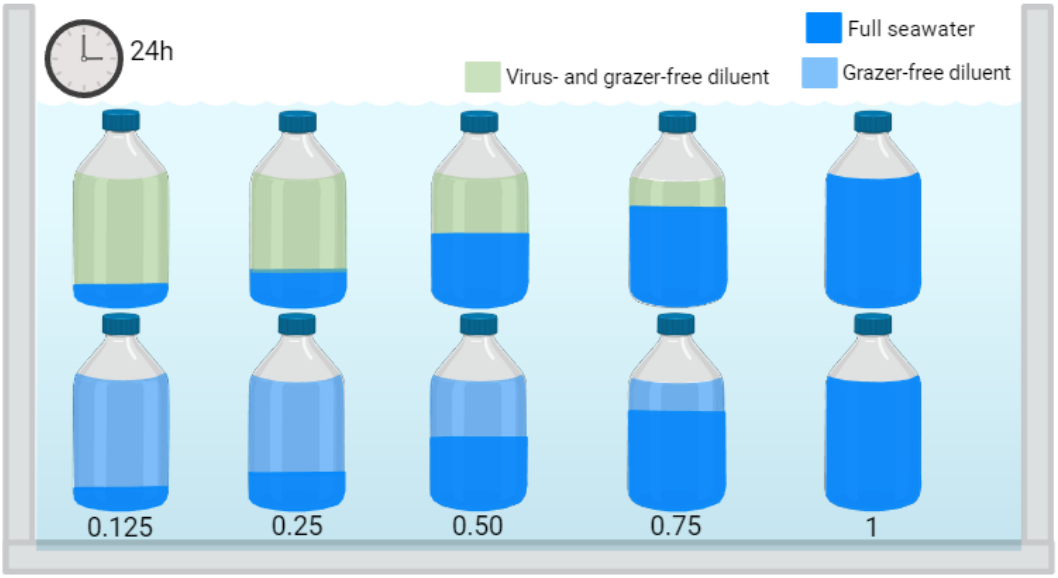

Figure 3.3:Schematic representation of experimental treatments with different proportions of grazer-free diluent (light blue), virus- and grazer-free diluent (light green) and unfiltered seawater (dark blue).

3.6.Determination of chlorophyll *a* concentration

Chlorophyll *a* (Chl*a*) concentration was used as a proxy of phytoplankton biomass in experimental treatments with unfiltered seawater (1- and 1+) at the beginning of incubation. Its determination was made according to the spectrophotometric method of Parsons et al. (1984), in which 1 L of sample was filtered through GF/F glass fiber filters (0.7 µm). The filtration was made in low light conditions and the pressure used was less than 100 mm Hg in order to minimize damage or loss of cells. After filtration, the filters were placed in test tubes protected from light and frozen at -20 ºC until further procedure. Chl*a* was extracted from the filters overnight at 4 ºC with 90% acetone; after extraction,

the filters were centrifuged (2000 rpm, 10 min). Absorbances of the supernatant were then measured spectrophotometrically (Hitachi U-2000) at 750 and 665 nm, before and after acidification with HCl 1 M to correct for phaeopigments. Chl*a* values were calculated based on equation 1, where *v* represents the volume of acetone added (mL), *V* the volume of filtered sample (L), and *l* the thickness of the spectrophotometer cell (cm).

$$Chla = 26.7 \times (A665b - A665a) \times v \times (V \times l)^{-1} \quad (1)$$

Initial Chl*a* concentrations were later used to estimate the initial biomass of phytoplankton (Bi) (µg C/L), according to equation 2 (Sathyendranath et al., 2009).

$$\log(Bi) = 1.81 + 0.63 \times \log(Chla) \quad (2)$$

3.7. Nutrients analysis

Dissolved inorganic macronutrients (nitrate NO_3^-, nitrite NO_2^-, ammonium NH_4^+, silicate SiO_4^{4-}, and phosphate PO_4^{3-}) concentrations were determined according to the spectrophotometric methods described by Hansen and Koroleff (1999), in which water samples were first filtered through cellulose acetate filter with a 0.45 µm nominal pore diameter and stored in scintillation vials that were kept in the fridge (4 ºC) until analysis. The concentration of nutrients present in the water samples was determined through the colorimetric methods, in which, basically, the concentration of each type of nutrient is calculated through the formation of a colored solution and its molecular absorption in spectrophotometry, using a Hitachi U-2000 spectrophotometer.

3.8. Quantification of heterotrophic prokaryotes

The abundance of heterotrophic prokaryotes at the beginning and the end of incubation was estimated using epifluorescence microscopy (Daley & Hobbie, 1975). In this method, an adequate volume (1-5 mL) of each fixed sample was filtered onto a black polycarbonate membrane with 0.2 µm nominal pore diameter (Nucleopore), placed with the shiny side upwards on a cellulose acetate filter with a 0.45 µm nominal pore diameter (membrane diameter 25 mm) that served as support filter to ensure homogeneous distribution of cells. The filtration pressure used was less than 100 mm Hg in order to minimize damage or loss of cells. The membrane filter was stained with acridine orange

solution and, after three minutes, the stain was removed by filtration. After filtration, the membrane filter was placed on a slide with a drop of non-fluorescent immersion oil Cargille type A. The slides were stored in the freezer until observation at the microscope. All slides were examined within 1 month of sample collection.

Slide observation was made, using a Leica DM LB epifluorescence microscope, equipped with blue and green light, at 1250× magnification and under dark conditions. Counts of heterotrophic prokaryotes were made under blue light and, because Cyanobacteria and heterotrophic prokaryotes can be confused since both are prokaryotes, the distinction between autotrophic prokaryotes (Cyanobacteria) and heterotrophic prokaryotes was made using green light. The cells were counted using a graticule (New Porton G12) (May, 1965) mounted on the eyepiece (previously calibrated with microscope calibration slide); at least 20 random visual fields and 300 cells in total were counted. For each experiment, the abundance of heterotrophic prokaryotes was estimated at the beginning and end of incubation using equation 3, where *x* represents the total cell count, *A* the area filtered (mm^2), *d* the dilution factor due to the addition of fixatives, *a* the area of the visual field (mm^2), *n* the number of visual fields counted, and *v* the volume filtered (L).

$$Abundance\ (cell\ L^{-1}) = \frac{x \times A \times d}{a \times n \times v} \quad (3)$$

During observation, one randomly selected cell per field, in the experimental treatments with unfiltered seawater (1- and 1+), was also measured using the New-Porton calibrated graticule to calculate the cellular volume, mean carbon content, and biomass of the heterotrophic prokaryotes. The initial biomass (BBi) (µg C/L) of heterotrophic prokaryotes was then estimated, based on the abundance of heterotrophic prokaryotes at the beginning of the incubation and the mean carbon content per cell (MCC) (fg C/cell), according to equation 4. The mean carbon content per cell (MCC) represents the mean of the estimated cellular carbon content (CC) (fg C/cell) values for each of the cells measured per field. These cellular carbon contents were obtained using equation 5 (Simon and Azam, 1989), in which VC corresponds to cellular volume (μm^3/ cell) calculated by applying equation 6 (Fuhrman, 1981). In this last equation, L (µm) corresponds to the major axis dimension and W the minor axis dimension, measured using the circles of the graticule.

$$BBi\ (\mu g\ C\ L^{-1}) = Abundance \times MCC \quad (4)$$

$$CC = 92.92 \times VC^{0.59} \quad (5)$$

$$VC = \frac{\pi}{4} \times\ W^2 \times (L - \frac{W}{3}) \quad (6)$$

3.9. Quantification of phytoplankton

The choice of the technique associated with the dilution method to determine changes in prey populations must be adapted to the aim of the study. The most widely used to estimate phytoplankton growth rates is the determination of changes in chlorophyll a, because of its greater simplicity and promptness to obtain the results. However, it only provides microzooplankton impact on phytoplankton without further details on the taxonomic composition of the prey consumed. To determine changes in other components of plankton communities due to microzooplankton feeding impact, cell counts by microscopy were used. The abundance of pico- (< 2 µm) and nanophytoplankton (2-20 µm) was estimated through epifluorescence microscopy, whilst the abundance of microzooplankton (20-200 µm) was estimated through inverted microscopy, following the methods of Haas (1982) and Utermöhl (1958), respectively. According to Domingues et al. (2008), the quantitative analysis of the whole phytoplankton community's abundance, biomass, and composition should be undertaken using both techniques, in order to account for all phytoplankton different sizes. Barbosa (2006) also highlighted the need to use epifluorescence microscopy, in conjunction with inversion microscopy, to quantitatively analyze the entire phytoplankton community in Ria Formosa coastal lagoon.

In the epifluorescence microscopy, the procedure was similar to the one mentioned above, but here an adequate volume (1-5 mL) of each fixed sample was first stained with proflavine (20 µL per 1 mL of sample) for three minutes and then vacuum-filtered (pressure <100 mm Hg) onto a black polycarbonate membrane with 0.4 µm nominal pore diameter (Nucleopore). After filtration, the membrane filter was placed on a slide with a drop of non-fluorescent immersion oil Cargille type A, and the slides were immediately observed at the microscope. Slide observation was made under dark conditions (to minimize loss of fluorescence) and using an epifluorescence microscope

(Zeiss Axio Imager A1), equipped with blue and green light, at 1000× magnification. Counts of specific phytoplankton groups were also made under blue light and using a graticule (New Porton G12) mounted on the eyepiece. Phytoplankton was identified into the following groups: Cyanobacteria, eukaryotic picophytoplankton, cryptophytes, and other plastidic nanoflagellates.

In the inverted microscopy method, or Utermöhl method, the samples previously preserved with Lugol's solution were added to sedimentation columns of 5-50 mL (depending on the dilution), where phytoplankton cells were allowed to settle in sedimentation chambers by gravity over a 24 h period. The sedimentation columns were sealed with a cover glass and sedimentation chambers were protected from light during settling. After settling, the sedimentation chambers were observed under an inversion microscope (Zeiss Axio Observer A1) at 400x magnification. During observation, cells were identified as diatoms, dinoflagellates, and euglenophytes (flagellates). Since most dinoflagellates are considered mixotrophic (Jeong et al., 2010; Stoecker, 1999), all dinoflagellates, except *Protoperidinium* were considered phytoplankton, although they also are potential grazers.

In both methods, at least 50 random visual fields, 400 cells in total, and 100 cells of the most abundant species were counted. Assuming that the cells were randomly distributed, the counting precision was ±10 % (Venrick, 1978). For each experiment, the abundance of the different taxonomic groups was estimated at the beginning and end of incubation using equation 3, where x represents the total cell count for that group, A the area filtered (mm^2), d the dilution factor due to the addition of fixatives, a the area of the visual field (mm^2), n the number of visual fields counted, and v the volume filtered (L). The abundances of the whole phytoplanktonic community were obtained through the sum of each of the specific groups of phytoplankton.

3.10. Quantification of microzooplankton

Microzooplankton, dominated by phagotrophic protists in the Ria Formosa coastal lagoon (Barbosa, 2006; Thiele-Gliesche, 1992), were also quantified to evaluate their relationship with the estimated potential instantaneous growth rates (μ_0) and grazing rates (g) of heterotrophic prokaryotes and phytoplankton. Growth or mortality of grazers may occur during incubation and can severely alter grazing rate estimates, thus should be accounted for to obtain a proper evaluation of microzooplankton grazing rates (Dolan et al., 2000). Cells were identified as aplastidic nanoflagellates in epifluorescence

microscopy and ciliates in inverted microscopy. Nanoflagellates were identified as >1 µm cells with a bright green color; in dilution experiments, only aplastidic nanoflagellates were identified by the absence of autofluorescence of chlorophyll a. Since a significant part of marine nanoflagellates are mixotrophic (Caron, 2000; Rychert, 2006), by extension also those found in Ria Formosa, no distinction was made between plastidic and aplastidic nanoflagellates in modified dilution experiments.

3.11. Data analysis

Assuming exponential growth, the apparent prey growth rates (r, d^{-1}) (= net growth rates) were calculated for each experimental treatment as the change in abundance during the incubation period. The calculation was made according to equation 7, where N_t and N_{t0} are the final and initial abundances, and t is the duration of the incubation.

$$r = \frac{\ln\left(\frac{Nt}{Nt0}\right)}{t} \tag{7}$$

Then, linear regression analysis was used to analyze the relationship between apparent growth rate and each of the dilution experimental series. The apparent growth rate in nutrient-enriched experimental treatments was plotted against the dilution factor, and the potential instantaneous growth rates of prey (μ_0) and grazing rates (g) were obtained as the y-intercept (growth in 100% dilution, i.e., in absence of grazers) and the slope (when negative, absolute value is considered; for other situations, see below) of the linear regression line, respectively (see Figure 1.2) (Landry and Hassett, 1982). Analysis of variance (ANOVA) was used to test the significance of the slopes (Dytham, 2011). In the case of statistically non-significant slopes, this is, slopes not significantly different from zero, the grazing rate was assumed zero and the instantaneous growth rate of prey was estimated as the average value of the apparent growth rate of all nutrient-enriched experimental treatments (as per Calbet et al., 2011; Chen et al., 2009; Domingues et al., 2021; Jiang et al., 2021; Murrell et al., 2002; Twiss and Smith, 2012). When inverted V-shaped responses were observed, it was used several intermediate dilution levels to determine if there was a range of dilution where the response was linear and negative, and it was used the slope in this region to calculate the grazing coefficient (Stoecker et al., 2015). In the case of significant non-negative slopes (e.g. positive slopes), the mortality rates were considered to be undeterminable (since negative grazing rates are

theoretically impossible) (Calbet et al., 2011; Twiss and Smith, 2012; York et al., 2011). In the case of V-shaped or L-shaped responses, phytoplankton potential instantaneous growth rate (μ_0) was estimated as the regression intercept of the linear (non-saturation range) portion of the dilution plot (generally dilution factor $\leq$ 0.7), and the microzooplankton grazing rate (g) was estimated according to equation 8 (Domingues et al., 2021).

$$g = \mu 0 - r_{DIL1+} \quad (8)$$

Because the apparent growth rates were derived from nutrient-enriched experimental treatments, the potential instantaneous growth rate of prey (μ_0) is an estimation of prey growth when nutrients are not limiting and can be overestimated. To obtain a growth rate representative of in situ conditions and to control for possible nutrient stimulation of growth in the experiments, the in situ instantaneous growth rate of prey (μ_{is}) (growth in the absence of grazers and with no nutrient enrichment) was estimated as the difference between potential instantaneous growth rates of prey (μ_0) and variation of apparent prey growth rates between undiluted samples with nutrients (1+) and without nutrients (1-) (Δr), expressed in equation 9 (Calbet et al., 2011). Standard errors (SE) for μ_{is} were estimated using the corresponding error propagation equations (http://julianibus.de/index.html).

$$\mu is = \mu 0 - \Delta r \quad \text{or} \quad \mu is = r_{DIL1-} + g \quad (9)$$

Net production of heterotrophic prokaryotes (NBP) (µg C L^{-1} d^{-1}) and particulate net phytoplankton production (pNPP) (µg C L^{-1} d^{-1}) were obtained by applying equations 10 and 11, respectively, which used the mean heterotrophic prokaryotes initial biomass in experimental treatments with unfiltered seawater (BBi) (µg C L^{-1}), and the mean phytoplankton initial biomass in experimental treatments with unfiltered seawater (Bi) (µg C/L); t corresponds the duration of the incubation.

$$NBP = BBi \times \left(e^{\mu is \times t} - 1\right) \quad (10)$$

$$pNPP = Bi \times \left(e^{\mu is \times t} - 1\right) \quad (11)$$

Finally, the grazing impact (I) on both phytoplankton and heterotrophic prokaryotes was estimated as the percentage of the daily production of prey removed by grazers, by combining grazing rates (g) and the in situ instantaneous growth rate of prey (μ_{is}), according to equation 12. The g:μ ratio is defined by Calbet and Landry (2004) as a reasonable proxy for the percentage of prey production consumed by microzooplankton.

$$I = \frac{g}{\mu is} \times 100 \quad (12)$$

For the modified dilution experiment, the abundance and apparent growth rates (r, d^{-1}) of heterotrophic prokaryotes were calculated using equations 2 and 3, respectively. The apparent growth rate in each dilution was then plotted against the dilution factor, thus obtaining the dilution curves for both the original dilution method and the modified dilution method. The viral lysis mortality rate (v) was obtained by calculating the difference in the slopes of the regression lines between the two dilution series (see Figure 1.3).

