BÁRBARA ANDREIA DOS SANTOS DE SOUSA E SÁ

TROPHIC DYNAMICS BETWEEN PHYTOPLANKTON AND MICROZOOPLANKTON IN THE RIA FORMOSA COASTAL LAGOON SYSTEM

UNIVERSITY OF ALGARVE

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Thesis to obtain the degree of Master in Marine Biology

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Thesis authorship declaration

I declare to be the author of this thesis which is original and inedited. The authors and articles consulted are properly cited in the text and are included in the references list.

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(Bárbara Andreia dos Santos de Sousa e Sá)

Acknowledgments and Dedication

To Dr. Professor Ana Barbosa and Dr. Rita Domingues by the orientation.

To Vanessa Viegas and Dr. Professor Duarte Duarte for the help in the field and laboratory.

To Joana Duarte by the friendship, support, motivation and the laughs.

To my parents by the patience, support, motivation and friendship.

I dedicate this work to my parents and friends

Resumo

O fitoplâncton constitui a base da rede alimentar da maior parte dos ecossistemas aquáticos, e apresenta um importante contributo na produção de oxigénio e consumo de dióxido de carbono, sendo por isso um grupo planctónico com grande relevância. A sua abundância reflete a interação entre dois modos distintos de regulação, o *bottom-up* e o *top-down* (como por exemplo, a herbivoria). A predação exercida pelo microzooplâncton é atualmente considerada a principal fonte de mortalidade do fitoplâncton. Assim, a compreensão da dinâmica trófica entre estes dois grupos planctónicos é essencial para um melhor entendimento do funcionamento e variabilidade dos ecossistemas aquáticos. A dinâmica trófica entre o fitoplâncton e o microzooplankton no sistema costeiro lagunar Ria Formosa (sul de Portugal) foi analisada por uma amostragem sazonal, durante um ano. A Ria Formosa é um sistema costeiro lagunar raso, e um dos ecossistemas mais importantes e vulneráveis em Portugal, devido a ser influenciado por diferentes fatores que afetam a sua produtividade primária. No entanto, é um ecossistema muito produtivo e que consegue fornecer serviços ecológicos, económicos e culturais. A metodologia aplicada nas experiências foi a técnica de diluição, com e sem adição de macronutrientes inorgânicos, em dois locais distintos da Ria Formosa (zona interior do sistema lagunar e zona de contacto com a região costeira adjacente), e em diferentes períodos do ano (primavera e outono). A análise da biomassa fitoplanctónica foi realizada por fluorimetria, enquanto as análises da composição e abundância de fitoplâncton e microzooplâncton foram por microscopia de epifluorescência (< 20 µm), e microscopia de inversão (> 20 µm). Os dados obtidos foram usados na determinação das taxas de crescimento instantâneo potencial e *in situ* do fitoplâncton, a produção fitoplanctónica e a herbivoria, sob a comunidade geral e grupos funcionais específicos de fitoplâncton. A temperatura e salinidade tiveram variações sazonais, não se correlacionaram positivamente e não ocorreu estratificação da coluna de água. No entanto, os seus valores estiveram dentro dos padrões normais da Ria Formosa. A estação interior da Ria Formosa teve elevados valores de turbidez e de PAR, enquanto a estação exterior teve valores reduzidos. A nínel sazonal, a turbidez foi superior no outono e o PAR foi superior na primavera. A Chl *a* teve grandes variações sazonais e entre estações de amostragem, de tal forma que os valores obtidos durante a primavera de 2015 seguiram os padrões de Chl *a* para a Ria Formosa, enquanto os valores da primavera de 2016 não seguiram de todo os padrões. As Cianobactérias dominaram a comunidade fitoplanctónica em ambas as estações do ano. Das seis experiências realizadas, quatro resultaram em respostas inesperadas relativamente à relação entre o crescimento aparente da comunidade fitoplanctónica e os fatores de diluição, tendo sido obtidas regressões lineares não significativas ou regressões lineares positivas. A abundância das Cianobactérias correlacionou-se positivamente com a temperatura, no entanto, as taxas de crescimento e de predação foram inferiores aos restantes grupos fitoplanctónicos, assim as Cianobactérias não foram uma presa preferencial para o microzooplânton. No entanto, o impacto do microzooplâncton na produção das Cianobactérias foi superior a 100%, logo a predação por protistas fagotróficos é suficiente para controlar o crescimento destes organismos. O Picofitoplâncton eucariótico teve taxas de crescimento e de predação semelhantes às das Cianobactérias, no entanto as Cianobactérias foram dominantes. As Criptofíceas foram pouco abundantes e apresentaram a menor taxa de crescimento fitoplanctónica. Para além disso, a diluição teve um efeito negativo no seu crescimento. A sua predação foi superior na primavera, tal como a predação dos outros nanoflagelados plastidicos, então parece que a

preferência de nanoflagellados plastídicos ocorre apenas na primavera, quando a abundância destes organismos é maior. Dos flagelados plastídiscos, os outros nanoflagelados foram os que tiveram maior taxa de crescimento, porém esta taxa foi inferior à das diatomáceas. A abundância dos outros nanoflagelados plastidicos correlacionou-se positivamente com a abundância de Ciliados, por isso este grupo foi um exemplo de que a predação pode estimular o crescimento do fitoplâncton. A abundância de Euglenofíceas foi maior na primavera e correlacionou-se positivamente com a temperatura e intensidade luminosa. A sua taxa de crescimento foi inferior à dos restantes grupos fitoplanctónicos, excepto as Criptofíceas. E relativamente à taxa de predação houve uma variação sazonal, dado que na primavera o impacto do microzooplâncton foi de 70%, enquanto durante o outono foi superior a 100%, assim durante a primavera outros processos de remoção de biomassa deverão ter ocorrido. Os Dinoflagelados Plastidicos foram dominados por um Gymnodinoide, provavelmente tóxico. A abundância de dinoflagelados plastidicos correlacionou-se positivamente com a temperatura e intensidade luminosa, e negativamente com as diatomáceas. O impacto do microzooplâncton foi superior na estação exterior, no entanto a abudância destes fitoplanctontes foi inferior no interior da Ria Formosa, então provavelmente, outros processos de remoção de biomassa terão ocorrido na estação interior. As taxas de crescimento e predação dos dinoflagelados plastidicos foram inversamente proporcionais às das Euglenofíceas, então parece que entre estes grupos específicos, o microzooplâncton prefere o mais abundante, sendo por isso oportunista. As diatomáceas cêntricas e pinuladas foram os fitoplanctontes com a maior variabilidade sazonal. A taxa de crescimento das diatomáceas cêntricas foi superior no outono e coincidiu com um declínio de dinoflagelados aplastidicos, demonstrando que estes predadores são essenciais para controlar as diatomáceas cêntricas. A taxa de predação corelacionou-se positivamente e significativamente com a abundâncias de Ciliados, demostrando que estes foram os principais predadores das diatomáceas cêntricas. A abundância e a taxa de crescimento das diatomáceas pinuladas correlacionaram-se positivamente com a temperatura e com um declínio de dinoflagelados aplastidicos, demonstrando que estes predadores são essenciais para controlar as diatomáceas pinuladas. As diatomáceas pinuladas tiveram uma taxa de crescimento superior à das cêntricas, a nível sazonal, no entanto a abundância de cêntricas foi superior. Para além disso, o impacto do microzooplâncton foi superior nas cêntricas, logo outros processos de remoção de biomassa terão ocorrido sobre as pinuladas para justificar a abundância inferior apesar do elevado crescimento. Geralmente, os predadores preferem pequenos fitoplanctontes, no entanto neste estudo, as diatomáceas cêntricas e pinuladas foram o grupo fitoplanctónico mais predado. O microzooplâncton removeu entre 44.83% e valores superiores a 100% da produção fitoplanctónica por dia. A taxa de crescimento do fitoplâncton foi entre 0.05 d^{-1} e 2.22 d^{-1}.

Palavras-chave: fitoplâncton, microzooplâncton, mortalidade, predação, método de diluição, Ria Formosa

Abstract

Phytoplankton is a planktonic group with great importance to the aquatic ecosystems, because constitutes the base of the food web, and have an important play role in the oxygen production and carbon dioxide consumption. Grazing by phagotrophic protists (microzooplankton) is considered the major mortality source of phytoplankton in the oceans. Thus, understanding the trophic dynamics between these two planktonic groups is essential for a better understanding of the functioning and variability of aquatic ecosystems. Trophic dynamics between phytoplankton and microzooplankton in the Ria Formosa coastal lagoon system (south Portugal) was studied through a seasonal sampling, with a period of one year. The methodology used in the experiments was the dilution technique, with and without enrichment of inorganic macronutrients, in two distinct places of Ria Formosa (inner station of the lagoon system and an outer station which is in contact with the adjacent coastal region) and in different periods of time (spring and autumn). The analyses of phytoplankton biomass were done through fluorimetry, while the phytoplankton and microzooplankton composition and abundance were through epifluorescence microscopy (< 20 µm) and inverted microscopy (> 20 µm). The temperature and salinity values were under the Ria Formosa normal standers.
Chl *a* had high seasonal variations, such that values obtained during the spring of 2015 followed the Chl *a* standards for the Ria Formosa, while values for the spring of 2016 did not. Cyanobacteria dominated the phytoplankton community in both seasons. It were performed six sets of experiments, and four of them had unexpected responses regarding the relationship between dilution factors and the apparent growth rate of phytoplankton. It was obtained non-significant linear regressions and positive linear regressions, showing that sometimes the dilution has a negative effect on phytoplankton. Microzooplankton removed, daily, between 44.83% and more than 100% of phytoplankton production. The growth rate of phytoplankton was between 0.05 d^{-1} and 2.22 d^{-1}.

Keywords: phytoplankton, microzooplankton, mortality, grazing, dilution method, Ria Formosa

Contents

1. Introduction
- **1.1.** Phytoplankton 2
- **1.2.** Microzooplankton 3
- **1.3.** Techniques for measuring microzooplankton grazing 4
- **1.4.** Ria Formosa: processes and plankton 6
- **1.5.** Study objectives 8

2. Materials and Methods
- **2.1.** Study area 11
- **2.2.** Sampling strategy 12
- **2.3.** Experimental design 13
- **2.4.** Quantification of phytoplankton and microzooplankton
  - **2.4.1.** Chlorophyll *a* concentration 14
  - **2.4.2.** Abundance and composition of phytoplankton and microzooplankton 15
- **2.5.** Phytoplankton community and group-specific growth rate and microzooplankton grazing 17
- **2.6.** Statistical analyses 19

3. Results
- **3.1.** Initial conditions
  - **3.1.1.** Temperature, salinity and water transparency 21
  - **3.1.2.** Chlorophyll *a* concentration 22
  - **3.1.3.** Abundance and composition of phytoplankton and microzooplankton 23
- **3.2.** Final conditions
  - **3.2.1.** Phytoplankton community growth rate and microzooplankton grazing 27
  - **3.2.2.** Phytoplankton group-specific growth rates and microzooplankton grazing 33
  - **3.2.3.** Microzooplankton growth 39

4. Discussion
- **4.1.** Critical evaluation of the experimental strategy 43
- **4.2.** Initial conditions
  - **4.2.1.** Temperature, salinity and water transparency 44
  - **4.2.2.** Chlorophyll *a* concentration 46
  - **4.2.3.** Abundance and composition of phytoplankton and microzooplankton 48
- **4.3.** Final conditions
  - **4.3.1.** Phytoplankton community growth rate and microzooplankton grazing 49
  - **4.3.2.** Phytoplankton group-specific growth rates and microzooplankton grazing 52
    - **4.3.2.1.** Cyanobacteria *Synechococcus* 55
    - **4.3.2.2.** Eukaryotic Picophytoplankton 57
    - **4.3.2.3.** Cryptophyceae 59
    - **4.3.2.4.** Other Plastidic Nanoflagellates 60