3.12. Statistical analysis

Dilution experiments were also used to assess potential nutrient limitation, by comparing phytoplankton apparent growth rates between nutrient-enriched and nutrient-unenriched undiluted treatments. To test nutrient limitation in dilution experiments, the significance of the difference between the apparent growth rates in undiluted treatments with and without nutrients was determined using a one-tailed t-test. If there are no significant differences between the undiluted treatments, there was no nutrient limitation at the time of sampling; significant differences between these treatments indicate potential nutrient limitation. For the modified dilution experiments, the significance of the difference between the slopes of the original and modified dilution method regression lines was determined using a one-tailed t-test (Fowler and Cohen, 1990). If the slopes are significantly different, the magnitude of viral mortality was assessed. A comparison between the regression lines obtained in each experiment was made using analysis of covariance (ANCOVA) to check for statistical differences in potential instantaneous growth rates and grazing rates among experiments, groups of phytoplankton, and, between rates of heterotrophic prokaryotes and total phytoplankton community in each experiment. The ANCOVAs were followed by one-tailed t-tests and confidence interval comparison to identify which groups were significantly different from the others

(Dytham, 2011). A significance level of 0.05 is set for all data analyses. All statistical analyses were performed using MS Excel 2022 (version 2206) and IBM SPSS_ Statistics v.28 software.

4. Results

4.1. Dilution technique experiments – grazing by microzooplankton

4.1.1. Characterization of initial environmental conditions and microbial assemblages

Three experiments using the dilution technique were carried out in order to estimate both growth and mortality rates due to grazing by microzooplankton of heterotrophic prokaryotes, total phytoplankton community, and specific phytoplankton groups in the Ria Formosa coastal lagoon over a seasonal cycle. The experiments were conducted under typical late spring (June), summer (August), and late summer-early autumn (October) conditions. The characterization of the abiotic environment at the sampling site in the Ria Formosa coastal lagoon, which includes values of salinity, temperature, and Secchi depth, at the time of sampling for the different experiments, is represented in Table 4.1. Water temperature presented lower values in June (mean = 17.7 ºC) and higher values in August (mean = 20.6 ºC), while salinity presented lower values in June (mean = 35.4) and higher values in October (mean = 36.5). The Secchi depth varied between 2.1 m in August and 3.3 m in October. The vertical profiles of salinity and temperature obtained during the experiments indicated that no significant vertical stratification was detected, suggesting a well-mixed or vertical homogeneous water column in Ria Formosa coastal lagoon during the respective sampling periods.

Table 4.1: Abiotic variables in the sampling site at the time of sampling

	Depth (m)	Temperature (ºC)	Salinity	Secchi depth (m)
June	0	17.8	35.3	2.8
	1	17.7	35.4	
	2	17.6	35.5	
August	0	20.7	35.7	2.1
	1	20.6	35.9	
	2	20.5	36	
October	0	19.8	36.5	3.3
	1	19.9	36.6	
	2	19.9	36.5	

The concentrations of dissolved inorganic macronutrients (nitrate NO_3^-, nitrite NO_2^-, ammonium NH_4^+, silicate SiO_4^{4-}, and phosphate PO_4^{3-}) at the beginning of the

experiments are represented in Table 4.2. Higher concentrations of silicate and phosphate were observed in October, while higher concentrations of nitrate and ammonium were observed in June and August, respectively. The concentration of nitrite did not vary between August and October.

Table 4.2: Concentrations of dissolved inorganic macronutrients at the beginning of the experiments (dl – detection limit)

	NO_3^- (µM)	NO_2^- (µM)	NH_4^+ (µM)	SiO_4^{4-} (µM)	PO_4^{3-} (µM)
June	1.30	<0.09	<dl	1.72	0.58
August	0.51	0.34	1.76	2.88	0.59
October	0.94	0.34	0.10	5.54	0.73

The initial abundance and biomass of planktonic heterotrophic prokaryotes at the sampling location varied between experiments, with abundance and biomass values of 1.03 x 10^9 cell/L and 24.27 µg C/L in June, 4.27 x 10^9 cell/L and 64.25 µg C/L in August, and 2.57 x 10^9 cell/L and 35.26 µg C/L in October. Initial chlorophyll-a concentration (Chl*a*) revealed mean values of 0.46 µg L^{-1} in June, 1.59 µg L^{-1} in August, and 0.93 µg L^{-1} in October. For the same period, the initial abundance and biomass of the phytoplankton assemblage was 9.29 x 10^6 cell/L and 39.68 µg C L^{-1} in June, 5.45 x 10^6 cell/L and 68.27 µg C L^{-1} in August, and 3.82 x 10^6 cell/L and 37.17 µg C L^{-1} in October. In terms of phytoplankton community structure, Cyanobacteria were the dominant group in terms of abundance with a relative contribution of 87% (8.08 x 10^6 cell/L), 63% (3.44 x 10^6 cell/L), and 66% (2.53 x 10^6 cell/L) in June, August and October, respectively. Eukaryotic picophytoplankton were the second most abundant group of phytoplankton, with abundances ranging between 6.91 x 10^5 in June and 1.38 x 10^6 in August (cell/L), contributing between 7% in June and 25% in August to the total phytoplankton abundance. Other phytoplankton groups were present but showed relatively lower contributions to the community abundance, namely plastidic nanoflagellates (4-5%), cryptophytes (1-4%), diatoms (0-4%), dinoflagellates (<1%), and euglenophytes (<1%). The initial abundance of phagotrophic protists, potential grazers of phytoplankton and heterotrophic prokaryotes, was higher during August and lower during June, with values of 8.22 x 10^4 cell/L in June, 4.10 x 10^5 cell/L in August and 1.16 x 10^5 cell/L in October,

with the aplastidic nanoflagellates being the major contributors to the measured abundance of phagotrophic protists at the beginning of all experiments. The abundance of ciliates ranged from 8.25 x 10^2 to 1.12 x 10^3 cell/L and those of aplastidic nanoflagellates ranged from 8.10 x 10^4 to 4.10 x 10^5 cell/L.

4.1.2. Growth and grazing rates of heterotrophic prokaryotes

The results of the linear regression analysis used to analyze the relationship between heterotrophic prokaryotes apparent growth rate and each of the dilution experimental series in the three dilution experiments are represented in Figure 4.1.

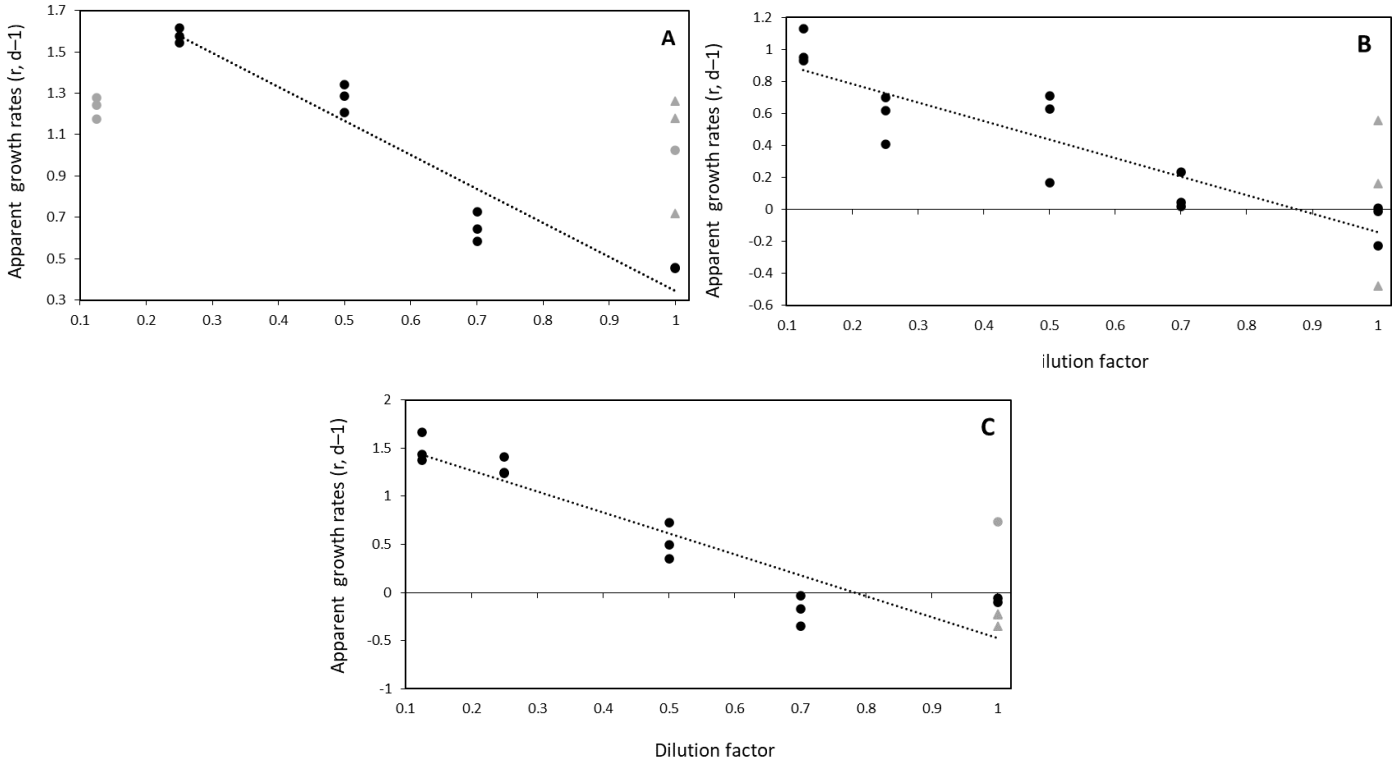

Figure 4.1: Relationship between dilution factor and apparent growth rate (r, d^{-1}) of heterotrophic prokaryotes in June (A), August (B), and October (C), in experimental treatments enriched with nutrients (circles) and in non-enriched treatments (triangles). Data points represented in grey were not used for linear regression.

The linear regressions obtained show a negative relationship between the apparent growth rate of heterotrophic prokaryotes in nutrient-enriched experimental treatments and the dilution factor in all the dilution experiments, as expected. Mean values of potential instantaneous growth rates (μ_0), grazing rates (g), in situ instantaneous growth rate (μ_{is}), net bacterial production (NBP), and the grazing impact (I) on heterotrophic prokaryotes in nutrient-enriched experimental treatments, and respective standard errors (± SE) are

presented in Table 4.3. Relevant statistical information of the linear regression analysis, namely coefficient of determination (R^2), number of values (n), and p-value of the regression slope (p-value) are also provided.

Table 4.3: Summary of results (± SE) of potential instantaneous growth rates (μ_0, d^{-1}), grazing rates (g, d^{-1}), in situ instantaneous growth rate (μ_{is}, d^{-1}), net bacterial production (NBP, µg C L^{-1} d^{-1}) and grazing impact (I, %) on heterotrophic prokaryotes in nutrient-enriched experimental treatments, and relevant statistical information of the linear regressions. (a) outlier(s) removed for compliance with dilution assumptions.

	μ_0	g	μ_{is}	NBP	I	R^2	p-value	n
June	1.99 ± 0.11	1.64 ± 0.17	2.59 ± 0.20	291.95	63.59	0.92	<0.001	11 (a)
August	1.02 ± 0.10	1.16 ± 0.16	1.17 ± 0.33	152.76	98.58	0.81	<0.001	15
October	1.70 ± 0.15	2.17 ± 0.26	1.52 ± 0.15	132.18	143.28	0.86	<0.001	14 (a)

Significant grazing rates of heterotrophic prokaryotes (i.e., significant negative slopes in the dilution experiments) were observed in all experiments. In June, a grazing rate of 1.64 ± 0.17 d^{-1} and a potential instantaneous growth rate of 1.99 ± 0.11 d^{-1} were determined from the linear regression equation. For the August and October experiments, the grazing rates corresponded to 1.16 ± 0.16 d^{-1} and 2.17 ± 0.26 d^{-1}, and the potential instantaneous growth rates of heterotrophic prokaryotes corresponded to 1.02 ± 0.10 d^{-1} and 1.70 ± 0.15 d^{-1}, respectively. Heterotrophic prokaryotes grazing rates presented a mean value of 1.66 ± 0.29 d^{-1}, slightly higher than the mean potential instantaneous growth rate (1.57 ± 0.29 d^{-1}). Statistical analysis showed significant differences between grazing rates ($p < 0.001$) and potential instantaneous growth rates ($p < 0.001$) across experiments. The potential instantaneous growth rate of heterotrophic prokaryotes was higher during June, and the grazing rate was higher during October. Furthermore, results also showed a higher in situ instantaneous growth rate of heterotrophic prokaryotes during June and a higher impact of grazing, this is, a higher percentage of daily heterotrophic prokaryotes production grazed by phagotrophic protists, during October. In situ instantaneous growth rate of heterotrophic prokaryotes varied between 2.59 ± 0.20 d^{-1} in June, 1.17 ± 0.33 d^{-1} in August, and 1.52 ± 0.15 d^{-1} in October. Heterotrophic prokaryotes daily production, varied between 132.18 and 291.95 µg C L^{-1} d^{-1}, showing a higher value

in June and a lower value in October. Grazing by phagotrophic protists removed 63.59% of heterotrophic prokaryotes daily production in June, 98.58% in August, and 143.28% in October. So, the grazing impact was higher during October and lower in June.

4.1.3. Growth and grazing rates of phytoplankton

The results of the linear regression analysis for the whole assemblage or specific phytoplankton functional groups in the dilution experiments conducted under typical late spring (June), summer (August), and late summer-autumn (October) conditions are represented in Figures 4.2, 4.3, and 4.4, respectively. Relationships between apparent phytoplankton growth rates and dilution factor in enriched dilutions were not always negative and linear as expected, with some non-linear or positive responses observed. Of the twenty-four plots obtained for phytoplankton, V-shaped or L-shaped saturated response curves were observed in only one plot (Figure 4.4.h), and inverted V-shaped responses were observed in five plots (Figure 4.2.c, 4.2.d, 4.3.d, 4.4.c, and 4.4.d), occurring more frequently for plastidic nanoflagellates and eukaryotic picophytoplankton. Positive slopes were only observed for dinoflagellates during June and August experiments, ergo, no grazing rates were determined in these two specific cases.

Mean values of potential instantaneous growth rates (μ_0), grazing rates (g), in situ instantaneous growth rate (μ_{is}), particulate net phytoplankton production (pNPP), and the grazing impact (I) on the entire phytoplankton community and specific phytoplankton functional groups in nutrient-enriched experimental treatments, and respective standard errors (± SE) are presented in Table 4.4 and 4.5, respectively. Relevant statistical information of the linear regression analysis, namely coefficient of determination (R^2), number of values (n), and p-value of the regression slope (p-value) are also provided.

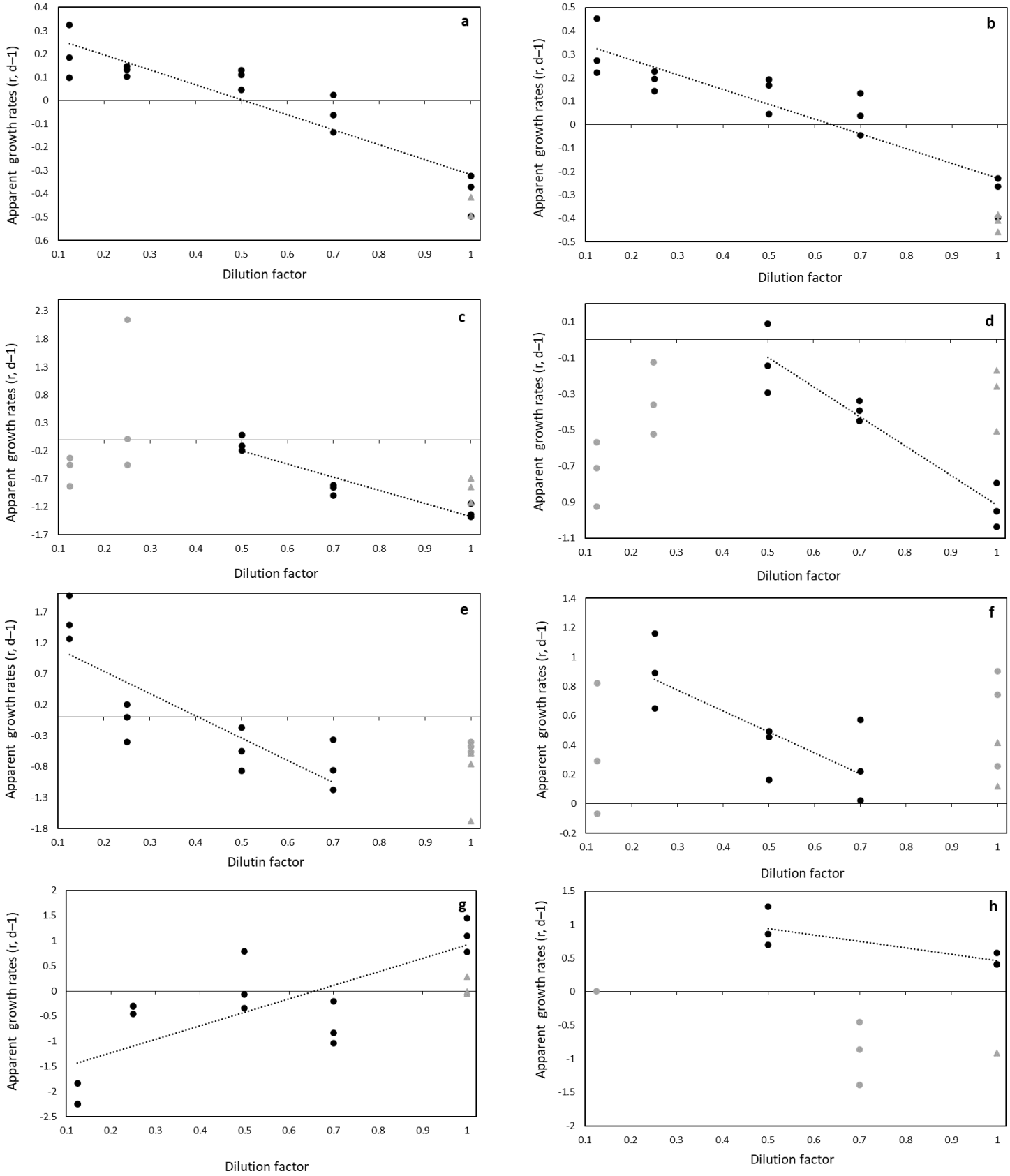

Figure 4.2: Relationship between dilution factor and apparent growth rate (r, d^{-1}) of (a) total phytoplankton community, (b) Cyanobacteria, (c) eukaryotic picophytoplankton, (d) plastidic nanoflagellates, (e) cryptophytes, (f) diatoms, (g) dinoflagellates, and (h) euglenophytes in June, in experimental treatments enriched with nutrients (circles) and in non-enriched treatments (triangles). Data points represented in grey were not used for linear regression.

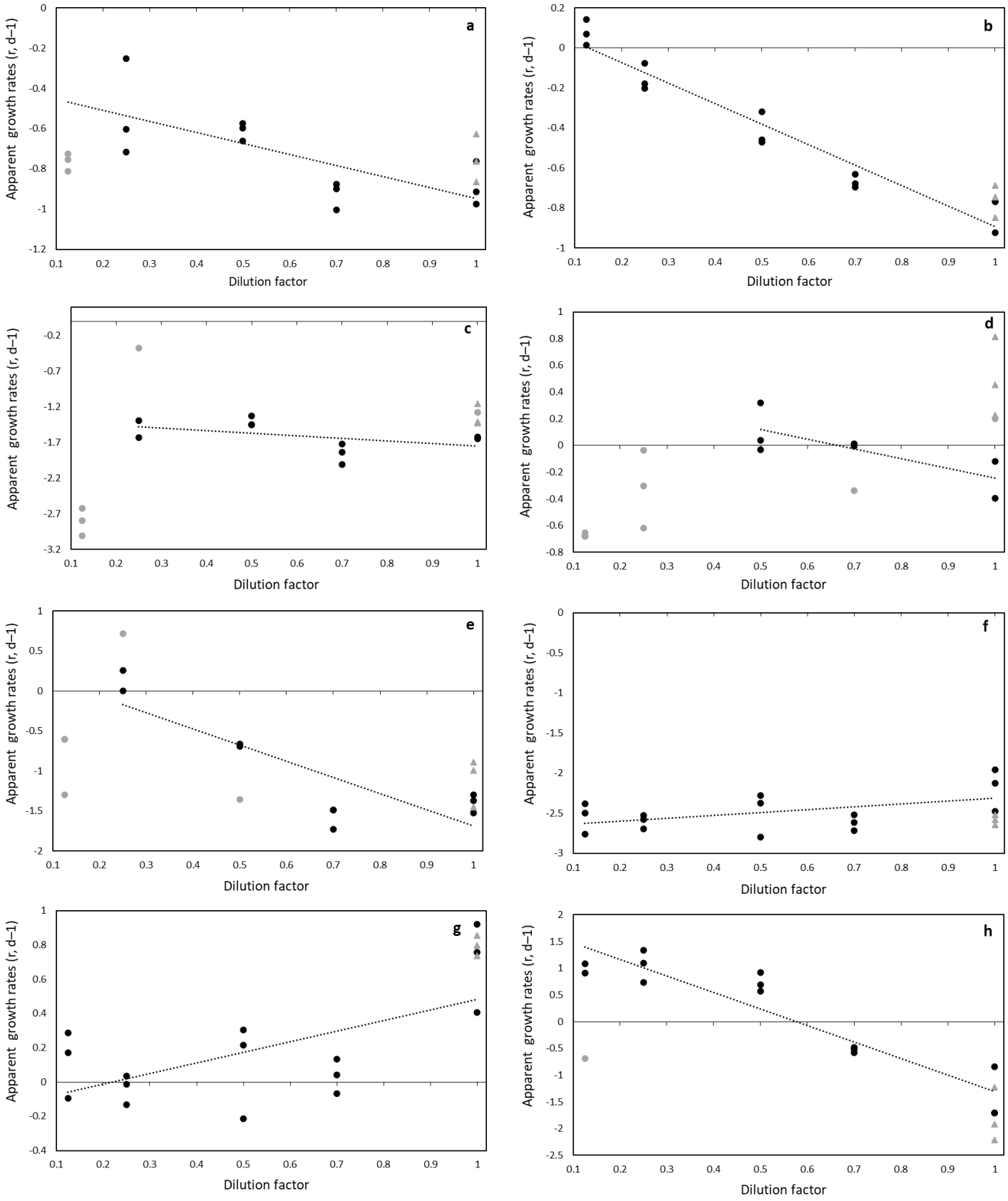

Figure 4.3: Relationship between dilution factor and apparent growth rate (r, d^{-1}) of (a) total phytoplankton community, (b) Cyanobacteria, (c) eukaryotic picophytoplankton, (d) plastidic nanoflagellates, (e) cryptophytes, (f) diatoms, (g) dinoflagellates, and (h) euglenophytes in August, in experimental treatments enriched with nutrients (circles) and in non-enriched treatments (triangles). Data points represented in grey were not used for linear regression.

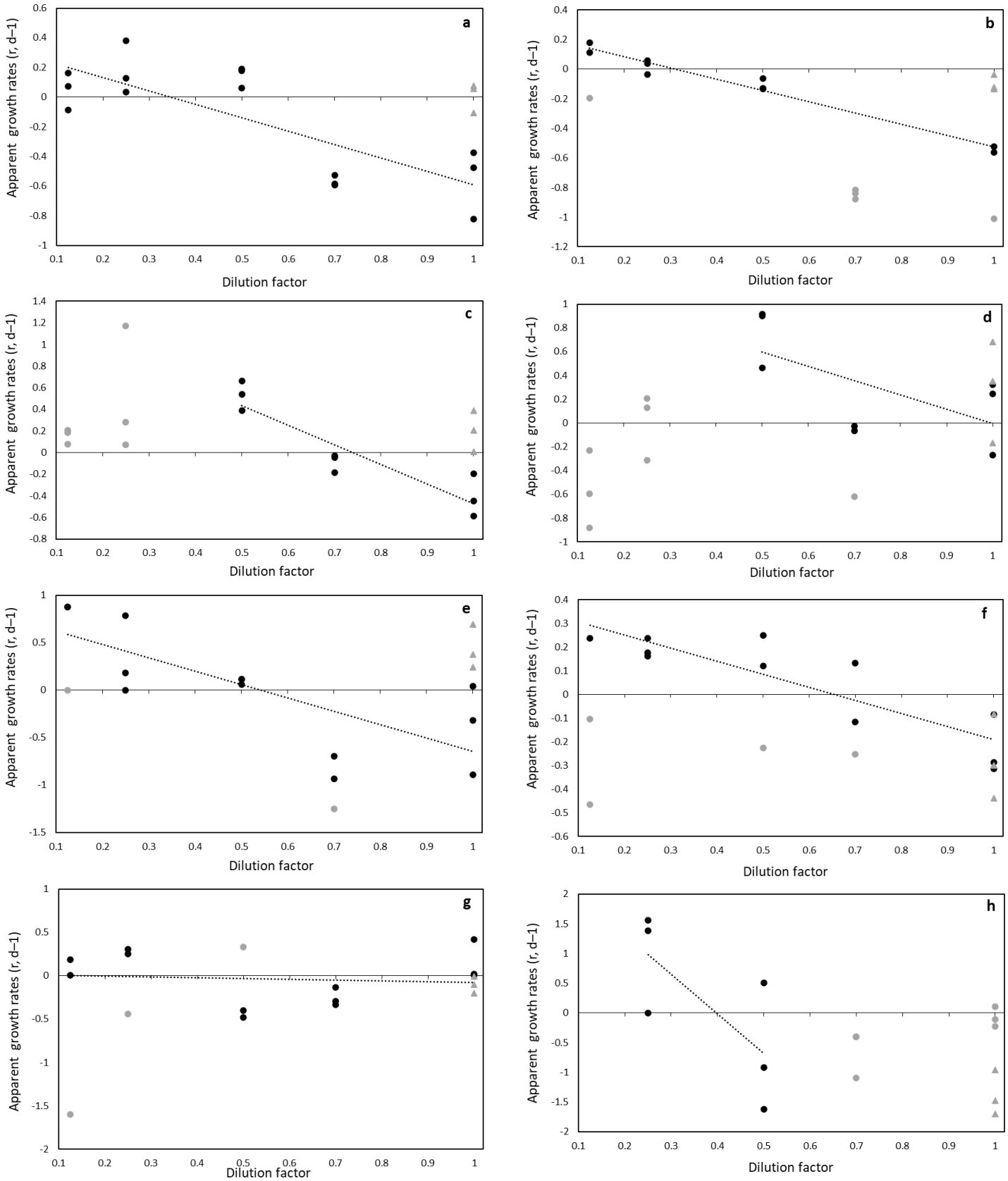

Figure 4.4: Relationship between dilution factor and apparent growth rate (r, d^{-1}) of (a) total phytoplankton community, (b) Cyanobacteria, (c) eukaryotic picophytoplankton, (d) plastidic nanoflagellates, (e) cryptophytes, (f) diatoms, (g) dinoflagellates, and (h) euglenophytes in October, in experimental treatments enriched with nutrients (circles) and in non-enriched treatments (triangles). Data points represented in grey were not used for linear regression.

Table 4.4: Summary of results (± SE) of potential instantaneous growth rates (μ_0, d^{-1}), grazing rates (g, d^{-1}), in situ instantaneous growth rate (μ_{is}, d^{-1}), particulate net phytoplankton production (pNPP, µg C L^{-1} d^{-1}), and grazing impact (I, %) for the entire phytoplankton community in nutrient-enriched experimental treatments. The regression coefficient of determination (R^2), number of values (n), and significance level of the regression slope (p-value) are also provided. (a) Outlier(s) removed for compliance with dilution assumptions. Negative potential instantaneous growth rates (μ_0), and resultant negative values, were assigned with a value of zero.