4.3.2.5. Euglenophyceae 63
4.3.2.6. Plastidic Dinoflagellates 64
4.3.2.7. Centric Diatoms 67
4.3.2.8. Pennate Diatoms 69
4.3.3. Microzooplankton growth 72
4.3.3.1. Aplastidic Nanoflagellates 72
4.3.3.2. Ciliates 74
4.3.3.3. Aplastidic Dinoflagellates 76

5. Conclusions 79

6. References 86

Annex 115

List of figures

Chapter 2

Figure 2.1 – Location of the two sampling stations in the Ria Formosa coastal lagoon (image adapted from Barbosa. 2006). The arrow indicates the inner station and the rectangle the outer station. 12

Figure 2.2 – Schematic representation of experimental treatments with different proportions of diluent (white) and sample (blue). The four different sample dilutions were (A) +0.10, (B) +0.25, (C) +0.50, (D) +1.00 and (E) -1.00. The (+) means enriched with nutrients and the (-) means no addition of nutrients (from Barbosa & Domingues, 2009). 14

Chapter 3

Figure 3.1 – Chlorophyll *a* concentration with the standard error in the two station of Ria Formosa at the sampling day. NA: not available. 23

Figure 3.2 – (A) Annual percentage of phytoplankton group specific in the inner station of Ria Formosa at the sampling day. (B) Annual percentage of phagotrophic protists in the inner station of Ria Formosa at the sampling day. 25

Figure 3.3 – (A) Annual percentage of phytoplankton group specific in the outer station of Ria Formosa at the sampling day. (B) Annual percentage of phagotrophic protists in the outer station of Ria Formosa at the sampling day. 26

Figure 3.4 – (A) Apparent growth rate of phytoplankton community and dilution factors in the inner zone of the Ria Formosa in spring 2015. (B) Apparent growth rate of phytoplankton community and dilution factors in the outer zone of the Ria Formosa in spring 2015. The open circles represent data that was not used to adjust the regression lines. 28

Figure 3.5 – (A) Apparent growth rate of phytoplankton community and dilution factors in the inner zone of the Ria Formosa in autumn 2015. (B) Apparent growth rate of phytoplankton community and dilution factors in the outer zone of the Ria Formosa in autumn 2015. 30

Figure 3.6 – (A) Apparent growth rate of phytoplankton community and dilution factors in the inner zone of the Ria Formosa in spring 2016. (B) Apparent growth rate of phytoplankton community and dilution factors in the outer zone of the Ria Formosa in spring 2016. 32

Figure 3.7 – (A) Potential instantaneous growth rate of phytoplankton group-specific in the Ria Formosa in spring 2015. (B) Grazing rate of phytoplankton group-specific in the Ria Formosa in spring 2015. NA: not available. 34

Figure 3.8 – (A) Potential instantaneous growth rate of phytoplankton group-specific in the Ria Formosa in autumn 2015. (B) Grazing rate of phytoplankton group-specific in the Ria Formosa in autumn 2015. NA: not available. 36

Figure 3.9 – (A) Potential instantaneous growth rate of phytoplankton group-specific in the Ria Formosa in spring 2016. (B) Grazing rate of phytoplankton group-specific in the Ria Formosa in spring 2016. NA: not available. 38

Figure 3.10 – (A) Microzooplankton growth in the inner station of the Ria Formosa during spring 2015. (B) Microzooplankton growth in the outer station of the Ria Formosa during spring 2015. NA: not available. 39

Figure 3.11 – (A) Microzooplankton growth in the inner station of the Ria Formosa during autumn 2015. (B) Microzooplankton growth in the outer station of the Ria Formosa during autumn 2015. 40

Figure 3.12 – (A) Microzooplankton growth in the inner station of the Ria Formosa during spring 2016. (B) Microzooplankton growth in the outer station of the Ria Formosa during spring 2016. NA: not available. 41

Chapter 4

Figure 4.1 – Five types of responses between the apparent growth of phytoplankton and dilution factors: (A) insignificant, (B) negative linear, (C) negative saturated, (D) saturated increasing and (E) positive linear (from Dix & Hanisak, 2015). 50

List of tables

Chapter 3

Table 3.1 – Physical and chemical variables *in situ* and estimated in the two stations of Ria Formosa at the sampling day. 22

Table 3.2 – Group specific composition of phytoplankton and microzooplankton and abundance. N.A.: not available. All the numbers must be multiple by 10^3 to have the real value (cel.L^{-1}). 24

List of abbreviations

Chl *a*	Chlorophyll a
DON	Dissolved organic nitrogen
DOP	Dissolved organic phosphate
Im	Average light intensity in the mixed layer
PAR	Photosynthetic active radiation

Chapter 1

1. Introduction

1.1. Phytoplankton

Phytoplankton is a group of prokaryotic and eukaryotic photosynthetic organisms that drift in the water column with the currents (Bidle & Falkowski, 2004; Ajani & Rissik, 2009). Phytoplankton includes Cyanobacteria, diatoms, dinoflagellates, coccolithophore and others flagellates (Ajani & Rissik, 2009). Phytoplankton can also be classified according to cell size, as Picophytoplankton (0.2 to 2.0 µm), Nanophytoplankton (2 to 20 µm) and microphytoplankton (20 to 200 µm). Nevertheless, some taxa attain up to 4000 µm (Ajani & Rissik, 2009). Cell dimensions are relevant because they control, directly and indirectly, the pathways and efficiencies of energy transfer from primary producers to consumers (aquatic food webs), including those sustaining upper trophic levels (Cloern & Dufford, 2005). This microscopic algae have many distinct biochemical contents (Cloern & Dufford, 2005), thus they can be organized into functional groups, pico-autotrophs, nitrogen-fixers, calcifiers, silicifiers and dimethyl sulfide (DMS) producers (Nair *et al.*, 2008).

Phytoplankton is the main primary producer of marine ecosystems, that is, the producers of autochthonous organic material that will fuel aquatic food webs. However, they are as well important to the Earth's primary production, because phytoplankton can fix about 50 Gt of carbon per year, as much as the tropical rainforests, thus representing almost half of global primary production of the planet (Falkowski *et al.*, 2004). So, phytoplankton is a key player for aquatic systems' functioning. Besides this function, phytoplankton has significant impacts on water quality and play vital roles in many ecosystem processes, such as in biogeochemical processes, mediating cycling, sequestration and exportation of inorganic and organic compounds. Moreover, phytoplankton is an excellent model systems to address fundamental ecological questions (Litchman & Klausmeier, 2008; Pereira Coutinho *et al.*, 2012), and paleoenvironmental reconstructions (Barbosa, 2009). Then this planktonic group plays a key role in regulating the ecological conditions and changes, and can be used to understand and predict the functioning and production of aquatic ecosystems and the possible responses to natural and anthropogenic-induced changes (Cloern & Dufford, 2005; Smetacek & Cloern, 2008).

Even though phytoplankton is biologically and functionally very diverse, it is regulated in the same way, by environmental factors that regulate phytoplankton growth (bottom-up regulation) or phytoplankton loss (top-down regulation). However, it is also affected by anthropogenic activities, such as eutrophication and climate change. Thus the spatial and temporal variability of phytoplankton in aquatic ecosystems reflects the interaction between abiotic and biotic factors (Domingues, 2010).

Bottom-up regulation of phytoplankton includes the effects of resources that control cell replication, such as nutrients, light, temperature, pH, salinity and oxygen concentration, and phytoplankton cells compete among each other for these resources (Domingues, 2010). Nutrients are usually considered the most important factor regulating phytoplankton growth, because they are essential for cell growth, some in relatively large amounts, the macronutrients (e.g., C, H, O, N, P, Si, Mg, K, Ca), and others in much smaller quantities, the micronutrients or trace elements (e.g., Fe, Mn, Cu, Zn, Ba, Na, Mo, Cl, V, Co) (Parsons *et al.*, 1984). Most of these elements are available in sufficient amounts in marine and freshwaters, but others, particularly nitrogen (N), phosphorus (P) and silicon (Si, required only by Si-containing cells such as diatoms), may occur in natural waters in extremely low concentrations for phytoplankton growth. Therefore, these elements, which are taken up by cells mostly in their inorganic form, will often limit phytoplankton growth (Parsons *et al.*, 1984; Domingues, 2010).

Top-down regulation of phytoplankton involves mortality and other loss processes that decrease the number of phytoplankton cells by mortality or removal (Reynolds, 1997). These processes include grazing, cell lyses, viral lyses, cell apoptosis, advection and sinking. Grazing by phagotrophic protists (microzooplankton) are considered the major mortality source of phytoplankton in the oceans (Calbet & Landry, 2004).

1.2. Microzooplankton

Microzooplankton is a group of heterotrophic and mixotrophic organisms that attain up to 200 µm. Includes protists (e.g., ciliates), dinoflagellates and ameboid forms (e.g., foraminifera), small metazoans (e.g., copepods nauplii), and some meroplanktonic larvae (Redden *et al.*, 2009).

Usually, microzooplankton is classified as a primary consumer (herbivore), because these organisms occupy a key position in marine food webs as large consumers of primary production (Calbet & Landry, 2004), consuming, on average, 62% of the daily production of phytoplankton (Schmoker *et al.*, 2013), is an important link between primary producers and higher trophic levels (e.g. copepods) in sub-polar and polar waters as well as in temperate and tropical waters (Levinsen & Nielsen, 2002; Calbet & Saiz, 2005; Campbell *et al.*, 2009; Sherr *et al.*, 2013), and as key components of the microbial loop (Sherr & Sherr, 2002).

The temporal variability of microzooplankton herbivory can determine the onset, duration and termination of phytoplankton blooms, sometimes dominated by relatively large and/or toxic cells (Sautour *et al.*, 2000; Strom *et al.*, 2001; Calbet *et al.*, 2003; Odate & Imai, 2003; Clough & Strom, 2005; Sun *et al.*, 2013).

1.3. Techniques for measuring microzooplankton grazing

The impact of the microzooplankton grazing on marine phytoplankton started to be measured through an indirect estimation, from production budgets of phytoplankton (Riley, 1956) and energetic requirements of organisms based on their size (Beers & Stewart, 1971). Then the direct estimations were developed, like the extrapolation from laboratory-determined feeding relationships to field situations of known species abundance of microzooplankton and size composition of potential prey (Heinbokel & Beers, 1979). However, this approach is only viable in cases of well-known feeding rates, behavior and prey preferences, and the available data is not extensive neither accurate, therefore the method is laborious and unsuited for the estimation of total microzooplankton impact on phytoplankton (Landry & Hassett, 1982).

A more direct technique was presented by Capriulo and Carpenter (1980), where the natural assemblage of plankton is divided into two size components, one fraction contains a few microzooplankton, but the majority is their preferred food and serves as a control, and the other fraction contains only plankton with more than 35 µm. Then grazing rates are measured, relative to the control, in a mixture of the smaller and larger size fractions. Nevertheless, this method has two limitations, one is that phytoplankton abundance and size composition differ between experimental and control containers,

thus the interpretation of grazing impact from general measures of phytoplankton biomass is ambiguous. The second is that the technique measures grazing impact only for the microzooplankton community bigger than 35 µm (Landry & Hassett, 1982).