	μ_0	**g**	**μ_{is}**	**pNPP**	**I**	**R^2**	**p-value**	**n**
June	0.33 ± 0.05	0.65 ± 0.08	0.28 ± 0.08	12.89	229.28	0.83	<0.001	15
August	- 0.40 ± 0.11	0.55 ± 0.16	-0.27 ± 0.15	-20.29	-204.86	0.53	0.007	12 (a)
October	0.31 ± 0.12	0.90 ± 0.20	0.88 ± 0.21	87.28	102.43	0.62	<0.001	15

A range in whole phytoplankton assemblage grazing and potential instantaneous growth rates over the experiments of June (g= 0.65 ± 0.08 d^{-1}; μ_0= 0.33 ± 0.05 d^{-1}), August (g= 0.55 ± 0.16 d^{-1}; μ_0= - 0.40 ± 0.110 d^{-1}) and October (g= 0.90 ± 0.20 d^{-1}; μ_0= 0.31 ± 0.12 d^{-1}) was observed. The grazing rate of the phytoplankton community presented an average value of 0.699 ± 0.106 d^{-1}, considerably higher than the average potential instantaneous growth rate (0.080 ± 0.240 d^{-1}). Significant grazing rates were observed in all experiments. The highest grazing rate was recorded in October while the lowest was in August. No significant differences in grazing rates were observed between experiments ($p = 0.267$). The potential instantaneous growth rate of the phytoplankton assemblage was higher in June and lower in August. There were significant differences in the potential instantaneous growth rates of phytoplankton between experiments ($p < 0.001$), which were more apparent between June and August, and between August and October. No significant differences were observed between June and October. In situ instantaneous growth rates of total phytoplankton community were also estimated, with values ranging from 0.28 d^{-1} in June, -0.267 d^{-1} in August, and 0.881 d^{-1} in October. Particulate net phytoplankton production results showed great variability in total phytoplankton community daily production between experiments, with values of 12.89 µg C L^{-1} d^{-1} in June, -20.29 µg C L^{-1} d^{-1} in August and 87.28 µg C L^{-1} d^{-1} in October. Grazing impact was higher during June, in which 229.28% of total phytoplankton community daily production was removed per day. During August and October, grazing impact was -204.86% and 102.43%, respectively.

Table 4.5: Summary of results (± SE) of potential instantaneous growth rates (μ_0, d^{-1}), grazing rates (g, d^{-1}), in situ instantaneous growth rate (μ_{is}, d^{-1}), and grazing impact (I, %) for the different specific groups of phytoplankton in nutrient-enriched experimental treatments. The regression coefficient of determination (R^2), number of values (n), and significance level of the regression slope (p-value) are also provided. (a) Outlier(s) removed for compliance with dilution assumptions; (b) V-shaped or L-shaped saturated response curves; (c) inverted V-shaped responses curves; (**) grazing rate values marked by asterisks are not significantly different from zero (i.e. statistically non-significant slopes) and are assumed as zero; (nd) grazing rate not determined because of positive slope.

		μ_0	g	μ_{is}	I	R^2	p-value	n
Cyanobacteria	**June**	0.40 ± 0.05	0.63 ± 0.08	0.28 ± 0.08	224.31	0.82	<0.001	15
	August	0.13 ± 0.05	1.02 ± 0.08	0.19 ± 0.09	538.60	0.93	<0.001	15
	October	0.24 ± 0.03	0.76 ± 0.05	0.68 ± 0.05	111.57	0.97	<0.001	10 (a)
Eukaryotic picophytoplankton	**June**	0.96 ± 0.26	2.33 ± 0.34	1.37 ± 0.30	170.91	0.87	<0.001	9 (c)
	August	1.74 ± 0.17	0.36 ± 0.25**	-1.44 ± 0.19	-	0.20	0.197	10 (a)
	October	1.34 ± 0.23	1.82 ± 0.30	2.06 ± 0.29	88.21	0.84	<0.001	9 (c)
Plastidic nanoflagellates	**June**	0.72 ± 0.16	1.63 ± 0.21	1.33 ± 0.21	122.52	0.90	<0.001	9 (c)
	August	0.48 ± 0.20	0.73 ± 0.27	1.24 ± 0.29	58.79	0.59	0.043	7 (c)
	October	1.21 ± 0.58	1.21 ± 0.45**	1.39 ± 0.56	-	0.42	0.084	8 (c)
Cryptophytes	**June**	1.47 ± 0.35	1.95 ± 0.35	0.95 ± 0.49	206.06	0.69	<0.001	12 (b)
	August	0.34 ± 0.33	2.03 ± 0.46	0.63 ± 0.39	321.61	0.71	0.002	10 (a)
	October	0.77 ± 0.23	1.41 ± 0.37	1.59 ± 0.42	88.66	0.57	<0.001	13 (a)
Diatoms	**June**	1.20 ± 0.23	0.57 ± 0.30	0.89 ± 0.34	64.12	0.60	0.014	9 (b)
	August	-2.49 ± 0.06	-0.36 ± 0.18**	-2.88 ± 0.20	-	0.24	0.061	15
	October	0.36 ± 0.07	0.55 ± 0.10	0.32 ± 0.15	174.25	0.76	<0.001	11 (a)
Dinoflagellates	**June**	-1.77 ± 0.38	nd	-2.80 ± 0.46	-	0.59	0.014	15
	August	-0.14 ± 0.13	nd	-0.03 ± 0.23	-	0.40	0.012	15
	October	-0.15 ± 0.13	0.09 ± 0.28**	-0.39 ± 0.21	-	0.01	0.755	12 (a)
Euglenophytes	**June**	0.15 ± 0.26	0.96 ± 0.36**	-1.23 ± 0.27	-	0.64	0.056	6 (a)
	August	1.78 ± 0.22	3.08 ± 0.35	1.42 ± 0.51	217.75	0.87	<0.001	14 (a)
	October	-0.36 ± 0.99	6.62 ± 3.18**	1.34 ± 1.28	-	0.52	0.106	6 (b)

Regarding specific phytoplankton groups, values estimated varied according to the phytoplankton group considered and to the different experiments. In general, mean values of potential instantaneous growth rates, grazing rates, in situ instantaneous growth rate, and grazing impact on daily phytoplankton production ranged between -0.49–1.52 d^{-1}, 0.56–3.08 d^{-1}, -1.08–1.06 d^{-1}, and 90.65–291.50%, respectively. The results indicated significant differences in both grazing rates and the potential instantaneous growth rates in all experiments between the different specific groups of phytoplankton. Some of the

specific phytoplankton groups (eukaryotic picophytoplankton, plastidic nanoflagellates, diatoms, dinoflagellates, and euglenophytes) showed grazing rates not significantly different from zero in part of the experiments. Eukaryotic picophytoplankton and cryptophytes showed higher average grazing rates, whereas eukaryotic picophytoplankton showed higher average potential instantaneous growth rates. From the three regression lines obtained for dinoflagellates, two had a positive slope and one showed a non-significant slope, therefore, no grazing rates were obtained for dinoflagellates in this study. Regarding the different seasons, cryptophytes had the highest potential instantaneous growth rate during June, while euglenophytes had the highest values during August and eukaryotic picophytoplankton during October. Cryptophytes showed the highest grazing rate during June, and euglenophytes and eukaryotic picophytoplankton had the highest grazing rate during August and October, respectively. Grazing rates of the different groups of phytoplankton were generally always higher, when estimated, than their potential instantaneous growth rates (Figure 4.5). The mean grazing impact of Cyanobacteria and cryptophytes was higher, with average values of 291.50% and 250.44%, respectively. The highest grazing impact value was observed for Cyanobacteria in August.

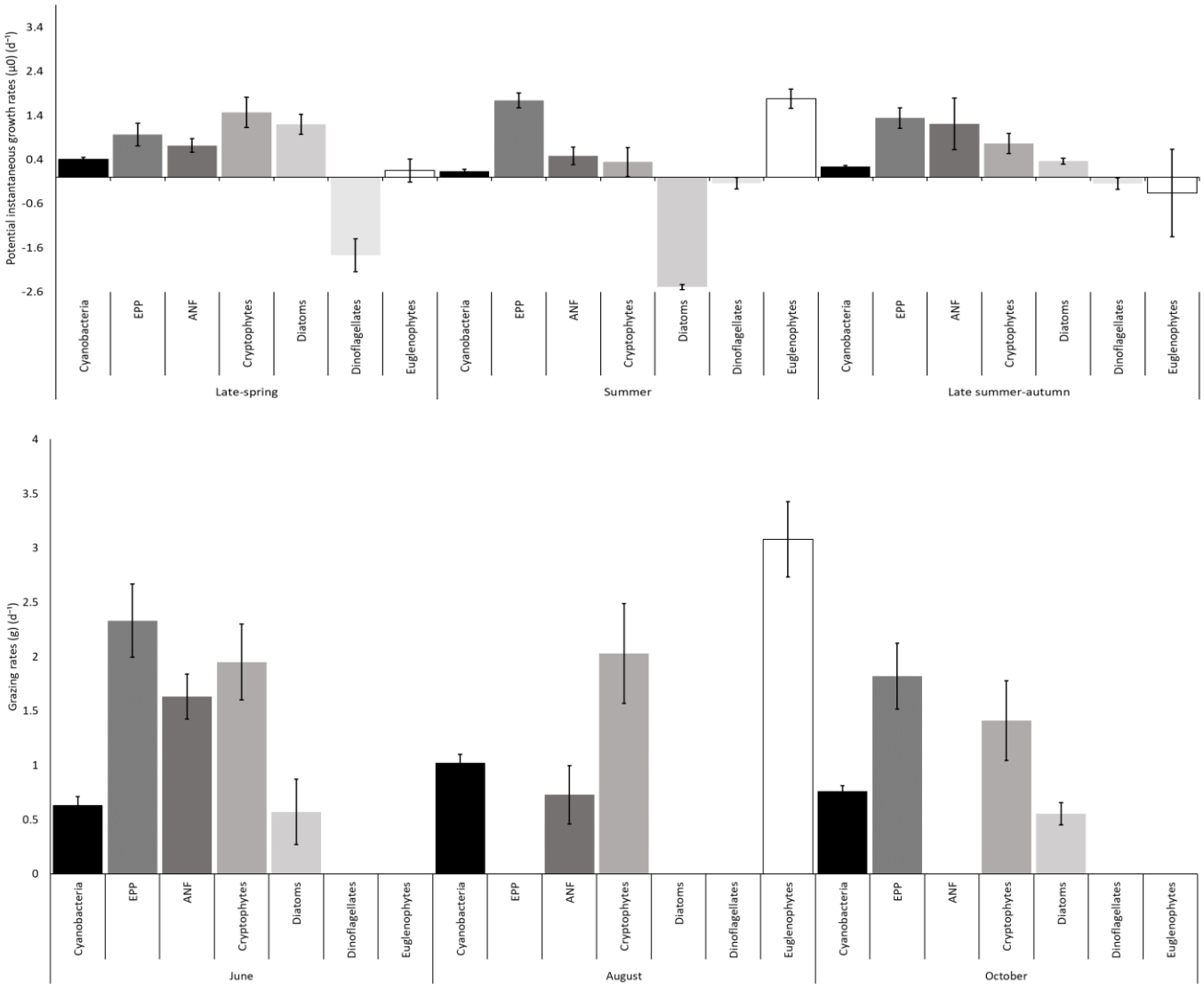

Figure 4.5: Variation of potential instantaneous growth rates (μ_0, d^{-1}) and grazing rates (g, d^{-1}) for the different phytoplankton groups in June, August, and October. EPP- Eukaryotic picophytoplankton and ANF- Plastidic nanoflagellates. Vertical lines represent ± 1

4.1.4. Comparison of growth and grazing rates of heterotrophic prokaryotes and phytoplankton

Grazing and potential instantaneous growth rates were significantly different between heterotrophic prokaryotes and total phytoplankton community in all experiments. Growth and grazing rates obtained for heterotrophic prokaryotes were significantly higher than those of total phytoplankton community during each experiment considered (Figure 4.6). Despite this clear difference between rates, the variability between experiments was the same for both microbial components, with higher growth rates in June and lower in August, and higher grazing rates in October and lower in

August. Grazing rates of the total phytoplankton community were generally higher than their potential instantaneous growth rates, whereas growth and grazing rates of heterotrophic prokaryotes showed no trend.

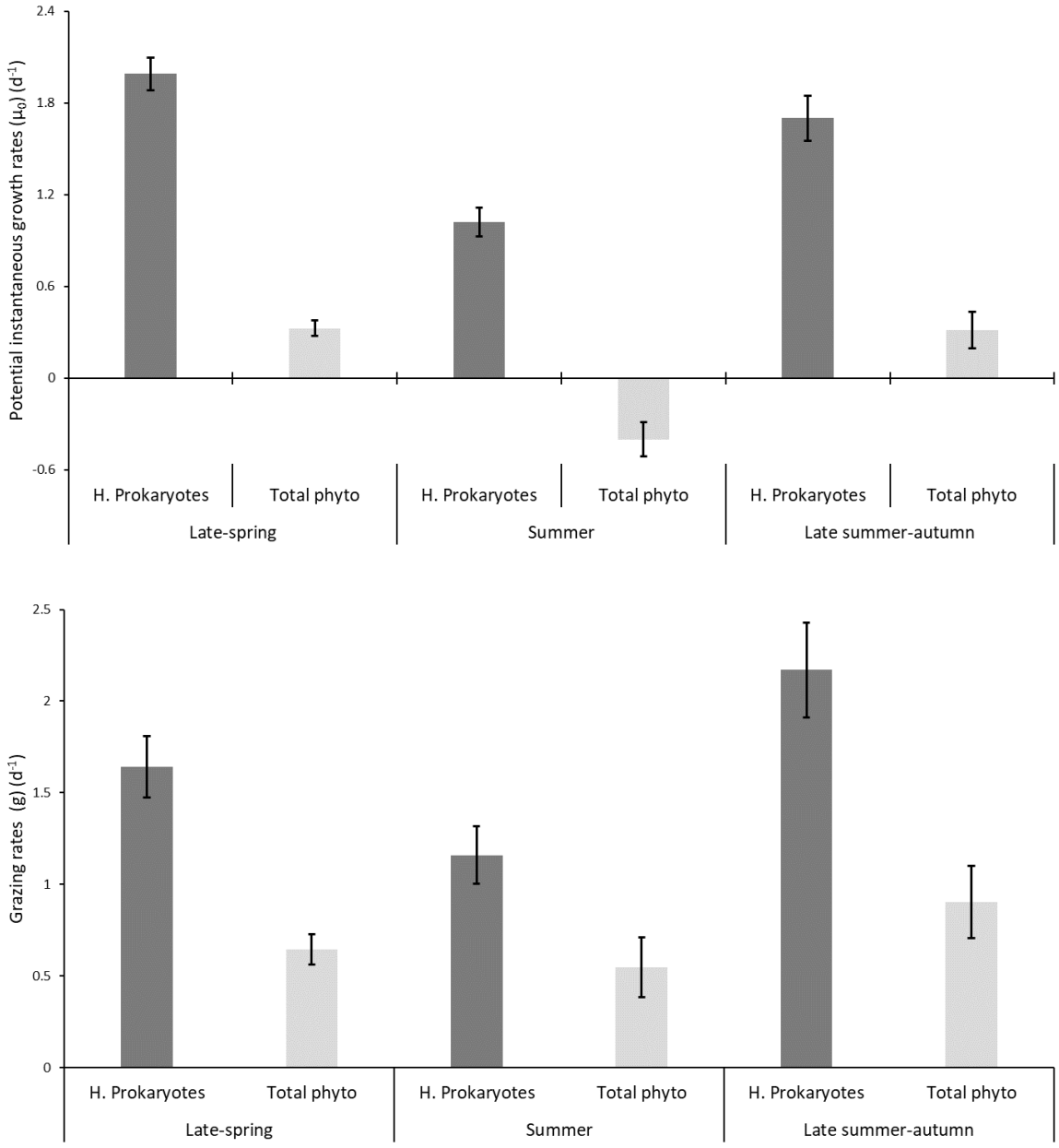

Figure 4.6: Variation of potential instantaneous growth rates (μ_0, d-1) and grazing rates (g, d-1) for heterotrophic prokaryotes and phytoplankton in June, August, and October. Vertical lines represent ± 1 SE.

4.1.5. Evaluation of potential nutrient limitation

The comparison between heterotrophic prokaryotes apparent growth rates in undiluted samples with and without nutrient addition revealed no significant differences between the two treatments in all experiments, and so no effects of nutrient addition on

growth rates of heterotrophic prokaryotes were detected. Curiously, significant differences between the two treatments were obtained in October ($t(2) = 3.84$, $p = 0.009$) for the total phytoplankton community, indicative of nutrient limitation, however the apparent growth rates in undiluted treatments without added nutrients were higher than with added nutrients. Furthermore, more differences between the two dilution treatments were observed for the different phytoplankton groups, but as the apparent growth rates in undiluted treatments without added nutrients were higher than with added nutrients, no nutrient limitation was considered. Potential nutrient limitation was observed for Cyanobacteria in June ($t(2) = 2.18$, $p = 0.047$) diatoms in August ($t(2) = 2.54$, $p = 0.032$), dinoflagellates in June ($t(2) = 4.69$, $p = 0.005$) and euglenophytes in June ($t(2) = 18.75$, $p = 1.65 \times 10^{-4}$) and in October ($t(2) = 5.38$, $p = 0.003$).

4.1.6. Changes in phagotrophic protists abundance

Phagotrophic protists counts conducted at the beginning (T_0) and end (T_{24}) of incubation showed alterations in ciliate and aplastidic nanoflagellates abundances. Growth rates in the different dilutions are reported in Table 4.6. Aplastidic nanoflagellates abundance increased in the undiluted samples in all experiments, while a decline in ciliate abundance, indicative of mortality during incubation, was observed in June and October experiments. A decrease in ciliate abundance was always recorded in the 50% and 25% dilution treatments.

Table 4.6: Apparent growth rates of ciliates and aplastidic nanoflagellates based on microscope counts on samples collected at T_0 and T_{24} from the experimental bottles.

	Experiment	**1-**	**1+**	**0.7**	**0.5**	**0.25**	**0.125**
Ciliates	**June**	-0.970	-0.741	-0.188	-0.606	-0.454	0.354
	August	0.155	0.163	-1.05	-0.929	-0.097	nd
	October	-0.300	-0.470	0.441	-0.138	-0.580	nd
Aplastidic nanoflagellates	**June**	1.56	1.21	0.540	0.511	0.031	-1.10
	August	0.735	0.839	1.43	1.67	1.59	0.180
	October	0.990	1.47	1.25	2.22	1.17	0.765

4.2. Modified dilution technique experiments – viral lysis.

4.2.1. Characterization of initial samples

The modified dilution method was used to estimate rates of heterotrophic prokaryotes grazing and viral lysis in the Ria Formosa coastal lagoon. The initial abundance of heterotrophic prokaryotes and nanoplankton in the Ria Formosa varied from 4.38 x 10^9 cell/L to 1.89 x 10^6 cell/L and from 1.29 x 10^8 cell/L to 2.73 x 10^3 cell/L, respectively. The highest abundances of heterotrophic prokaryotes occurred during October and the highest abundances of nanoflagellates occurred during March, regarding the sampling period.

4.2.2. Heterotrophic prokaryotes growth and mortality

For each experiment, dilution curves of both the original dilution method and the modified dilution method were obtained by plotting the apparent growth rate of heterotrophic prokaryotes against the dilution factor, and are represented in Figure 4.7.

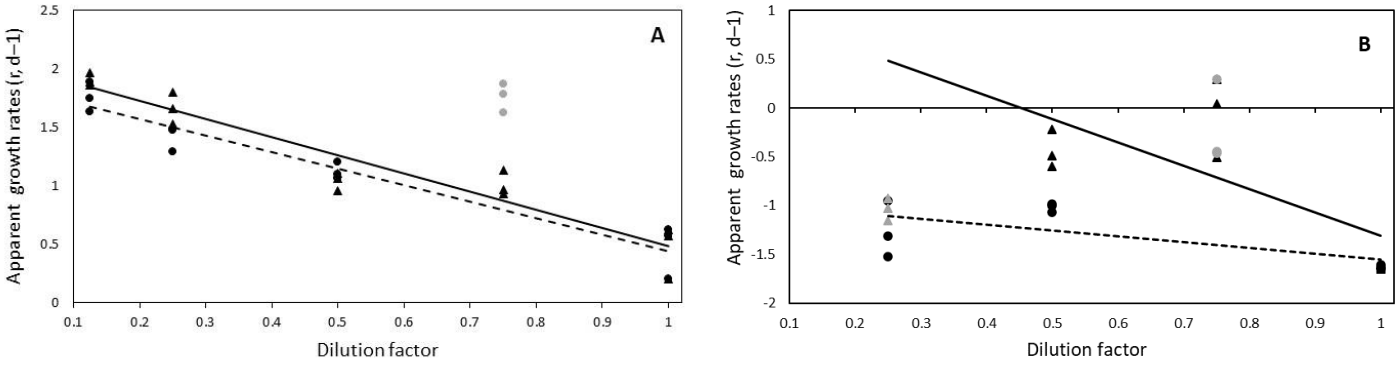

Figure 4.7: Dilution plots of apparent growth rate (r, d^{-1}) of heterotrophic prokaryotes versus dilution factor in March (A) and October (B). Triangular symbols with solid black line (▲—) represent the dilution curves for the modified dilution me method (grazer and virus-free dilution series) and the circular symbols with dashed black line (● - -) represent the dilution curves for the original dilution method (grazer-free dilution series). Grey symbols (● and ▲) were not used for linear regression.

Comparing heterotrophic prokaryote abundance in different dilutions at the beginning of the experiment, the values show that the abundance increased with the fraction of undiluted seawater, suggesting that experimental dilutions with grazer-free diluent and grazer and virus-free diluent successfully produced gradients of heterotrophic prokaryote abundance. During the incubation period, the results show that the abundance of heterotrophic prokaryotes increased in all experimental treatments during the March experiment, reflected by positive values of apparent growth rate, however in October experiment the abundance decreased in some experimental treatments. Mean values of

potential instantaneous growth rates (μ_0), grazing rates (g), and viral lysis mortality rate (v) on heterotrophic prokaryotes, and respective standard errors (± SE) are presented in Table 4.7. Relevant statistical information of the linear regression analysis, including regression coefficient of determination (R^2), number of values (n), and significance level of the regression slope (p-value) are also provided.

Table 4.7: Summary of results (± SE) of potential instantaneous growth rates (μ_0, d^{-1}), grazing rates (g, d^{-1}), and viral lysis mortality rate (v, d^{-1}) on heterotrophic prokaryotes obtained by original dilution method (grazer-free dilution series) and by the modified dilution method (grazer and virus-free dilution series). The regression coefficient of determination (R^2), number of values (n), and significance level of the regression slope (p-value) are also provided. (a) Outlier(s) removed for compliance with dilution assumptions; (**) grazing rate values marked by asterisks are not significantly different from zero (i.e. statistically non-significant slopes) and are assumed as zero.

		μ_0	**g**	**R^2**	**p-value**	**n**	**v**
March	**Grazer-free dilution series**	1.84 ± 0.072	1.40 ± 0.125	0.926	5.61E-07	12 (a)	0.149
	Grazer and virus-free dilution series	2.03 ± 0.086	1.55 ± 0.140	0.904	5.58E-08	15	
October	**Grazer-free dilution series**	-1.03 ± 0.170	0.597 ± 0.265**	0.421	0.059	9 (a)	1.79
	Grazer and virus-free dilution series	1.08 ± 0.731	2.39 ± 0.940	0.480	0.039	9 (a)	

Significant grazing rates of heterotrophic prokaryotes (i.e. significant negative slopes in the dilution experiments) were observed in all dilution series, except for the grazing rate estimated in October for grazer-free dilution series, therefore, at that time of year, grazing was not a significant source of mortality. The negative regression lines obtained for the grazer-free dilution series indicated potential instantaneous growth rates ranging from -1.03 ± 0.170 d^{-1} in October to 1.84 ± 0.072 d^{-1} in March, while the values obtained for grazer- and virus-free dilution series ranged from 1.08 ± 0.731 d^{-1} in October to 2.03 ± 0.086 d^{-1} in March. This clearly shows higher growth rates of heterotrophic prokaryotes during March than in October. In October, the slopes of the grazer-free and the grazer- and virus-free dilution series regressions lines were significantly different ($t(14) = 1.83$), with mortality due to grazing in the presence of viral lysis (2.39 ± 0.940 d^{-1}) being greater than mortality due to grazing alone. Therefore, viral lysis mortality on heterotrophic prokaryotes estimated was 1.79 d^{-1} and the impact of viruses appeared to increase heterotrophic prokaryotes mortality. In October, the rate of mortality of

heterotrophic prokaryotes was observed to be higher than their growth rates. In March, the slopes of the two dilution series regressions were not significantly different ($t(23) = 0.791$) and grazing was the only significant source of mortality ($1.40 \pm 0.125\ d^{-1}$), and at a lower rate than the growth rate. Thus, during October heterotrophic prokaryotes experienced both significant grazing and viral lysis, whilst in March heterotrophic prokaryotes only experienced significant mortality due to grazing.

4.2.3. Nanoplankton growth and mortality rates

Linear regression trend lines produced from the plots of apparent growth rate (d^{-1}) versus the dilution factor for both the grazer-free diluent and grazer- and virus-free diluent are represented in Figure 4.8.

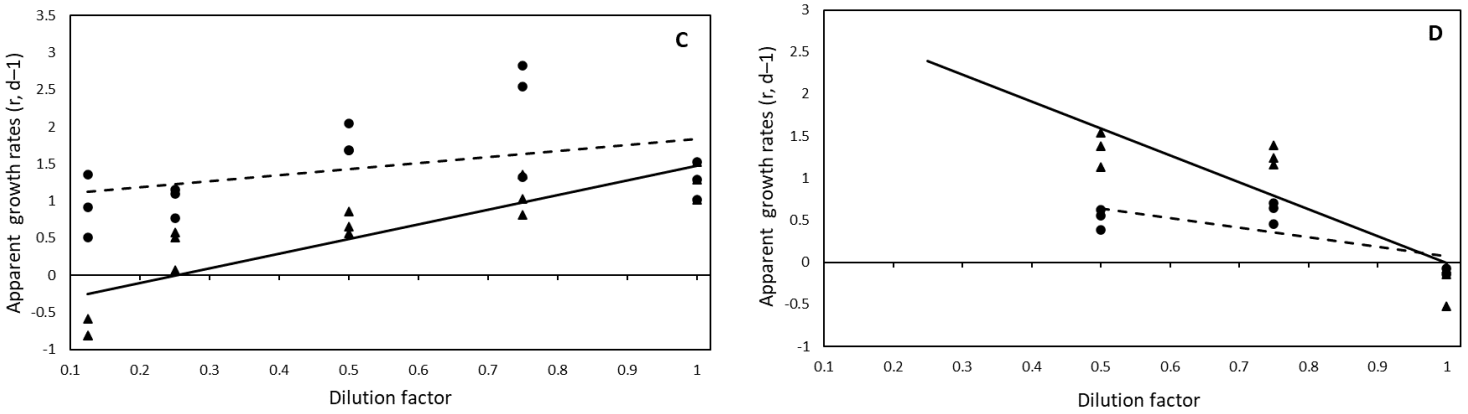

Figure 4.8: Dilution plots of apparent growth rate (r, d^{-1}) of nanoplankton versus dilution factor in March (C) and October (D). Triangular symbols with solid black line (▲—) represent the dilution curves for the modified dilution method (grazer and virus-free dilution series) and the circular symbols with dashed black line (● - -) represent the dilution curves for the original dilution method (grazer-free dilution series).

The regression lines obtained for nanoplankton in March had positive slopes and no grazing rates were obtained, thus making it impossible to estimate the mortality due to viral lysis in this experiment. Comparing nanoplankton abundance in different dilutions at the beginning of the March experiment, the values show that the abundance of nanoplankton increased with fraction of the undiluted seawater, suggesting that experimental dilutions with grazer-free diluent and grazer and virus-free diluent successfully produced gradients of nanoplankton abundance. The plots also show that, during the incubation period, the abundance of nanoplankton increased in the majority of experimental treatments during the March experiment, reflected by positive values of apparent growth rate. Mean values of potential instantaneous growth rates (μ_0), grazing rates (g), and viral lysis mortality rate (v) on heterotrophic prokaryotes, and respective

standard errors (± SE) are presented in Table 4.8. Relevant statistical information of the linear regression analysis like regression coefficient of determination (R^2), number of values (n), and significance level of the regression slope (p-value) are also provided.

Table 4.8: Summary of results (± SE) of potential instantaneous growth rates (μ_0, d^{-1}), grazing rates (g, d^{-1}), and viral lysis mortality rate (v, d^{-1}) on nanoplankton obtained by original dilution method (grazer-free dilution series) and by the modified dilution method (grazer and virus-free dilution series). The regression coefficient of determination (R^2), number of values (n), and significance level of the regression slope (p-value) are also provided. (a) Outlier(s) removed for compliance with dilution assumptions; (nd) grazing rate not determined because of positive slope.

		μ_0	**g**	**R^2**	**p-value**	**n**	**v**
March	**Grazer-free dilution series**	1.02 ± 0.296	nd	0.758	0.116	15	-
	Grazer and virus-free dilution series	-0.505 ± 0.192	nd	0.179	2.43E-05	15	
October	**Grazer-free dilution series**	1.21 ± 0.330	1.14 ± 0.443	0.523	0.042	8	2.06
	Grazer and virus-free dilution series	3.19 ± 0.536	3.20 ± 0.689	0.755	0.002	9	

In the October experiment, the slopes of all regression lines were significant and were significantly different from one another ($t(13) = 2.51$), indicating that an estimate of viral mortality on nanoplankton at a level of significance of $p < 0.05$ could be calculated. For grazer-free diluent and grazer-and virus-free diluent, the potential instantaneous growth rates ranged from 1.21 ± 0.330 to 3.19 ± 0.536 d^{-1}. Mortality due to grazing in the presence of viral lysis (3.20 ± 0.689 d^{-1}) was observed to be greater than mortality due to grazing alone (1.14 ± 0.443 d^{-1}), such that the mortality rate due to viral lysis estimated was 2.06 d^{-1} and here too, the impact of viruses appeared to increase nanoplankton mortality.