According to Landry and Hassett (1982), the techniques developed until then to determine the grazing exerted by microzooplankton were problematic. Therefore they developed a new technique to estimate the herbivory by microzooplankton in natural seawater communities, the dilution method. This approach is the most commonly used, and is a useful method to assess the microzooplankton grazing impact and phytoplankton growth rates (Strom *et al.*, 2001; Moigis & Gocke, 2003; Calbet & Landry, 2004; McManus *et al.*, 2006; Paterson *et al.*, 2007 and 2008).

This technique consists in the manipulation of the encounter rates between phytoplankton and their microzooplankton grazers through a series of different dilutions, which is prepared using particle-free water from the same source, to estimate potential and *in situ* instantaneous growth rate of phytoplankton, and grazing rate exerted by microzooplankton (Landry & Hassett, 1982).

Also the changes in phytoplankton abundance can be determined by the instantaneous coefficients of population growth and mortality by predation. This is a common assumption in most studies when regarding grazing of phytoplankton. Taking this information into account, it is expected to obtain an inverse relationship between the dilution factor and the growth rate of phytoplankton. This inverse relationship will generate a negative slope, which is the predation coefficient.

The approach relies on three basic assumptions concerning the nutrients, phytoplankton and microzooplankton. The first states that individual phytoplankton growth rate is limited neither by density dependent nor by nutrients during the course of the experiment, which implies that instantaneous growth rate of the prey community is assumed to remain constant throughout the dilution series. For that reason, in the dilution series, nutrients were added in the samples to compare and to correct for nutrient-replete growth rates. The second assumes that phytoplankton grow is exponential. The third assumption arrogates that the probability of a phytoplankton cell being consumed is directly related to the rate of contact between consumers and preys.

This means that consumers are not saturated with natural density of prey and the number of prey ingested by a particular consumer is linearly related to prey density (Landry & Hassett, 1982). Other implication is that grazer abundance relative to dilution level does not change over the incubation period (Dix & Hanisak, 2015).

The advantages of the dilution method are that is a simple method and requires little manipulation of the natural communities, except the dilution itself and the addition of nutrients to satisfy the assumption that the phytoplankton growth rate is not limited by nutrients nor by density dependence (Dolan *et al.*, 2000). Furthermore, with the development of this technique it was possible the determination of the phytoplankton saturation, this is when grazing by microzooplankton becomes irrelevant (Redden *et al.*, 2002). Another possible study is the observation of specific mortality of phytoplankton by grazing. In studies of Obayashi and Tanoue (2002), it was found that the microzooplankton has a preference for green microalgae.

Since its introduction, the dilution technique has been widely applied and used in combination with taxon-specific pigment analysis by high-performance liquid chromatography (HPLC) (e.g. Burkill *et al.*, 1987; Latasa *et al.*, 1997; Landry *et al.*, 1998; Obayashi & Tanoue, 2002; Selph *et al.*, 2011) and with flow cytometry (e.g. Landry *et al.*, 1995a; Kuipers & Witte, 2000; Liu *et al.*, 2002; Selph *et al.*, 2011). Both combinations can provide growth and mortality rates associated with specific groups of phytoplankton, which allows the understanding of trophic interactions in complex food webs and subsequent carbon dynamics. However in this study, neither combination was used.

1.4. Ria Formosa: processes and plankton

The Ria Formosa is a shallow coastal lagoon system (Andrade *et al.*, 2004; MCOA, 2008), located at the interface between land and sea, consequently is influenced by different factors that affect the primary productivity of these systems, such as nutrients inputs (Brito *et al.*, 2010). Shallow coastal lagoons are dynamic and highly valuable systems in the land-sea interface, and normally have a strong salinity range from salty to brackish waters, depending on the freshwater inputs and the level of water exchange with the sea (Kjerfve *et al.*, 1996). There are occasions when lagoons have hypersaline

waters due to evaporation, which is very common in systems such as Ria Formosa (Kjerffve *et al.*, 1996; Brito *et al.*, 2010).

Given the dynamic conditions of the coastal lagoons, especially in terms of the physical characteristics and salinity regime, the number of species present in these lagoons is very restricted when compared to more stable habitats, such as the marine habitats (Joint Nature Conservation Committee: JNCC; Pecqueur *et al.*, 2011). This kind of habitat can provide valuable ecosystem services and, in conjugation with the fact that shallow coastal lagoons are relatively uncommon in Europe, justify the classification of coastal lagoons as priority habitats in the European Union (Gönenç and Wolfin, 2005).

Ria Formosa is a very productive ecosystem (Santos *et al.*, 2004; Newton & Icely, 2006; Cunha & Duarte, 2007), with an average primary production of ~1400 g C $m^{-2}.yr^{-1}$ (Sprung *et al.*, 2001), and the phytoplankton is the main contributor to this mean (Duarte *et al.*, 2008). Due to its high productivity, this lagoon system can provide many ecological, (e.g. a breeding, wintering and staging area for various species of water birds and nursery for aquatic species), economical (e.g. nursery for aquatic commercial species like cephalopods, fishes, crustaceans and bivalves, aquaculture and salt extraction) and cultural (e.g. esthetic value for tourism) goods and services (MCOA, 2008; Ribeiro *et al.*, 2008; Anthony *et al.*, 2009; Barbosa, 2010; Brito *et al.*, 2010).

The multiple services which Ria Formosa can offer, allows an increase of urban development, tourism and agriculture, and that can lead to a deterioration of the water quality (decreased oxygen saturation, increased concentration of fecal coliform and others, and increased organic matter) and eutrophication (increased concentration of dissolved inorganic nutrients in the water column), due to the discharge of untreated or partially treated domestic and industrial sewage, and agricultural runoff (Dionísio *et al.*, 2000; Newton *et al.*, 2003; Newton & Mudge, 2003; Santos *et al.*, 2004; Mudge *et al.*, 2007; Mudge *et al.*, 2008; Cabaço *et al.*, 2008). However, the status of this lagoon system can vary from "bad" to "good" depending on the criteria. Based on the European Environmental Agency (nutrients concentration) has a "poor" to "bad" status, but according with the United States Estuarine Eutrophication Assessment has a "good" status (Newton *et al.*, 2003). Other two criteria to classify the status of the lagoon is

with the diversity of benthic macrofauna and the dissolved oxygen, and to have a "good" status, they have to be high (Gamito, 2008; Newton *et al.*, 2009).

The processes that reduce the abundance of phytoplankton in the Ria Formosa include pelagic and benthic predation and exportation with tidal currents. However, predation by microzooplankton is the main source of mortality of phytoplankton, and their impact is more significant for the Eukaryote Picophytoplankton and Plastidic Nanoflagellates (Barbosa, 2006). When this reduction processes fails, and there is no limitation of nutrients, it may occur a harmful algal bloom (HAB). Which will increase the toxins concentration in the water column, and consequently the capture of bivalves will be prohibited, because the intake of contaminated bivalves can cause serious health problems (FAO, 2011).

Despite the relevance, there are only two studies addressing microbial trophic dynamics, including phytoplankton herbivory, in the Ria Formosa: Thiele-Gliesche (1992) and Barbosa (2006). Other studies addressing phytoplankton in the Ria Formosa were regarding the stressed spatial and seasonal variability of phytoplankton composition, biomass and production (Loureiro *et al.*, 2006), the influence of nutrient enrichment (Falcão & Vale, 2003; Edwards *et al.*, 2005; Loureiro *et al.*, 2005; Newton & Mudge, 2005) and the phytoplankton growth and microzooplankton grazing under increased temperature (Barreto, 2012).

1.5. Study objectives

The objectives of this study were to determine the phytoplankton group-specific growth and grazing rates and the grazing impact of microzooplankton on phytoplankton in the Ria Formosa coastal lagoon, a protected coastal ecosystem. In order to have a better analysis of the trophic dynamics in Ria Formosa, it were chosen two distinct zones, an inner zone of the lagoon system and outer zone, and two different seasons, spring and autumn. What makes these zones so distinct are their locations, one is more protected while the other is not, and is therefore more influenced by the adjacent coastal zone. This factor will change the biotic and abiotic conditions between seasons. For the seasons, only spring and autumn were chosen because the study period was not

sufficient for an annual study but also because these seasons are distinct periods of time, excellent for seasonal comparison. The method used was the dilution technique.

Chapter 2

2. Materials and Methods

2.1. Study area

The Ria Formosa is located on the south coast of Portugal and extends approximately 55 km from east to west, and 6 km from north to south, and has an average depth of 2 m (Andrade *et al.*, 2004; MCOA, 2008). It is characterized by a semi-closed aquatic ecosystem with a semi-diurnal tidal regime and a mesotidal system of multiple water inlets, thus being very dynamic, and is partially separated by barriers Ocean Islands (Newton & Mudge, 2003; Barbosa, 2010). This coastal lagoon is considered priority area for conservation within the international legislation, being part of the Ramsar and the Natura 2000 European conservation networks (Ramsar; European Comission).

The climate of the study area is Mediterranean, with wet winters and hot dry summers. The atmospheric temperature varies between 8°C and 30°C, with average values between 16ºC and 20ºC. The annual insolation ranges between 3000 and 3200 hours, while the precipitation is concentrated from November to February, ranging between 400 and 600 mm (Serpa *et al.*, 2005). This ecosystem is in a region that was classified by the Intergovernmental Panel on Climate Change (IPCC, 2007) as being very vulnerable to climate change.

The Ria Formosa is one of the most important and vulnerable ecosystems in Portugal (Domingues *et al.*, 2015), and it is strongly subjected to anthropogenic activities and natural nutrient inputs. The various anthropogenic factors are urbanization, intensive agriculture, aquaculture and coastal engineering (Newton *et al.*, 2003). The natural nutrient inputs are due to regular upwelling events that occur in the coastal area adjacent to the lagoon system, and which influence the outer area of the Ria Formosa and may extend to the inland areas. These events are most often between March and October (Loureiro *et al.*, 2006; Relvas *et al.*, 2007; Barbosa, 2010; Cravo *et al.*, 2014).

2.2. Sampling strategy

Sampling was conducted between May 2015 and May 2016, and two stations were sampled: an inner zone and an outer zone (see Fig.2.1). The inner station is located in a confined area of the western sector of the Ria Formosa (Faro beach), has an average depth of 2.5 m and is located in an area of subtidal stands of *Cymodocea nodosa*. The outer station is located at the main inlet (Barra Faro-Olhão), next to the navigation buoy nº 2, in contact with the adjacent coastal zone and presents an average depth of 15 m. The samples were collected in different tidal stages ebb-tide for the inner station and flood-tide for the outer station.