5. Discussion

Given the limited number of studies investigating microbial mortality in the Ria Formosa coastal lagoon, the present study aimed to assess growth and grazing rates of heterotrophic prokaryotes, whole phytoplankton assemblage, and specific phytoplankton groups, in different seasons, using the dilution technique. Notably, the findings revealed substantial variability in the estimated rates between seasons and between the different microbial components. During the study period, it as observed clear seasonal effects on the microbial dynamics within Ria Formosa coastal lagoon, although further experiments would be necessary to confirm this. It is important to note that no dilution experiment was conducted during the winter season, limiting the ability to draw conclusions regarding the full annual variability of grazing rates. Previous studies have already provided growth and mortality rates of the phytoplankton assemblage in the Ria Formosa coastal lagoon (Barbosa, 2006; Thiele-Gliesche, 1992), with some studies employing the dilution method (Domingues et al., 2021; Sá, 2017). However, this study represents the second attempt to estimate mortality rates on heterotrophic prokaryotes due to microzooplankton grazing in Ria Formosa coastal lagoon and the first to employ the dilution method for this purpose. Additionally, in addressing the understudied aspect of heterotrophic prokaryote mortality due to viral lysis in the lagoon, the present study provides novel insights into the contribution of viruses to heterotrophic prokaryote mortality in the Ria Formosa coastal lagoon. As far as we know, no previous information regarding this aspect is available for this ecosystem. The following analysis and interpretation of the results obtained, intended to discuss the implications and potential ecological significance of the results, focus on the different microbial components considered and the broader implications for microbial dynamics in the Ria Formosa coastal lagoon.

5.1. Initial microbial community

The physico-chemical and biological variability observed in Ria Formosa over temporal and spatial scales results from the strong salinity and temperature gradients, shallow waters, close coupling between benthic and pelagic domains, and restricted connections to the adjacent sea, characteristic of coastal lagoons (Loureiro et al., 2006). Phytoplankton and heterotrophic prokaryotes biomasses showed maxima during summer. Unimodal cycles with summer maxima and winter minima are frequently reported for

temperate shallow ecosystems in which growth is driven by increased temperature and light availability (Cebrián and Valiela, 1999), including other temperate coastal ecosystems in Portugal (e.g. Gameiro et al., 2007) and the inner regions of the Ria Formosa coastal lagoon (Barbosa, 2010, 2006; Domingues et al., 2017a, 2015). Chlorophyll-a concentrations and total phytoplankton abundances estimated were lower than values previously reported for the Ria Formosa (Domingues et al., 2023, 2017a, 2015), although high abundances of phytoplankton community during spring have also been reported (Domingues et al., 2017a). In terms of abundance, Cyanobacteria dominated the phytoplankton community in all seasons analyzed, with values within the range recently reported for the Ria Formosa coastal lagoon (Domingues et al., 2023, 2017a, 2017b, 2015). However, the cited studies often observed greater abundances of eukaryotic picophytoplankton, that frequently dominates the phytoplankton community in terms of numbers. For example, Domingues et al. (2017a) observed a phytoplankton community dominated by eukaryotic picophytoplankton under typical spring conditions (75.2%), fairly surpassed Cyanobacteria (2.1%), while Domingues et al. (2015) observed dominance of eukaryotic picophytoplankton under typical summer conditions (83%). The competitive ability of eukaryotic picophytoplankton under low nutrient concentrations and increase temperature and light intensity have been used to explain this dominance during summer (Vaquer et al., 1996). Indeed, the contribution of eukaryotic picophytoplankton to the total phytoplankton abundance was higher during summer (25%), but Cyanobacteria still dominated the community. However, in agreement with this study, Domingues et al. (2017b) also found a phytoplankton community dominated by Cyanobacteria (94%) under typical summer conditions, and Galvão et al. (2019) found dominance of these microorganisms under a widespread seasonal distribution. The numerical importance of Cyanobacteria and eukaryotic picophytoplankton in the Ria Formosa had been previously recognized by Barbosa (2006) and also in other coastal environments (Bec et al., 2005), being associated with their small size and high surface-to-volume ratio that provides them a competitive advantage to acquire nutrients in resource-limited environments (Agawin et al., 2000; Raven, 1998). Cyanobacteria possess an array of qualities that may favor them in conditions that usually limit the growth of other competing phytoplankton groups, enabling them to grow and dominate the phytoplankton community, as maximum growth rates at high temperatures, fewer cell losses by sedimentation and grazing, N and P storage capacity, ability to grow at low light levels, halotolerance and regulation of buoyancy, that had been associated to

Cyanobacteria dominance in other temperate coastal ecosystems (e.g. Guadiana estuary) not only during summer but also in the autumn and winter (Domingues et al., 2007, 2005; Domingues and Galvão, 2007; Galvão et al., 2012). Thus, the observed dominance of Cyanobacteria during the study period suggests a significant variability of the dominant phytoplankton group probably in response to a set of variable bottom-up and top-down influences, resultant of species-specific physiological tolerances, resource uptake strategies, and key ecophysiological attributes. Moreover, factors other than competitive traits of Cyanobacteria may have been responsible for eukaryotic picophytoplankton lower abundances, such as increasing grazing pressure (further discussed) and/or other top-down processes, not addressed in this study. Besides, lower counts of eukaryotic phytoplankton reported also can be related to identification issues, since this phytoplankton group is very difficult to identify using conventional methods due to its very small size and characteristics. Larger phytoplankton groups, as plastidic nanoflagellates, dinoflagellates and diatoms were present in initial samples at lower abundances than he picosized fraction but also at lower values than previously described for the Ria Formosa (Barbosa, 2006; Domingues et al., 2023, 2017a, 2015; Loureiro et al., 2006), although the abundance of diatoms in late summer-autumn was very similar to that reported in Domingues et al. (2017a). The heterotrophic prokaryotes abundances were maximum in summer – late summer-autumn (August-October) and minimum in early-spring (March), and were similar to the average annual abundances reported in other studies, although biomass values are lower (Barbosa, 2006; Galvão et al., 2019). Initial abundance of phagotrophic protists were also in the range of values recently reported for the Ria Formosa (Domingues et al., 2021).

5.2. Potential nutrient limitation

The nutrient environment is a major abiotic variable controlling phytoplankton growth, in particular for Ria Formosa coastal lagoon, where light limitation rarely occurs (Domingues et al., 2017a). In dilution experiments, Landry (1993) recommends adding sufficient nutrients to experimental treatments when nutrient availability is known or expected to be limiting. A nutrient-limited phytoplankton community should respond to increase nutrient loading by increasing phytoplankton growth rates. A comparison between prey apparent growth rates in undiluted treatments with and without added nutrients was performed in order to control for potential nutrient stimulation of growth in

the dilution experiments that could lead to overestimation of growth rates. Recent nutrient-enrichment experiments clearly showed that nutrient limitation, particularly by N, is a common occurrence in Ria Formosa coastal lagoon during all seasons (Domingues et al., 2017a, 2015), and especially for diatoms during summer (Domingues et al., 2017b, 2015), period when nutrients concentrations are typically lower (Barbosa, 2010). Diatoms nutrient limitation during summer was, indeed, an observation in this study and supports previous findings. N limitation was previously reported by Loureiro et al. (2006, 2005), that observed nutrient limitation at inner lagoon locations and suggested that N as the most likely limiting nutrient for phytoplankton growth in Ria Formosa, pointing out significant increases in diatom growth rates after N enrichment. Other dilution experiments have also observed an enhancement of diatom growth in nutrient-enriched experiments (Juhl and Murrell, 2005; Modigh and Franze, 2009). As diatoms also depend on Si availability, it is important to consider the potential contribution of Si to nutrient limitation during summer. Nutrient limitation concerning other specific groups of phytoplankton demonstrated that nutrient limitation is very variable across seasons and phytoplankton functional groups. Further nutrient enrichment experiments carried out in the Ria Formosa coastal lagoon revealed that co-limitation by N and P is frequently observed throughout the seasonal cycle, beyond the limitation by single N and P also observed (Domingues et al., 2023), while the present study observed no potential nutrient limitation of phytoplankton community. Furthermore, it also reveals non-consistent results with experimental data obtained from previous nutrient enrichment experiments, probably because these experiments evaluated the occurrence of nutrient limitation of the phytoplankton community derived from responses of chlorophyll-*a* concentration, and in this study, there may have been artifacts related to deriving the total phytoplankton responses based on the sum of the abundances of the different specific groups. As explained by Domingues et al. (2008), abundance and chlorophyll *a* are two different phytoplankton metrics, and can therefore induce different responses.

5.3. Top-down control on microbial components

5.3.1. Microzooplankton grazing and transfer through the microbial food web

Most of the knowledge on the role of microzooplankton in marine food webs has been obtained following the establishment of the grazing dilution method (Landry and Hassett,

1982), which has been used for approximately 40 years and is still used for a wide variety of ecosystems. The dilution experiments conducted in the Ria Formosa coastal lagoon revealed notable seasonal variations in the growth and grazing rates of heterotrophic prokaryotes and phytoplankton. Specifically, it was observed a significantly higher growth rate (μ_0) in June and a higher grazing rate (g) in October. These findings do not align with a previous study by Barbosa (2006), which applied the differential filtration technique to samples from the Ria Formosa and reported maximum rates of growth and grazing rates on both microbial components during late-spring, with mean growth rates of 0.030 ± 0.004 h^{-1} for heterotrophic prokaryotes and of 0.040 ± 0.008 h^{-1} for phytoplankton assemblage, and mean grazing rates of 0.019 ± 0.004 h^{-1} for heterotrophic prokaryotes and of 0.003 h^{-1} for phytoplankton assemblage. Barbosa (2006) focuses on estimating grazing rates by phagotrophic protists <100 μm and <10 μm, without considering the contribution of larger microzooplankton. It is crucial to acknowledge the potential influence of all microzooplankton <200 μm in the grazing dynamics. The grazing impacts obtained in this study were higher than the mean annual removal of 47% of daily phytoplankton production and 65% of the daily heterotrophic prokaryotes production per day, estimated by Barbosa (2006). This suggests that phagotrophic protists within the size range of 100 μm and 200 μm exert significant control over heterotrophic prokaryotes and phytoplankton in the Ria Formosa coastal lagoon. The inclusion of these larger grazers in this study provided a more comprehensive understanding of the grazing dynamics within the Ria Formosa coastal lagoon. Still, unlike Barbosa (2006), water samples here were not pre-filtrated through a 200 μm mesh, which means that larger grazers were also present in water samples and may have somehow affected directly or indirectly the results obtained. While the results of this study shed light on the growth and grazing rates of heterotrophic prokaryotes and phytoplankton assemblage, it is essential to acknowledge that the rates reported in this study were estimated using the dilution method, which is obviously not free of problems (Dolan, and McKeon, 2005; Dolan et al., 2000; Gallegos, 1989).

One impression arising from browsing the available data is that, despite the increasing use of the dilution method to estimate grazing on microbial communities, apparently, it has been applied to a lesser extent in coastal lagoons, and so the role of microzooplankton grazing remains poorly understood in these ecosystems. Heterotrophic prokaryotes growth and grazing rates in this study (1.02-1.99 d^{-1} and 1.16-2.17 d^{-1}, respectively) are within the range reported during dilution experiments made by Pecqueur et al. (2022) in

a Mediterranean coastal lagoon in the South of France (Thau Lagoon) (μ_0 =1.63-2.43 d^{-1}; g = 1.33-2.28 d^{-1}), that found maximum growth and grazing rates of heterotrophic prokaryotes during late-spring. The estimates for phytoplankton community are within the range of the values recently estimated by a dilution experiment conducted in Ria Formosa coastal lagoon during winter (μ_0= 0.85 ± 0.07 d^{-1}; g= 0.46 ± 0.11 d^{-1}; I= 87.9%) (Domingues et al., 2021), although the combination of the two studies is suggestive of higher grazing rates during late summer-autumn and higher growth rates of the phytoplankton community during winter, contrary to observations of Barbosa (2006), although the significance of the differences between rates obtained through the two dilution methods applied in Ria Formosa was not tested. Furthermore, estimates for phytoplankton community are within the range of values estimated for other temperate coastal lagoons (Banu, 2012; Bec et al., 2005; Esqueda-Escárcega et al., 2013; Hlaili et al., 2007). The estimations for heterotrophic prokaryotes and phytoplankton assemblage also are within the same range of values of other temperate coastal ecosystems (McManus and Ederington-Cantrell, 1992; Modigh and Franze, 2009; Rychert, 2022).

Table 5.1: Estimations of potential instantaneous growth rates (μ_0, d^{-1}) and grazing rates (g, d^{-1}) for heterotrophic prokaryotes and phytoplankton assemblage in several temperate coastal ecosystems, including coastal lagoons.

System (location)	Heterotrophic prokaryotes		Phytoplankton		Reference
	μ_0	g	μ_0	g	
Ria Formosa (Portugal)	1.02 – 1.99	1.16 – 2.17	-0.40 – 0.33	0.55 – 0.90	This study
Thau Lagoon (France)	1.63 - 2.43	1.33 - 2.28	-	-	Pecqueur et al. 2022
Coastal zone of the Gulf of Gdansk (Baltic Sea)	0.52 - 1.34	0.58 - 1.37	-	-	Rychert, 2022
Ria Formosa (Portugal)	-	-	0.85	0.46	Domingues et al., 2021
Ensenada de la Paz (México)	-	-	-	0.38 - 0.86	Esqueda-Escárcega et al., 2013
Homa Lagoon (Turkey)	-	-	1.32 - 4.51	0.20 - 3.34	Banu, 2012
Gulf of Naples (Italy)	-	-	-1.22 - 1.96	0.15 - 1.53	Modigh & Franze, 2009
Bizerte Lagoon (Tunisia)	-	-	0.72 - 1.04	0.54 - 0.70	Hlaili et al., 2007
Thau Lagoon (France)	-	-	-0.23 - 2.63	-0.28 - 1.13	Bec et al., 2005
Chesapeake Bay (USA)	-	-	0 - 2.15	-0.22 - 1.60	McManus & Ederington-Cantrell, 1992

Over the study period, heterotrophic prokaryotes experienced significant variation and high growth rates, leading to changes in production (132.18 – 291.95 µg C L^{-1} d^{-1}) with relatively high values during all seasons. Likewise, phytoplankton also experienced significant variation in their growth rates, although with lower production (12.89 – 87.28 µg C L^{-1} d^{-1}) compared to heterotrophic prokaryotes. The growth rates of some specific phytoplankton groups were frequently higher than those of the whole phytoplankton community. This indicates that the specific phytoplankton groups experienced more rapid growth compared to the overall phytoplankton community, even when they were not the biomass dominant group, which supports the notion of heterogeneity in phytoplankton

growth, especially in optimal conditions – without nutrient limitation or grazing pressure. This could be attributed to specific adaptations of each of the phytoplankton groups, such as specific nutrient requirements, efficient utilization of light, competitive abilities in resource acquisition, etc. Furthermore, a wide range of grazing rates among phytoplankton groups is suggestive of selectivity of grazers among taxa. Most dilution experiments in coastal lagoons report growth and grazing rates for the entire phytoplankton community (Banu, 2012; Bec et al., 2005; Esqueda-Escárcega et al., 2013; Grinienė et al., 2016; Hlaili et al., 2007), which masks the complex aspects of each specific group. So, to obtain a comprehensive understanding of the ecological processes, it is essential to consider and analyze the specific dynamics of each phytoplankton group separately in future studies. In this study, prey growth rates were estimated as both the y-intercept of the nutrient amended growth curve (μ_0) and the sum of the grazing rate (i.e. the slope) and net prey growth in undiluted control incubations (μ_{is}). The latter rate, in theory, provides a more accurate estimate of prey in situ growth rate (Landry 1993), however, the values discussed here are those of μ_0 as it is the maximum rate of cell division that a group would have in an ideal situation (no predators or no nutrient limitation).