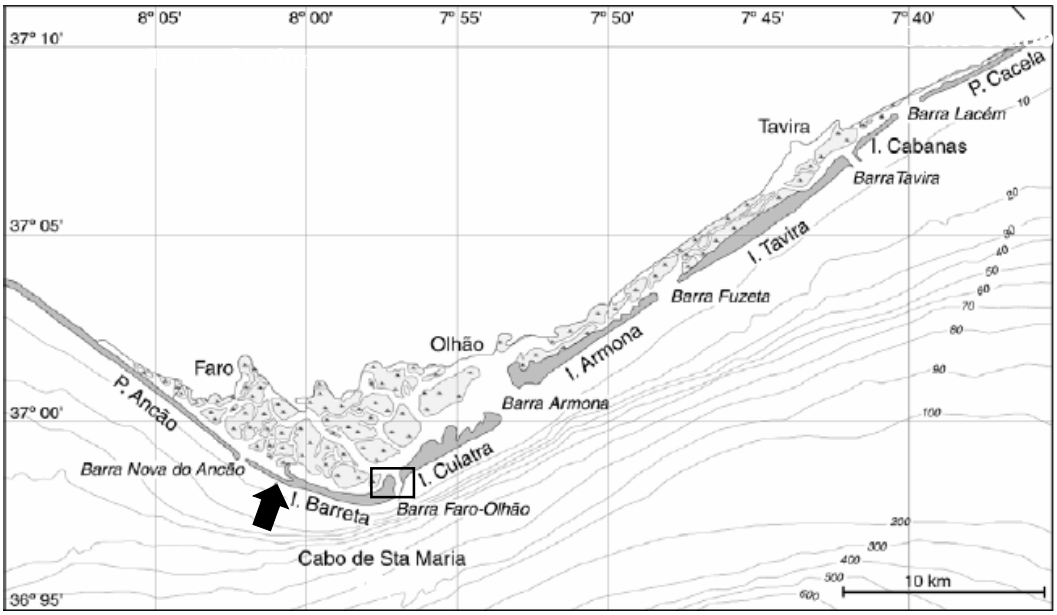

Figure 2.1 – Location of the two sampling stations in the Ria Formosa coastal lagoon (image adapted from Barbosa. 2006). The arrow indicates the inner station and the rectangle the outer station.

The Ria Formosa has a reduced depth and has an absence of stratification of the water column (Benoliel, 1984, 1985, 1989; Newton & Mudge, 2003), so the samples were collected only at the surface level, about 10 cm from the surface. Water samples were collected with the aid of a plastic collector of 5 L, previously washed 3 times with water from the station, sealed and transported to the laboratory. During the transportation, the bottles were protected from the sun light and turbulence. For each sampling, *in situ* water temperature and salinity were measured with a multiparameter sensor and the

Secchi depth was measured for subsequent determination of the average photosynthetic active radiation (PAR) light intensity in the mixed layer (I_m).

The I_m was considered as a percentage of the light intensity in the surface (I_0), using values of the mixed layer depth (Z_m) and the vertical light extinction coefficient (K_e), as the equation 1 (Kirk, 1986). So, for the outer station it was used the equation 2 (non-turbid, Z_s > 5 m; Poole & Atkins, 1929) and for the inner station was the equation 3 (turbid aquatic systems, Z_s < 5 m; Holmes, 1970), because the stations have different values of turbidity.

$$I_m = (I_0 \times (1 - e^{-K_e \times Z_m})) \times (\frac{1}{K_e \times Z_m}) \quad \text{Equation 1}$$

$$K_e = \frac{1.7}{Z_s} \quad \text{Equation 2}$$

$$K_e = \frac{1.4}{Z_s} \quad \text{Equation 3}$$

Where:

I_0 – PAR intensity at the surface (units: µmol photons.m^{-2}.s^{-1})

I_m – Average light intensity in the mixed layer (units: µmol photons.m^{-2}.s^{-1})

K_e – Vertical light extinction coefficient (units: m^{-1})

Z_m – Mixed layer depth (units: m^{-1})

Z_s – Secchi disk depth (units: m^{-1})

2.3. Experimental design: dilution method

The entire procedure was performed under low light conditions. Two different methods of filtration were used to prepare particle-free water (diluent), Whatman GF/F glass fiber filters with a pore of 0.7 µm and a cartridge. Four different sample dilutions were prepared (Dil. 0.10, 0.25, 0.50 and 1.0) in 10 L Thermo Scientific Nalgene bottles, and in order to avoid differences in nutrient concentration among dilutions during the experiment, the treatments were enriched with inorganic macronutrients (+5 µM of ammonia (NH_4^+), +20 µM of nitrate (NO_3^-), +25 µM of silicate (SiO_4^{4-}) and +1.6 µM of

orthophosphate (PO_4^{3-})). In a second set of samples, nutrients were not added to allow the analysis of the significance of them.

After the homogenization of the experimental treatments (sample dilutions), they were transferred to duplicate 2L polycarbonate bottles (see Fig.2.2), and sealed with parafilm. The bottles were placed randomly inside an incubation tank in a vertical position. The tank was placed outside the laboratory so it could be under natural insolation conditions, and was covered with nets, to simulate the average light intensity in the mixed layer (see equation 1), creating conditions similar to *in situ*. The incubation period was 24 hours. All water aliquots were drawn from a well-mixed carboy, including samples for initial concentrations of chlorophyll and microorganisms abundances.

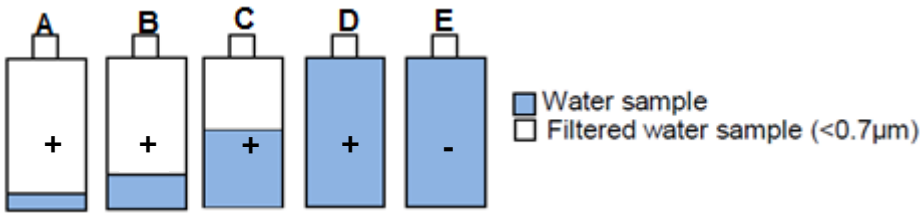

Figure 2.2 – Schematic representation of experimental treatments with different proportions of diluent (white) and sample (blue). The four different sample dilutions were (A) +0.10, (B) +0.25, (C) +0.50, (D) +1.00 and (E) -1.00. The (+) means enriched with nutrients and the (-) means no addition of nutrients (from Barbosa & Domingues, 2009).

2.4. Quantification of phytoplankton and microzooplankton

2.4.1. Chlorophyll *a* concentration

Chlorophyll *a* concentration (Chl *a*), as indicator of the total phytoplankton biomass, in all experimental treatments, at the beginning (t_0) and at the end of the incubation period (t_{24}), was analyzed using two methods, a semi-quantitative (Chl *a in vivo* fluorescence) and a quantitative (extracted Chl *a*). Chl *a in vivo* fluorescence was evaluated using a fluorimeter 10-AU-005-CE of Turner Designs Instruments and was analyzed only to have an estimation of the Chl *a* concentration. To quantify the Chl *a* concentration it was used the Lorenzen (1966) method. After sample filtration through a Whatman GF/F glass fiber filters (0.7 µm pore), filters were macerated with 5 mL of acetone 90%, then more 5 mL were added, and stored in a refrigerator. After 24 hours, the samples were centrifuged and analyzed in a fluorimeter, 10-AU-005-CE of Turner Designs

Instruments, before and after the addition of hydrochloric acid (HCl) to correct for phaeopigments. These pigments are Chl *a* degradation products, which are indicators of grazing activity (Jeffrey, 1980). To determined Chl *a* concentration it was used the equation 4 (see JGOFS, 1994).

$$Chl\ a\ (\mu g.L^{-1}) = (\frac{F_m}{F_m - 1}) \times (F_0 - F_a) \times K_x \times (\frac{Vol_{ex}}{Vol_{filt}}) \qquad \text{Equation 4}$$

Where:

F_m – Acidification coefficient

F_0 – Reading before acidification

F_a – Reading after acidification

K_x – Door factor from calibration calculations

Vol_{ex} – Extraction volume

Vol_{filt} – sample volume

2.4.2. Abundance and composition of phytoplankton and microzooplankton

It was taken two subsamples, before and after incubation period, then it was added the fixers, glutaraldehyde solution (final concentration of 1%) in one subsample, and lugol solution (0.3 mL per 100 mL of sample) in the other. The samples with glutaraldehyde solution were stored in dark glass bottles and placed in a refrigerator, while the ones with lugol solution were stored in plastic bottles and in the dark.

Afterwards, it was proceeded the preparation of the samples, with glutaraldehyde, to identify and quantify the abundance of Picophytoplankton, nanophytoplankton and phagotrophic nanoprotists (zooplankton) using the epifluorescence microscope of Zeiss Axio Observer.A1, with the addition of proflavine (20 µL per 1 mL of sample). This preparation had to be performed until 24h after the fixation of the sample.

The filters used were a cellulose acetate with 0.4 µm of pore (support filter to ensure homogeneous distribution), and a black polycarbonate membrane with 0.4 µm of pore. It was pipetted a given volume of sample, proflavine was added, and waited 3 min. Completing this period, the sample with proflavine was filtered with a pressure lower

than 100 mm, in order to minimize damage or loss of cells. It was placed into a slide a non-fluorescent oil drop, followed by the polycarbonate membrane which contained the cells, an extra non-fluorescent oil drop and a coverslip (Haas, 1982). The obtained preparations were stored into the freezer for later observation. During the observation, the phytoplankton was integrated into the following groups, Cyanobacteria *Synechococcus*, Eukaryotic Picophytoplankton and Plastidic Nanoflagellates, while the phagotrophic protists were into Aplastidic Nanoflagellates.

As for the samples with lugol solution, a known volume was settled in a sedimentation column (graduated cylinders) for 72h (4h per 1 cm). During the observation it was analyzed the microphytoplankton, such as plastidic dinoflagellates and diatoms, and the microphagotrophic protists (zooplankton), such as ciliates and aplastidic dinoflagellates, on the inverted microscope of Zeiss Axio Observer.A1(Sournia, 1978).

Both in epifluorescence and inverted microscopy were counted at least 400 cells in total, to have only 10 % error (Sournia, 1978). To identify the planktonic organisms it were used different identification books and websites. Phytoplankton was identified according to the Swedish Meteorological and Hydrological Institute (SMHI), and Dodge (1982). Microzooplankton was identify through an online guide from Strüder-Kypke *et at.*, Corliss (1979 and 1985), Dodge (1982), and Margulis *et al.* (1993).

To calculate the abundance of each of phytoplankton and microzooplankton species/group it was necessary different equations, one for the epifluorescence microscope (see equation 5) and another for the inverted microscope (see equation 6).

$$Abundance\ (cel.L^{-1}) = \frac{X \times A \times d}{a \times n \times V} \qquad \text{Equation 5}$$

Where:

X – Total number of enumerated cells

A – Area of the polycarbonate filter (mm^2)

d – Correction factor for sample dilution induced by the preservative

a – Area of field observed (mm^2)

n – Number of observed microscopic fields

V – Volume of the filtered preserved sample (L)

$$Abundance\ (cel.L^{-1}) = \frac{X \times A \times d}{V \times n \times a} \qquad \text{Equation 6}$$

Where:

X – Total number of enumerated cells

A – Area of the sedimentation chamber (mm^2)

d – Correction factor due to sample dilution by the preservative

V – Volume of the sedimented fixed sample (L)

n – Number of observed microscopic fields

a – Area of the microscopic field (mm^2)

2.5. Phytoplankton community and group-specific growth rate, microzooplankton grazing

The exact dilution factors were estimated based on the *in vivo* fluorescence of Chl *a* obtained at the beginning of the experiments (IVF0 observed). The apparent growth rate of phytoplankton community for each experimental treatment and replicate was determined assuming that the growth is exponential, as in equation 7. An identic strategy, based on abundance, was used to calculate the specific rates.

$$r = \frac{\ln IVF_{24} - \ln IVF_0}{t} \qquad \text{Equation 7}$$

Where:

IVF_{24} – *In vivo* fluorescence of Chl *a* at the end of the incubation period

IVF_0 – *In vivo* fluorescence of Chl *a* at the beginning of the incubation period

t – Incubation period (1 day)

A scatter plot was generated using the exact dilution factor represented on the x-axis, and the apparent growth rate of phytoplankton (r) in the y-axis, and alinear regression line was adjusted for each data set (sample, date and/or phytoplankton taxa). On the scatter plot it were used both set of samples, with and without nutrients. The potential

instantaneous growth rate of phytoplankton (μ_0) and microzooplankton grazing rates (g) were estimated as the regression intercept and regression slope, respectively.

The *in situ* instantaneous growth rate of phytoplankton (μ_{is}) of phytoplankton was determined according to the equation 8.

$$\mu_{is} = \mu_0 - (r_{Dil.1.0+} - r_{Dil.1.0-}) \qquad \text{Equation 8}$$

Where:

μ_0 – Potential Instantaneous growth rate of phytoplankton (after nutrient addition; d^{-1})

$r_{Dil.1.0+}$ – Apparent growth rate of phytoplankton in the non-diluted sample with nutrients

$r_{Dil.1.0-}$ – Apparent growth rate of phytoplankton in the non-diluted sample without nutrients

Net primary production of phytoplankton (NPP) was calculated according to equation 9. Phytoplankton biomass (B_0) was estimated using Chl *a* in non-manipulated samples assuming an C:Chl *a* ratio of 49 mg C for both the inner station and outer station (Barbosa, 2006; Domingues *et al.*, 2008).

$$NPP(\mu gC.L^{-1}.d^{-1}) = B_0 \times (e^{\mu \times t} - 1) \qquad \text{Equation 9}$$

Where:

B_0 – Phytoplankton biomass in the non-diluted samples (t_0; $\mu gC.L^{-1}$)

μ – *In situ* instantaneous growth rate of phytoplankton (d^{-1})

t – Time (d^{-1})

The grazing impact of microzooplankton on phytoplankton (I) was estimated as the percentage of the daily production of phytoplankton removed by microzooplankton, in accordance with equation 10.

$$I = 100 \times \frac{(B_0 \times e^{\mu t} - B_0) - (B_0 \times e^{(\mu - g)t} - B_0)}{B_0 \times e^{\mu t} - B_0} \qquad \text{Equation 10}$$

Where:

B_0 – Phytoplankton biomass in the non-diluted samples (t_0; $\mu gC.L^{-1}$)

μ_{is} – *In situ* instantaneous growth rate of phytoplankton

g – Microzooplankton grazing rate

2.6. Statistical analyses

All the statistical tests and numerical analysis were carried out using statistical program for Windows. The notation for the statistical parameters follows the normally used, where n is the number of observations, x the average, SE the standard error, R^2 the determination coefficient, F the statistical test of the analysis of variance and p the probability of a given null hypothesis (H_0), rejected for $p < 0.05$ (Sokal & Rohlf, 1995). The mean values were presented with the respective standard errors, preceded by the signal ± (x ± SE).

Chapter 3

3. Results

3.1. Initial conditions

3.1.1. Temperature, salinity and water transparency

The water temperature in the inner station, located in the west sector of the lagoon system (Faro beach), between May 2015 and May 2016, varied between 11.62ºC and 22.00ºC. In the outer station, located at the main inlet (Barra Faro-Olhão), in contact with the adjacent coastal region, the values varied between 15.80ºC and 18.50ºC (Table 3.1). Comparing the values of the water temperature between inner and outer station, the inner station had higher values, excepting during the autumn. At the seasonal level, the temperature presented a range of variation between 11.62ºC and 22ºC, with maximum values in the spring.

The salinity in the inner station, between May 2015 and May 2016, varied between 32.4 and 35.4. In the outer station, the values varied between 31.5 and 35.4 (Table 3.1). Comparing the values of salinity between inner and outer station, excepting the autumn, which had no differences, the inner zone during both springs was slightly more saline than the outer zone. At the seasonal level, the salinity presented a range of variation between 31.5 and 35.4, with lower values in periods of rainfall (April and May) and higher values in autumn. The water temperature and salinity correlated negatively and significantly in both seasons ($p>0.05$).

The values of the Secchi depth (Z_s) in the inner station, between May 2015 and May 2016, varied between 1.5 m and 2.2 m. In the outer station, the values varied between 2.5 m and 5.0 m (Table 3.1). These differences were reflected in the values of the vertical light extinction coefficient (K_e), which can represent the water turbidity, and presented values between 0.636 m^{-1} and 0.933 m^{-1}, and 0.340 m^{-1} and 0.680 m^{-1}, respectively. Comparing the values, the outer station had higher values of Secchi depth and lower values of water turbidity, while the inner station had the opposite, lower values of Secchi depth and higher values of water turbidity. At the seasonal level, the Secchi depth presented a range of variation between 1.5 m and 5 m, with higher values during spring, regarding the water turbidity, the values were between 0.340 m^{-1} and 0.933, with higher values during autumn.

The average light intensity in the mixed layer (I_m), which integrated the radiation incident to the surface, its attenuation velocity in the water column and the depth of the mixed layer, in the inner station, between May 2015 and May 2016, varied between 45.3 µmol photons.m^{-2}.s^{-1} and 56.6 µmol photons.m^{-2}.s^{-1}. In the outer station, the values varied between 14.7 µmol photons.m^{-2}.s^{-1} and 28.4 µmol photons.m^{-2}.s^{-1} (Table 3.1). Comparing the values of the average light intensity in the mixed layer between inner and outer station, the inner station had higher values. At the seasonal level, the average light intensity in the mixed layer presented a range of variation between 14.7 µmol photons.m^{-2}.s^{-1} and 56.6 µmol photons.m^{-2}.s^{-1}, with higher values during spring.

Table 3.1 – Physical and chemical variables *in situ* and estimated in the two stations of Ria Formosa at the sampling day.

Seasons and stations	Temperature (ºC)	Salinity	Z_s (m^{-1})	K_e (m^{-1})	I_m (µmol photons.m^{-2}.s^{-1})
Spring 2015 inner	22.00	32.4	2.2	0.636	56.6
Autumn 2015 inner	11.62	35.4	1.5	0.933	45.3
Spring 2016 inner	20.00	33.7	1.5	0.933	54.7
Spring 2015 outer	18.50	31.5	5.0	0.340	28.4
Autumn 2015 outer	15.80	35.4	2.5	0.680	14.7
Spring 2016 outer	16.00	33.0	3.5	0.486	20.4

3.1.2. Chlorophyll *a* concentration

The Chl *a* concentration, obtained with the Lorenzen method, in the inner station, between May 2015 and May 2016, varied between 0.20 µg.L^{-1} and 0.76 µg.L^{-1}. In the outer station, the values varied between 0.14 µg.L^{-1} and 1.56 µg.L^{-1}. The autumn of 2015 do not have all the data available, due to problems during the experiments (Fig. 3.1). Comparing the values of the Chl *a* concentration between inner and outer station, there are contradictions, both springs have opposite relations between stations and the values are discrepant. Regarding autumn it is not possible to compare. At the seasonal level, the Chl *a* concentration presented a range of variation between 0.14 µg.L^{-1} and 1.56 µg.L^{-1}, with a maximum value in the outer station during spring 2016.

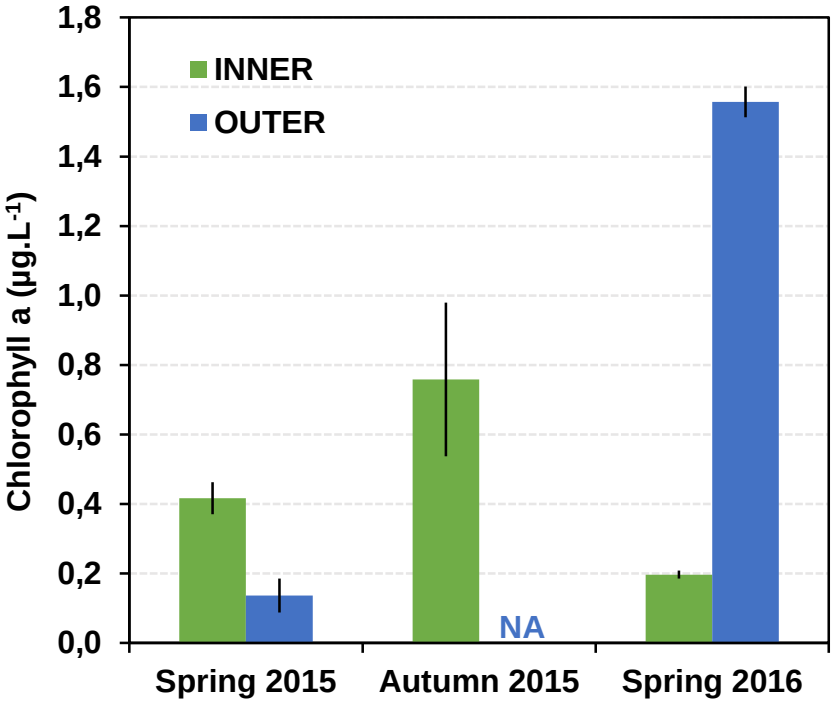

Figure 3.1 – Chlorophyll *a* concentration with the standard error in the two stations of Ria Formosa at the sampling day. NA: not available.

3.1.3. Abundance and composition of phytoplankton and microzooplankton

In the period of time between May 2015 and May 2016, the inner station had an average abundance of $140.30 \times 10^3 \pm 300.92 \times 10^3$ cel.L^{-1} of phytoplankton and of $103.55 \times 10^3 \pm 160.26 \times 10^3$ cel.L^{-1} of phagotrophic protists. The outer station had an average abundance of $278.62 \times 10^3 \pm 428.68 \times 10^3$ cel.L^{-1} of phytoplankton and of $188.45 \times 10^3 \pm 268.33 \times 10^3$ cel.L^{-1} of phagotrophic protists. Thus, the outer station had higher abundances; however, these results are an underestimation since not all the data were available.

The phytoplankton was divided in 8 groups: Cyanobacteria *Synechococcus*, Eukaryotic Picophytoplankton, Cryptophyceae, Other Plastidic Nanoflagellates, Euglenophyceae, Plastidic Dinoflagellates, Centric Diatoms and Pennate Diatoms. The phagotrophic protists were divided in 3 groups: Aplastidic Nanoflagellates, Ciliates and Aplastidic Dinoflagellates (Table 3.2). Regarding the abundance of these taxonomic and/or morphological groups considered, the Cyanobacteria *Synechococcus*, Eukaryotic Picophytoplankton, Other Plastidic Nanoflagellates, and Aplastidic Nanoflagellates were higher in the inner station. In the outer station were the same as in the inner station. Comparing the abundances between inner and outer station, it is possible to observe domination, in both, of picoplankton and nanoplankton.

Table 3.2 – Group specific composition of phytoplankton and microzooplankton and abundance. N.A.: not available. All the numbers must be multiple by 10^3 to have the real value (cel.L^{-1}).