Contrary to the initial hypothesis, the results of this study revealed that the mean growth rates of heterotrophic prokaryotes and total phytoplankton community were lower than their mean grazing rates. It would be expected that the growth rates of the microbial components considered exceeded their grazing rates because microzooplankton grazing is but one of many top-down processes (e.g., metazooplankton grazing, sinking, lysis, advection). In this study, heterotrophic prokaryotes grazing rates presented a mean value of 1.66 ± 0.29 d^{-1} and a mean potential instantaneous growth rate of 1.57 ± 0.29 d^{-1}, and the grazing rate of the phytoplankton community presented an average value of 0.70 ± 0.11 d^{-1}, considerably higher than the average potential instantaneous growth rate of 0.08 ± 0.24 d^{-1}. All the μ and g estimated for the total phytoplankton community are within the range of values reviewed in the literature (Calbet and Landry, 2004; Schmoker et al., 2013). It is uncommon for microzooplankton grazing to be higher than prey growth, which was frequently observed in this study, suggesting a decoupling of these processes characteristic of temperate coastal waters (Strom, 2002). One possible explanation for $\mu < g$, i.e. g:μ ratio > 1, could be nutrient limitation during incubation that would lead to an underestimation of instantaneous growth rates, yet nutrients were added to avoid this. This uncommon result was also observed by First et al. (2007) that suggested that the

higher grazing rates may have been caused by the decrease of top-down control of metazooplankton in microzooplankton, through the pre-filtration of the water samples. As water samples were not pre-filtrated to remove larger grazers, it does not seem to be associated with the observations. So, it can be suggested that microzooplankton consume a significant proportion of heterotrophic prokaryotes and phytoplankton community in the Ria Formosa coastal lagoon. In an estuarine or productive coastal environment, grazing response may be greater at times, due to higher standing stocks of microzooplankton from greater overall ecosystem productivity and biomass (Calbet and Landry, 2004). In addition, microzooplankton consumed >100% of heterotrophic prokaryote and phytoplankton daily production (PP) in the Ria Formosa coastal lagoon (obtained through g/μ ratio), with values well above the mean values (~60%) obtained by Calbet & Landry (2004) and Schmoker et al. (2013) for coastal systems, further emphasizing the importance of microzooplankton grazing in this ecosystem. The occurrence of this imbalance between growth and grazing rates, resulting in the frequent removal of > 100% of daily heterotrophic prokaryotes and phytoplankton production, suggests that other mechanisms of "top-down" control, such as tidal advection and grazing by metazooplankton, may not contribute as much as the microzooplankton grazing in Ria Formosa coastal lagoon, This was already emphasized in Barbosa (2006) and supports the understanding of the importance of microzooplankton grazing as a significant source of mortality for heterotrophic prokaryotes and phytoplankton in aquatic systems (Calbet and Landry, 2004; Sherr and Sherr, 2002).

Surprisingly, in August (summer), lower grazing rates of both heterotrophic prokaryotes and phytoplankton assemblage were observed despite maximum abundances of phagotrophic protists, particularly nanoflagellates, the most important grazer of heterotrophic prokaryotes in Ria Formosa (Barbosa, 2006). This could be indicative that microzooplankton may have other prey as an alternative food source. Indeed, several microzooplankton organisms are also known to feed on other microzooplankton, which releases some prey from their predators and therefore creates trophic cascades (Calbet, 2001; Calbet and Landry, 1999). In addition, Gallegos et al. (1996) suggested that the microzooplankton may be supported by an alternative food source, such as detritic material. Furthermore, a decrease in grazing rates as been reported in the case of a senescent phytoplankton bloom (the end of bloom situation) because phytoplankton cells are in poor health, and microzooplankton, even when abundant, may strongly reduce their grazing rate (Calbet et al., 2011).

Furthermore, high rates of heterotrophic prokaryotes growth and grazing indicated that the microbial loop contributes to a greater proportion of carbon flow in the Ria Formosa coastal lagoon. The high impact of microzooplankton in heterotrophic prokaryotes daily production observed in this study also highlights the importance of phagotrophic protists, in particular aplastidic nanoflagellates, as the main predators of heterotrophic prokaryotes in several aquatic systems (Sanders et al., 1992; Sherr and Sherr, 2016, 2002; Suthers et al., 2019). Furthermore, it highlights the importance of heterotrophic prokaryotes as alternative food sources and how this can be used to understand the low impact on primary production, sometimes denoted in dilution experiments. As grazers in dilution experiments have at their disposal several alternate potential prey items (e.g., heterotrophic prokaryotes), prey-switching may come into play and so, low impacts on primary production do not necessarily mean lower importance of microzooplankton in marine systems; rather, it can indicate that microzooplankton can feed on organisms other than phytoplankton.

In order to estimate the role of each specific phytoplankton group in carbon cycling in the Ria Formosa, it is necessary to investigate their growth capability as well as their losses due to microzooplankton grazing. The impact of grazing was clearly variable depending on the specific phytoplankton group considered, related to their ecological characteristics and interactions with grazers. The most significant prey losses due to grazers in this study concerned smaller phytoplankton groups. After all, small phytoplankton has the advantage in all functional traits related to growth and resource utilization, while the advantage of larger phytoplankton is related to resistance to grazing (Sommer et al., 2016). Large phytoplankton tend to have lower nutritional quality (except diatoms) and are more difficult to consume than small phytoplankton and thereby microzooplankton tend to graze selectively on more nutritious small phytoplankton (Branco et al., 2020). The grazing rates on smaller prey, such as Cyanobacteria, were also higher than for other larger phytoplankton groups in Thau Lagoon (Pecqueur et al., 2022). As hypothesized, the mean grazing rates of eukaryotic picophytoplankton (1.38 d^{-1}) and cryptophytes (2.35 d^{-1}) were significantly higher and the impact of grazing on these groups corresponded to an average removal of 129.55% and 264.12% of primary production per day, respectively. A previous application of the dilution method in the Ria Formosa detected higher grazing rates upon diatoms during winter (Domingues et al., 2021). Size is a critical factor in determining grazer-prey relationships. The traditional assumption is that larger grazers consume larger prey, which is reflected in early models

of the “microbial loop” (Azam et al., 1983), where aplastidic nanoflagellates (<20 μm) feed on picoplankton (heterotrophic prokaryotes and picophytoplankton <2 μm) and microzooplankton (mostly ciliates and aplastidic dinoflagellates, 20–200 μm) feed on nanoplankton. However, there are overlaps in the size ranges of prey consumed by different microzooplankton groups; for example, some aplastidic flagellates and ciliates eat phytoplankton almost as large as themselves (Sommer et al., 2016), resulting in complex interactions within the planktonic food web, such that establishing clear cause-effect relationships is difficult. Selective feeding by microzooplankton is common and widespread (Stoecker et al., 1981; Strom et al., 2007; Tillmann, 2004; Verity, 1991), and can effectively explain the higher grazing rates of eukaryotic picophytoplankton and cryptophytes in relation to other phytoplankton groups and even the most abundant, Cyanobacteria. Ciliates and nanoflagellates preferentially feed on cells of <20 μm (i.e., picoplankton and nanoplankton) (Fenchel, 1987; Jonsson, 1986; Sherr and Sherr, 2007, 1992). Picophytoplankton are potentially the most important food source for microzooplankton because of their small size (<2 μm) (James and Hall, 1998). Cryptophytes are known to be either preferred or optimal prey for numerous protists (Johnson et al., 2018), such as aplastidic and mixotrophic dinoflagellates (Johnson, 2015; Larsen, 1988) and ciliates (Jakobsen and Hansen, 1997; Weisse and Kirchhoff, 1997) that selectively feed on cryptophytes. Burkill et al. (1987) also found microzooplankton selective grazing on cryptophytes. Aplastidic nanoflagellates have also been observed to feed on cryptophytes (Kwon et al., 2017). Selective feeding on eukaryotic picophytoplankton has also been reported in coastal ecosystems (Bec et al., 2005; Samuelsson and Andersson, 2003; Worden et al., 2004). As aplastidic nanoflagellates were the most abundant microzooplankton in the Ria Formosa coastal lagoon during the study period, the higher grazing rates observed for eukaryotic picophytoplankton can also be attributed to the abundance and ecological interactions between these groups, as eukaryotic picophytoplankton are within the prey size range of aplastidic nanoflagellates (Jürgens and Massana, 2008). Even the plastidic nanoflagellates could be grazing on eukaryotic picophytoplankton during incubations, as in reality, most are mixotrophs (Sanders, 1991). These findings highlight the importance of considering the ecological interactions between microzooplankton and specific phytoplankton groups when evaluating grazing dynamics and nutrient cycling in the Ria Formosa coastal lagoon. Furthermore, euglenophytes appear also to be an essential component of microbial food webs and carbon flow, particularly during summer in Ria Formosa, when its grazing rate

was relatively high (g = 3.08 ± 0.35 d^{-1}), corresponding to the removal of 217.75% of their production per day. Regarding the g:μ ratio of each group, the number of cells that were grazed and transferred to higher trophic levels was greater than the number of cells accumulated (g:μ > 1) for the majority of the cases. This has an important implication for carbon cycling in Ria Formosa coastal lagoon, where most of heterotrophic prokaryotes and phytoplankton daily production on Ria Formosa seems to be mediated by microzooplankton, recycling and fueling the microbial food web, as well as transferring carbon to the higher trophic levels. Overall, considering not only the higher grazing rates of smaller size phytoplankton (pico- and nano-sized) but also the grazing impact on larger phytoplankton cells, this study highlights the importance of all microbial components in the overall functioning of the microbial food web and confirms the relevance of microzooplankton in the Ria Formosa coastal lagoon, effectively mediating carbon transfer to higher trophic levels. As energy loss occurs at each trophic level, with 50-70% of energy being lost with each additional step (Straile, 1997), microzooplankton grazing on phytoplankton, as opposed to direct grazing by metazooplankton, can substantially diminish the overall efficiency of food web energy transfer as a result of their position as trophic intermediaries.

However, contrary to what was hypothesized, euglenophytes and eukaryotic picophytoplankton were the phytoplankton groups with higher growth rates. In contrast, in Barbosa (2006), diatoms were the phytoplankton functional group with higher growth rates (0.048 ± 0.010 h^{-1}). In a previous dilution experiment conducted in the Ria Formosa by Domingues et al. (2021), diatoms also showed the highest growth rates between the different functional groups of phytoplankton, exhibiting growth rates of 1.29 ± 0.16 d^{-1}. Also through the application of dilution experiments in Ria Formosa, Sá (2017) observed higher growth rates of diatoms among the different phytoplankton groups during the spring and autumn of 2015, despite also observing high growth rates of euglenophytes. Euglenophytes are mixotrophic (Bicudo and Menezes, 2016; Yamaguchi et al., 2012) and can feed on picophytoplankton, which can be related to higher growth rates compared to other groups. The higher growth rates in the absence of grazers (μ0) of eukaryotic picophytoplankton found in this study may have been related to the assimilation and use of available resources during incubation. In fact, small phytoplankton, as eukaryotic picophytoplankton, can effectively utilize nutrients because of their larger surface-to-volume ratio which gives them an advantage in acquiring nutrients and absorbing light energy competing with larger cells (Raven, 1998). In addition to diatoms not having the

highest growth rates, as hypothesized, they were the functional group with one of the lowest grazing rates. Diatoms could be avoided by microzooplankton more than other phytoplankton functional groups, as observed in (Burkill et al., 1987) also using the dilution technique, due to their large size and grazing defenses, like spines, that can prevent and discourage microzooplankton from consuming them (Verity and Villareal, 1986), although, due to their varied feeding strategies (e.g. direct engulfment, pallium feeding, and tube feeding), aplastidic and mixotrophic dinoflagellates may consume large diatoms (Hansen and Calado, 1999; Menden-Deuer et al., 2005; Stoecker, 1999). Furthermore, it should be noted that diatoms sink faster than the other phytoplankton functional groups which, even if the bottles were mixed during the incubation period, may have also indirectly contributed to lower grazing rates.

5.3.2. Viral lysis

Considering that no information on the contribution of viruses to prokaryote mortality is available for the Ria Formosa coastal lagoon, this study is the first study to provide information on viral-induced mortality on heterotrophic prokaryotes and provides valuable insights that, as microzooplankton grazing, viral lysis is an important source of mortality of heterotrophic prokaryotes in the Ria Formosa. Although some limitations were unavoidable and further identified, the results of this study provide a first estimation of the potential impacts of viral communities on heterotrophic prokaryotes in the Ria Formosa. While there are surprisingly few published mortality rates using the modified dilution method in other temperate coastal lagoons, viral lysis estimates on heterotrophic prokaryotes in this study are within the range of values estimated for other temperate coastal waters using the modified dilution technique (Taira et al., 2009). Jacquet et al. (2005) applied the modified dilution method on Lake Bourget (France) to estimate viral mediated mortality on heterotrophic prokaryotes and obtained results (0.29 – 1.14 d^{-1}) lower than those reported in this study. In the marine zone of the Ria de Aveiro coastal lagoon, Almeida et al. (2001) found that viral lysis contributed, on average, to 36% of heterotrophic prokaryotes mortality. The results of the modified dilution experiments suggest that viral lysis was a relatively weak cause of heterotrophic prokaryotes mortality in March, but a significant source of mortality in October, even exceeding the grazing rates of microzooplankton, as hypothesized. Virus mediated mortality is typically only quantified when the slopes of the two dilution series regressions are significantly different

(Evans et al., 2003). In March, the regression slopes of the two dilution series did not significantly differ, indicating that viral infection had no detectable impact on heterotrophic prokaryotes mortality. So, no clear mortality impacts of viruses could be detected on heterotrophic prokaryotes, suggesting that viruses had no impact on heterotrophic prokaryotes during this period. The absence of viral lysis may be due to low successful encounters between viruses and heterotrophic prokaryotes resultant of host and viruses not being abundant enough during incubation, as suggested by Personnic et al. (2009). However, it is important to consider that viruses really had no impact, especially in periods of low metabolic activity such as early spring season (Personnic et al., 2009). Nonetheless, observed high potential instantaneous growth rate of heterotrophic prokaryotes in samples with manipulated viral abundance during both experiments indicates an impact of viral lysis. Conversely, in the October experiment, the viral induced mortality on heterotrophic prokaryotes was highly relevant. This observation highlights a potential seasonality of viral lysis, suggesting that its relative contribution to heterotrophic prokaryotes mortality can vary with changing environmental conditions, such as nutrient availability, temperature, and community composition. In addition, this study fortifies the importance of viral lysis as a loss factor in the Ria Formosa coastal lagoon. This has several implications for the flow of matter and energy through the food web, as viral lysis reduces the amount of organic matter flowing to higher trophic levels through grazing and increases the recycling of organic matter and nutrients within the microbial loop (Brum et al., 2014). Also considering its potential to be a useful tool for estimating the impact of viruses on phytoplankton populations, further application of the modified dilution technique is needed in the Ria Formosa coastal lagoon in order to gain a better understanding of the viral mediated mortality on microbial components in this ecosystem. As the rise in ocean temperature, which is a prominent and extensively documented consequence of climate change, is projected to persist and intensify in the future (Hansen et al., 2006), one area of interest and further investigation is the effect of temperature on the balance between microzooplankton grazing and viral lysis on planktonic microbes, as it would have significant impacts on microbial food webs, considering their different implications for the flow of matter and energy (Tjidens et al., 2008).