Date	Spring 2015		Autumn 2015		Spring 2016	
	Inner	Outer	Inner	Outer	Inner	Outer
Phytoplankton						
Cyanobacteria *Synechococcus*	N.A.	N.A.	1257.68 ± 5.26	1131.38 ± 5.26	N.A.	491.30 ± 6.22
Eukaryotic Picophytoplankton	N.A.	N.A.	544.64 ± 7.89	486.76 ± 2.63	N.A.	208.34 ± 3.11
Cryptophyceae	N.A.	N.A.	44.73 ± 2.63	28.94 ± 2.63	49.75 ± 1.55	46.64 ± 9.33
Other Plastidic Nanoflagellates	N.A.	N.A.	68.41 ± 5.26	221.01 ± 5.26	288.41 ± 0.78	1436.59 ± 6.22
Euglenophyceae	2.56 ± 0.20	0.00	6.07 ± 0.10	4.08 ± 0.10	4.10 ± 0.15	N.A.
Plastidic Dinoflagellates	18.52 ± 0.39	44.58 ± 1.02	26.87 ± 0.40	20.60 ± 0.10	38.28 ± 0.30	N.A.
Centric Diatoms	97.93 ± 0.59	26.29 ± 0.38	15.03 ± 0.30	18.71 ± 0.20	26.28 ± 0.15	N.A.
Pennate Diatoms	9.26 ± 0.20	8.38 ± 0.12	9.45 ± 0.10	5.67 ± 0.30	17.47 ± 0.46	N.A.
Phagotrophic protists						
Aplastidic Nanoflagellates	N.A.	N.A.	447.29 ± 5.26	265.74 ± 2.63	299.29 ± 0.78	755.61 ± 3.11
Ciliates	12.41 ± 12.02	46.08 ± 0.48	10.05 ± 0.10	23.88 ± 0.20	32.36 ± 0.15	N.A.
Aplastidic Dinoflagellates	7.49 ± 0.79	24.94 ± 0.21	15.22 ± 0.30	14.43 ± 0.10	4.25 ± 0.30	N.A.

The analysis of the annual percentage (May 2015 – May 2016) of each taxonomic and/or morphological group for total phytoplankton and microzooplankton abundance presented the importance of Cyanobacteria *Synechococcus* (49.8%) and of Aplastidic Nanoflagellates (90.1%) in the inner station (Fig. 3.2A), and of Cyanobacteria *Synechococcus* (38.8%), Other Plastidic Nanoflagellates (39.7%) and Aplastidic Nanoflagellates (90.3%) in the outer station (Fig. 3.3B).

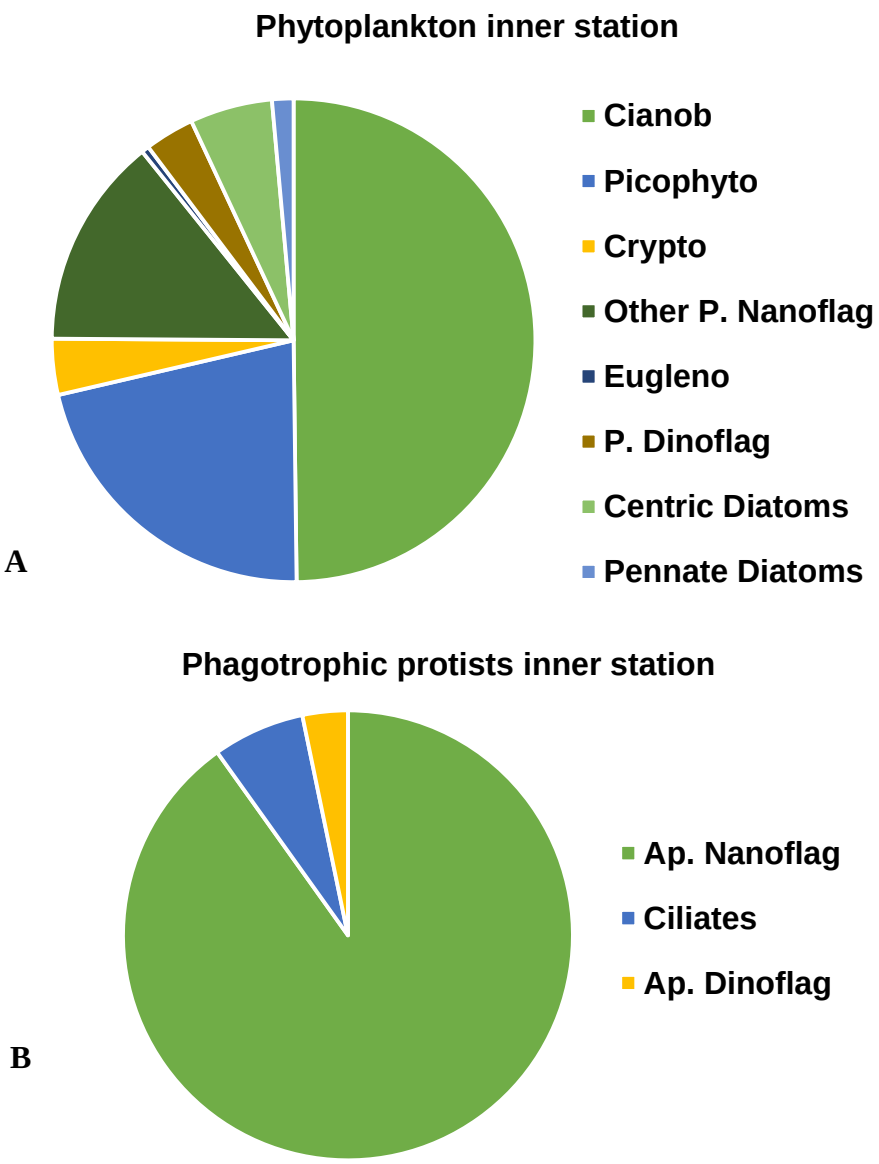

Figure 3.2 – (A) Annual percentage of phytoplankton group specific in the inner station of Ria Formosa at the sampling day. (B) Annual percentage of phagotrophic protists in the inner station of Ria Formosa at the sampling day.

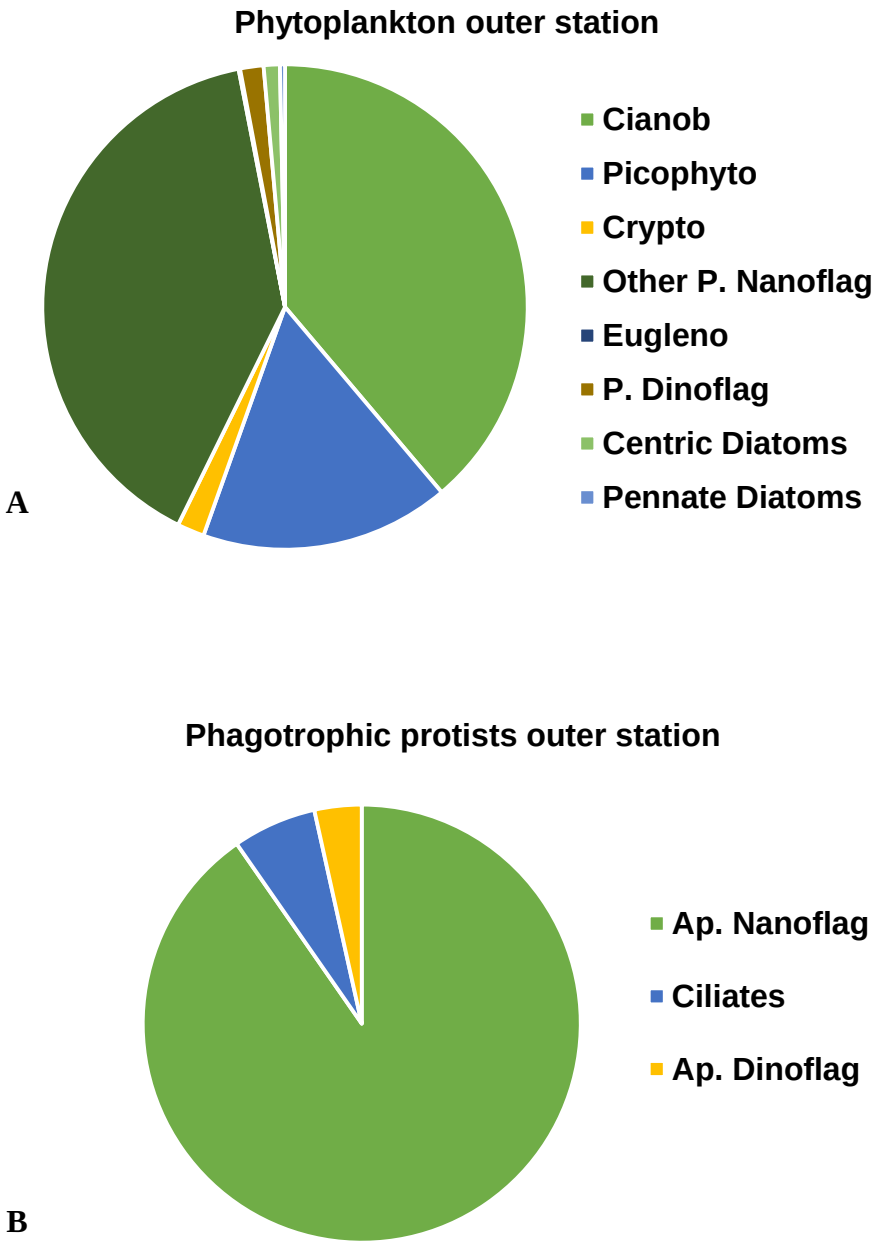

Figure 3.3 – (A) Annual percentage of phytoplankton group specific in the outer station of Ria Formosa at the sampling day. (B) Annual percentage of phagotrophic protists in the outer station of Ria Formosa at the sampling day.

3.2 Final conditions

All the data presented were obtained from the dilution experiments with the cartridge. The dilution obtained from the Whatman GF/F glass fiber filters with a pore of 0.7 µm was less efficient and accurate.

3.2.1. Phytoplankton community growth rate and microzooplankton grazing

The relationship between dilution factors and the apparent growth rate of phytoplankton (r) in spring of 2015 in the inner station had a positive linear regression. So it was impossible to determine the real growth and predation rate, because there were violations of the assumptions (Fig. 3.4A)

The relationship in spring 2015 in the outer zone had a negative linear regression. However, the slope did not have a different value from zero, so it was impossible to determine the real growth and predation rate (Fig. 3.4B). The plots, for both stations, obtain in the experiment without nutrients are in the annex.

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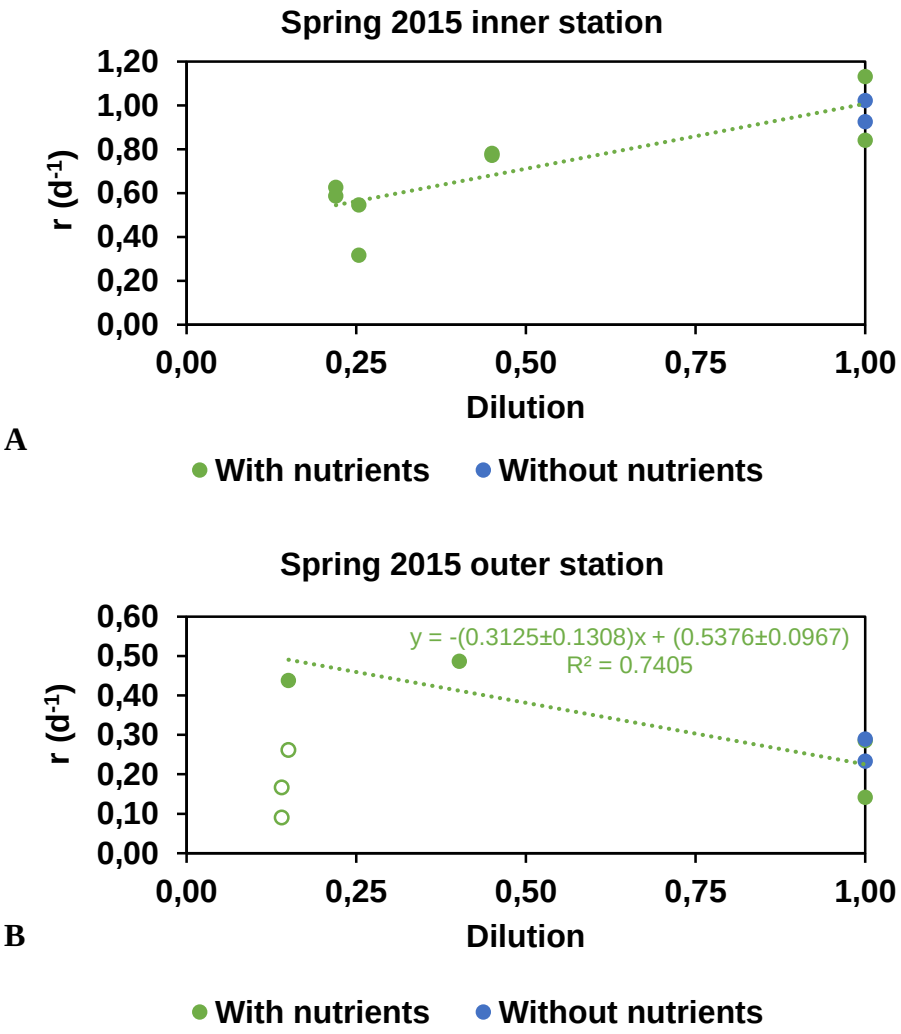


Figure 3.4 – (A) Apparent growth rate of phytoplankton community and dilution factors in the inner zone of the Ria Formosa in spring 2015. (B) Apparent growth rate of phytoplankton community and dilution factors in the outer zone of the Ria Formosa in spring 2015. The open circles represent data that was not used to adjust the regression lines.

The relationship between dilution factors and the apparent growth rate of phytoplankton in autumn of 2015 in the inner station had a positive linear regression. So it was impossible to determine the real growth and predation rate, because there were violations of the assumptions (Fig. 3.5A).

The relationship in autumn of 2015 in the outer station had a negative linear regression. The slope had a different value from zero, so it was possible to determine the real growth and predation rate. It did not occur nutrients effect, so there were no significant differences between the experiments with and without nutrients (Fig. 3.5B).

In the experiment with nutrients the values of the apparent growth rate of phytoplankton were between 0.75 d^{-1} and 3.63 d^{-1}, the predation rate was 3.36 d^{-1}, the potential instantaneous growth rate was 3.20 d^{-1}, the instantaneous growth rate of phytoplankton *in situ* was 3.29 d^{-1}, the net primary production of phytoplankton was 7.96 d^{-1}, and the percentage of daily phytoplankton production removed by microzooplankton was 100.24%. The plots, for both stations, obtain in the experiment without nutrients are in the annex.

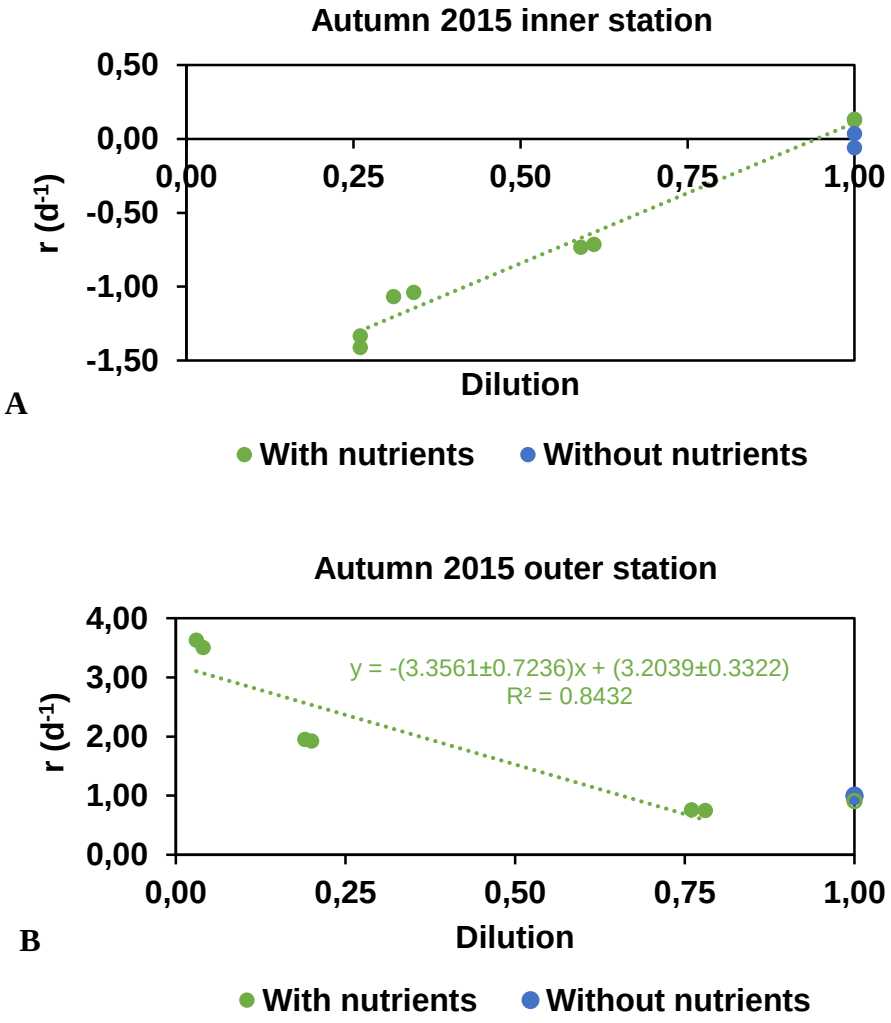


Figure 3.5 – (A) Apparent growth rate of phytoplankton community and dilution factors in the inner zone of the Ria Formosa in autumn 2015. (B) Apparent growth rate of phytoplankton community and dilution factors in the outer zone of the Ria Formosa in autumn 2015.

The relationship between dilution factors and the apparent growth rate of phytoplankton in spring of 2016 in the inner station had a negative linear regression. The slope had a different value from zero, so it was possible to determine the real growth and predation rate. It did not occur nutrients effect, so there were no significant differences between the experiments with and without nutrients (Fig. 3.6A).

In the experiment with nutrients the values of the apparent growth rate of phytoplankton were between 0.73 d^{-1} and 1.61 d^{-1}, the predation rate was 0.68 d^{-1}, the potential instantaneous growth rate was 1.40 d^{-1}, the instantaneous growth rate of phytoplankton *in situ* was 1.48 d^{-1}, the net primary production of phytoplankton was 3.14 d^{-1}, and the percentage of daily phytoplankton production removed by microzooplankton was 63.94%. The plots, for both stations, obtain in the experiment without nutrients are in the annex.

The relationship between dilution factors and the apparent growth rate of phytoplankton in spring of 2016 in the outer station had a positive linear regression. So it was impossible to determine the real growth and predation rate, because there were violations of the assumptions (Fig. 3.6B).

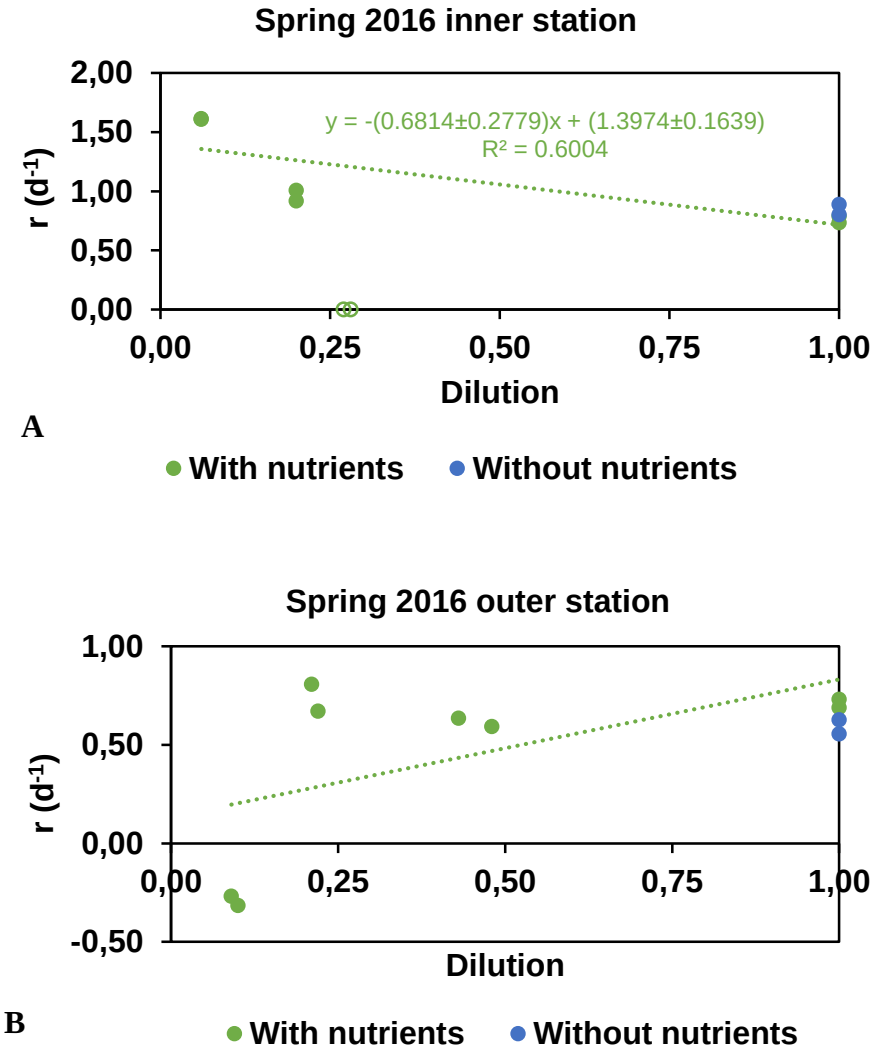


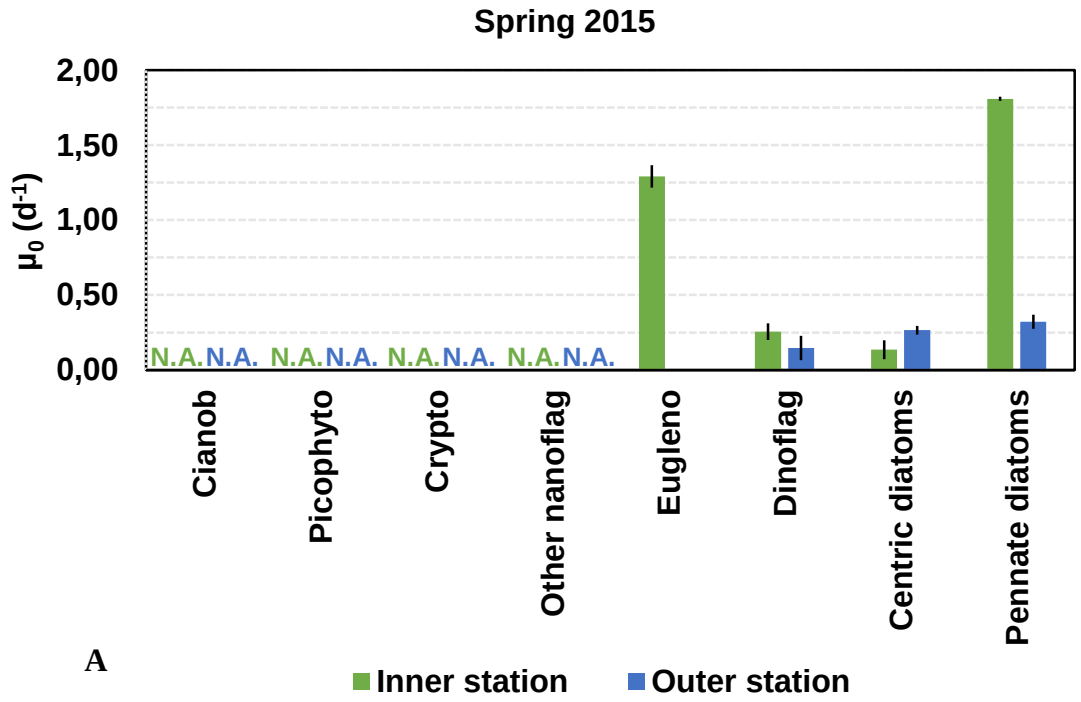
Figure 3.6 – (A) Apparent growth rate of phytoplankton community and dilution factors in the inner zone of the Ria Formosa in spring 2016. (B) Apparent growth rate of phytoplankton community and dilution factors in the outer zone of the Ria Formosa in spring 2016.