5.4. Potential mixotrophy interference

The dilution method, like any other method, is subjected to several limitations and challenges, extensively discussed in the literature (Calbet and Saiz, 2018, 2013; Schmoker et al., 2013). A new paradigm emerging in marine ecology is that many protist plankton are mixotrophs, combining phototrophy and phagotrophy (Flynn et al., 2019; Mitra et al., 2016). As a major process within the microbial food web (Gilbert and Mitra, 2022), mixotrophy can have important implications in dilution experiments as it is not taken into consideration by the dilution technique, being often neglected (Duarte Ferreira et al., 2021). Some uncommon results as positive slopes, in particular for dinoflagellates, were reported in this study. As dinoflagellates are mixotrophic (Andersen et al., 1996; Cloern and Dufford, 2005; Jeong et al., 2010), positive slopes in two out of three dilution experiments suggest that dinoflagellates benefit from the presence of grazers, i.e., dinoflagellates have higher prey encounter rates and thus higher feeding/growth rates in the least diluted treatments. This was probably caused by low growth rates at high dilution due to mixotrophy with obligate phagotrophy, as their growth rates were severely depressed when prey densities are highly diluted. According to McManus and Ederington-Cantrell (1992), these low growth rates observed at high dilution levels could be attributed to the slower growth of dinoflagellates when diluted, as the depletion of food resources is more important than release from grazing pressure; this suggests that dinoflagellates in the Ria Formosa are mostly mixotrophs rather than exclusively autotrophic, which presumably would respond to dilution like other phytoplankton. As the specific composition of dinoflagellates was not addressed in this study, this topic needs further assessment. Furthermore, negative potential instantaneous growth rates of dinoflagellates were reported in all dilution experiments, which indicates that in the absence of grazers dinoflagellate abundance decreases instead of increasing, which may suggest dinoflagellates grazing upon the microzooplanktonic component. Considering this, all dinoflagellates should have been considered as microzooplankton rather than phytoplankton, and as this distinction is crucial to understand the role of grazing in Ria Formosa coastal lagoon, future studies should address the grazing impact of dinoflagellates within the microzooplankton community. As it is recognized that most planktonic primary producers, excluding diatoms and Cyanobacteria, have also the potential to be mixotrophic (Flynn et al., 2013; Glibert and Mitra, 2022), it can add complexity to growth and grazing rates estimations of dilution experiments for other phytoplankton groups considered in this study (e.g., cryptophytes, nanoflagellates, etc).

If there was competition for certain inorganic nutrients during incubation, it is possible that mixotrophy could have also influenced the outcomes of the experiments, as it considered an efficient strategy of "eating the competition" (Thingstad et al., 1996), potentially leading to altered nutrient availability and availability of prey for other organisms. Future studies should quantify grazing rates and feeding preferences of mixotrophs, as well as explore their potential trophic interactions with other microbial components, such as phytoplankton and heterotrophic prokaryotes, therefore providing insights into the functional role and ecological significance of dinoflagellates as microzooplankton grazers in the Ria Formosa coastal lagoon.

5.5. Methodological constraints and future studies

5.5.1. Non-expected responses

Negative growth rates can be unexpected and intriguing, since at higher dilutions, the encounter rate between grazers and prey is lower, decreasing the grazing rate and increasing the growth rate of prey. However, negative potential instantaneous growth rates of phytoplankton and consequently negative derivative parameters (e.g. grazing impact (I)) were obtained and have been extensively reported in previous dilution experiments (Bec et al., 2005; Chen et al., 2009; Putland, 2000; Rivkin et al., 1999; Suzuki et al., 2002; York et al., 2011). These negative growth rates are not unrealistic and can be explained. It seems evident that for phytoplankton, the most immediate perturbation may result most likely from a change of light or nutrient conditions and negative growth rates were probably caused by inadequate incubation conditions and methodological artifacts. As reviewed by Zhou et al. (2013), light and temperature regimes during incubation, sampling error, contamination of particle-free water and nutrient limitation could be the possible reasons for the negative μ0 in dilution experiments. Additionally, Suzuki et al. (2002) indicated negative growth caused by incubation conditions, like inappropriate light levels or nutrient limitation. As nutrients were added in excess to dilution bottles to promote constant phytoplankton growth, nutrient limitation does not seem a reasonable reason for these unexpected results. However, light conditions probably limited phytoplankton growth during incubations as photosynthetically active radiation (PAR) intensity at which bottles were exposed during incubation (90 – 120 μmol photons m^{-2} s^{-1}) was lower than the mean light intensity in the mixed layer (Im) typically reported for the inner lagoon: 262 ± 183.3 μmol photons m^{-2} s^{-1} (Domingues, 2022); autumn= 300 μmol photons m^{-2} s^{-1}; winter = 275 μmol photons

m^{-2} s^{-1}; spring = 710 μmol photons m^{-2} s^{-1} (Domingues et al., 2017a); summer = 536 μmol photons m^{-2} s^{-1} (Domingues et al., 2017b). Better measures to mimic the in situ light intensity during incubation should have been taken, as the experimental set-up must be designed to provide illumination such that light neither limits nor inhibits phytoplankton growth. In situ incubation, commonly used in other studies, takes advantage of ambient light and temperature conditions and is recommended to be applied in future studies. The control bottles should be covered with different levels of screens to simulate the mean light intensity in the mixed layer (Domingues and Barbosa, 2009). The spectrum of solar irradiation impacting dilution bottles may further be manipulated based on the material used to make the bottle and/or the incubator (Brum et al., 2014). Furthermore, incubation temperature could have been another factor because the temperature was not regulated to values similar to the in situ conditions; the incubation tank was just left at ambient temperature. The transfer from in situ to incubation temperature could have induced a potential shock in the microbial communities and affected the rates measured (Menden-Deuer et al., 2018). According to Schlüter (1998), negative values of phytoplankton community can be produced by large chain-forming diatoms that dominated the community and formed visible aggregates that settled in incubation bottles. Despite, the negative μ_0 of total phytoplankton community and diatoms being observed in the same season (summer) in this study, this was probably not important because smaller cells, like Cyanobacteria, were dominant. Insufficient mixing before sampling (Zhou et al., 2013) may explain the negative μ_0 of diatoms obtained in summer. It is especially curious that, in the present study, negative phytoplankton growth rates were always observed accompanied with no detectable, non-significant or negative grazing. One should note that microzooplankton grazing rates from dilution experiments are obtained from the apparent growth rates of phytoplankton, which could explain this observation. Still, the also affected grazing rates do not exclude contamination of particle-free water as the reason for the negative μ0 of phytoplankton, despite all material used was acid-cleaned with 10% HCl and washed with deionized water between each use. If contamination occurred it would not only cause phytoplankton death, but also less microzooplankton grazing. Beyond the factors and processes referred, negative growth rates may also occur due to viral lysis, programmed cell death or other processes. Obviously the reason is not clear and this is a topic that needs further assessment. Combining experimental manipulations, field observations, and laboratoty analysis can provide further insights

about the underlying mechanisms driving negative growth rates of preys. Since the dilution method is one of the most used methods for research on microzooplankton grazing, knowing the characteristics of the parameters estimated, as well as measures that should be taken to avoid adverse effects are of great importance for the correct application of the dilution method and estimation of the microzooplankton grazing rates.

In addition to the negative growth rates, the linearity of the dilution equation is sometimes compromised, thus challenging this dilution method and making it not free of criticism. Despite the critical assumption that grazing is a linear function of prey density, other non-expected responses as non-linear or non-significant linear responses to dilution gradients are not uncommon (Grinienė et al., 2016; Modigh and Franze, 2009; Moigis, 2006; Teixeira and Figueiras, 2009). In this study, non-significant and non-linear relationships between apparent growth rates and the dilution factor were observed in some dilution plots. The second assumption of the dilution technique is that phytoplankton are consumed at a rate proportional to their concentration, resulting in a linear response. Non-linear responses may be detected when this assumption is violated, implying that microzooplankton is food-saturated or that microzooplankton abundance changes during the experiment (Dolan and McKeon, 2004). As observed in previous dilution experiments, microzooplankton can exhibit a maximum ingestion rate at high food concentrations, and this maximum rate can remain constant even as prey abundance continues to increase, leading to the occurrence of saturated feeding responses (Gallegos, 1989; Moigis, 2006; Teixeira and Figueiras, 2009). As saturated feeding responses of microzooplankton are generally only encountered in eutrophic waters (Gallegos, 1989), this cannot explain the non-linear responses observed. However, changes in microzooplankton abundance were reported in this study and have been also referred to as a potential explanation for nonlinear or nonsignificant dilution experiments (Dolan et al., 2000; Gallegos, 1989; Moigis, 2006). Furthermore, positive slopes also reported in other studies (Calbet et al., 2011; York et al., 2011) might result from mixotrophy (discussed above), trophic cascade effects during incubation, complex cycling of nutrients between internal and external pools, or filtration contamination (Calbet and Saiz, 2013). Although the mentioned non-expected responses cannot be completely ruled out in this study, considering the microzooplankton dynamics in each non-expected response in detail in future studies may reveal some interesting patterns.

5.5.2. Other methodological concerns

The dilution method relies on the assumption that grazing pressure is proportional to the dilution factor, assuming that the grazers do not experience significant growth or mortality during the incubation period. However, in several experiments, this assumption was not met as changes in the abundances of ciliates and aplastidic nanoflagellates were observed, indicating potential growth or mortality during incubations, possibly related to experimental conditions or interactions with other organisms. For example, nutrient addition has been shown to enhance the growth of grazers caused by an increment in phytoplankton during incubation, which induces changes in microzooplankton grazing pressure (Modigh and Franze, 2009). Trace metals like iron could also limit phytoplankton growth and as there is a lack of studies examining the impact of trace metals on phytoplankton in the Ria Formosa and trace metals were not added to the nutrient cocktail, this could have also somehow affected the outcomes of the dilution experiments (Moigis and Gocke, 2003). Moreover, it is noteworthy a discrepancy between the estimates of grazing mortality on heterotrophic prokaryotes obtained by the two experiments conducted in October, which appears to be attributed more to methodological factors than variations in community composition. In fact, the majority of the studies that applied the modified dilution technique, obtained the virus-free diluent through 30 kDA ultrafiltration (Beckett and Weitz, 2018; Mojica and Brussaard, 2020; Ortmann et al., 2011; Pasulka et al., 2015; Staniewski et al., 2017, 2012; Tsai et al., 2018, 2013), although 10 kDa filters have occasionally been used (Evans et al., 2003; Taira et al., 2009). In this study, the modified dilution technique was employed to estimate viral lysis in the absence of access to 30 kDA ultrafiltration, and an alternative approach was adopted. In this case, the virus-free diluent was obtained by autoclaving the samples. It should be noted that autoclaving, while effective in targeting viral particles, can have significant effects on the quality and bioavailability of DOC, namely DOC composition caused by changes in DOC compounds due to hydrolysis and denaturation of various compounds and colloids during autoclaving (Andersson et al., 2018). As heterotrophic prokaryotes rely on DOM as a carbon source, it is crucial to acknowledge that the use of autoclaving may have introduced artifacts in this study. In this study, autoclaving was chosen as a practical solution and future studies should consider the use of 30 kDA ultrafiltration, exploring alternative virus removal approaches to obtain virus-free diluent or employ complementary techniques to mitigate the potential drawbacks associated with autoclaving. Also concerning modified dilution experiments, the duration of incubation

may have contribute to inconsistent lysis measurements, as it may not cover the full lytic infection cycle of the virus (e.g. lytic cycle extending beyond a 24-h period) (Anderson et al., 2018).

In summary, if prey organisms experience stress due to inappropriate incubation conditions it may result in increased mortality, potentially affecting the outcomes derived from the dilution experiments. Considering this, it is crucial for future experiments to consider all possible sources of mortality beyond those under investigation and employ methodologies that minimize any resultant negative effect. Some assumptions underlying the dilution method may have not been fully met in experiments, highlighting the need for careful interpretation of the grazing rates estimated using this approach in future studies. Experimental artifacts are inevitable in both methodologies used, but it is important to acknowledge their potential impact on results in order to support the assumption underlying the dilution method.

6. Conclusion

Considering the fundamental ecological roles of microbial plankton, it is highly relevant to quantify their grazing and viral mortality rates in order to understand the dynamics of aquatic ecosystems. In common with several other studies, this study has shown microzooplankton grazing to be an important biomass removal process of phytoplankton and heterotrophic prokaryotes, where microzooplankton play a crucial role by consuming a significant portion of these microorganisms, even exceeding the daily production. Studying the majority of the components in the microbial food web simultaneously allows for a comparison of the growth and grazing mortality rates among these components, which provides valuable insights into the fate of these microorganisms within the food web, as well as their dynamics and interactions within the Ria Formosa coastal lagoon. High growth and grazing rates of heterotrophic prokaryotes indicate that the microbial loop contributes to a great proportion of carbon flow in the Ria Formosa coastal lagoon, with heterotrophic prokaryotes serving as a vital link between phytoplankton and higher trophic levels. The most significant prey losses due to grazers in this study concerned smaller phytoplankton groups, emphasizing size as a critical factor in determining grazer-prey relationships in the Ria Formosa, although larger phytoplankton cells were also highly consumed. So, this study highlights the importance of all microbial components in the overall functioning of the microbial food web and confirms the relevance of microzooplankton in the Ria Formosa coastal lagoon. Furthermore, viral lysis interacts with microzooplankton as sources of heterotrophic prokaryotes mortality in Ria Formosa. As far as is known, this is the first evidence showing that viruses are an important source of mortality of heterotrophic prokaryotes in the Ria Formosa coastal lagoon. Considering the relevance of this important ecosystem, future studies are needed to explore each of these mortality factors individually, under varying conditions and across time and space, to evaluate their relative magnitude and to improve models of carbon flow in the Ria Formosa coastal lagoon. Studying microbial growth and mortality processes uncovers valuable information about the roles microorganisms play in nutrient cycling, carbon sequestration, and energy transfer within the microbial food web, as it helps to understand the dynamics of microbial communities, their interactions with other organisms, and their impact on the overall productivity of aquatic ecosystems. In addition, it can contribute to improving models and predictions related to climate change, ocean acidification, and other global processes.

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