3.2.2. Phytoplankton group-specific growth rates and microzooplankton grazing

The values of the potential instantaneous growth rate (μ_0), during spring 2015, were higher for the Pennate Diatoms in the inner station, as well as in the outer station (Fig. 3.7A). Comparing the stations, there was a higher growth in the inner station. The dinoflagellates and centric diatoms did not show significant differences between inner and outer station ($p < 0.05$), however, the values of the grazing rates for the centric diatoms were significantly different between stations ($p > 0.05$). These organisms had low growth and higher grazing rate. The grazing rates were higher for the Class Euglenophyceae in the inner station and for both diatoms in the outer station (Fig. 3.7B). Comparing the stations, the grazing rate was higher in the outer station. Nevertheless, the results were uncomplete, so it is not possible to take accurate conclusions.

In the inner station, the potential instantaneous growth rates for Euglenophyceae, Dinoflagellates, Centric Diatoms and Pennate Diatoms were, 1.29 d^{-1}, 0.26 d^{-1}, 0.13 d^{-1} and 1.81 d^{-1}, respectively. The predation rates were 0.78 d^{-1}, 0.55 d^{-1}, 0.45 d^{-1} and 0.47 d^{-1}, respectively. The percentage of daily phytoplankton production removed by microzooplankton was 70.15%, 187.40%, 276.95% and 44.83%, respectively.

In the outer station, the potential instantaneous growth rates for Dinoflagellates, Centric Diatoms and Pennate Diatoms were 0.15 d^{-1}, 0.27 d^{-1} and 0.32 d^{-1}, respectively. The predation rates were 0.63 d^{-1}, 1.22 d^{-1} and 1.18 d^{-1}, respectively. The percentage of daily phytoplankton production removed by microzooplankton was 315.89%, 251.03% and 181.96%, respectively.



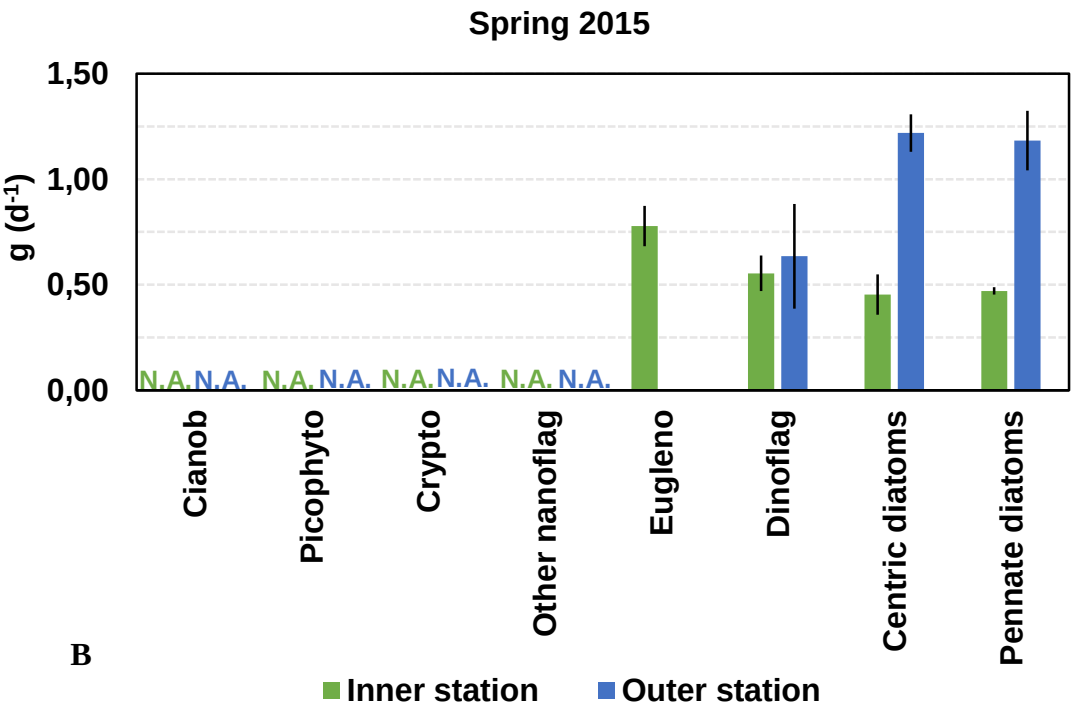


Figure 3.7 – (A) Potential instantaneous growth rate of phytoplankton group-specific in the Ria Formosa in spring 2015. (B) Grazing rate of phytoplankton group-specific in the Ria Formosa in spring 2015. NA: not available.

The values of the potential instantaneous growth rate (μ_0) during autumn 2015 were higher for the centric diatoms in the inner station and for both diatoms in the outer station (Fig. 3.8A). The Cyanobacteria *Synechococcus* and Eukaryotic Picophytoplankton had a positive linear regression in both station, so it was impossible to determine the real growth and predation rate, because there were violations of the assumptions. The Class Cryptophyceae had no significant growth ($p < 0.05$). The flagellates (Other P. Nanoflagellates, Class Euglenophyceae and P. Dinoflagellates) had a low growth and grazing rates (Fig. 3.8B). Both Diatoms had higher growth rates in the outer station, but regarding the grazing rate it was different, the Centric Diatoms had higher grazing rate at the inner station, while the Pennate Diatoms had similar grazing rates in both stations.

In the inner station, the potential instantaneous growth rates for Cryptophyceae, Other P. Nanoflagellates, Euglenophyceae, P. Dinoflagellates, Centric Diatoms and Pennate Diatoms were, 0.05 d^{-1}, 0.88 d^{-1}, 0.38 d^{-1}, 0.62 d^{-1}, 1.19 d^{-1} and 0.42 d^{-1}, respectively. The predation rates were 1.12 d^{-1}, 0.92 d^{-1}, 1.11 d^{-1}, 1.85 d^{-1}, 6.33 d^{-1} and 2.13 d^{-1}, respectively. The percentage of daily phytoplankton production removed by microzooplankton was 547.91%, 117.18%, 117.93%, 201.44%, 199.42% and 111.91%, respectively.

In the outer station, the potential instantaneous growth rates for Cyanobacteria *Synechococcus*, Eukaryotic Picophytoplankton, Euglenophyceae, P. Dinoflagellates, Centric Diatoms and Pennate Diatoms were 0.47 d^{-1}, 0.47 d^{-1}, 0.12 d^{-1}, 0.23 d^{-1}, 2.17 d^{-1} and 2.22 d^{-1}, respectively. The predation rates were 0.86 d^{-1}, 0.88 d^{-1}, 1.25 d^{-1}, 0.67 d^{-1}, 3.02 d^{-1} and 2.21 d^{-1}, respectively. The percentage of daily phytoplankton production removed by microzooplankton was 155.60%, 157.82%, 609.15%, 242.77%, 107.74% and 99.92%, respectively.

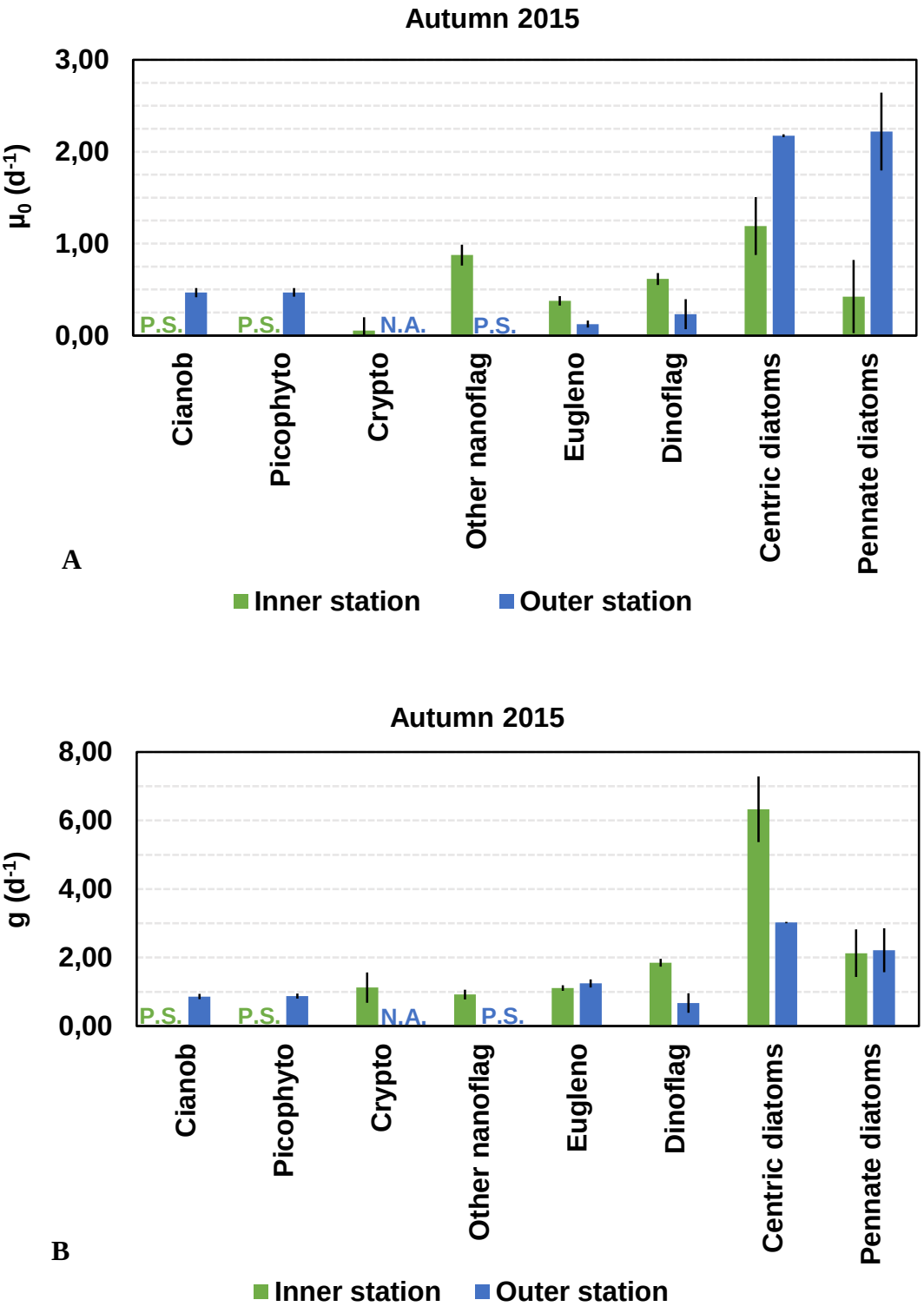


Figure 3.8 – (A) Potential instantaneous growth rate of phytoplankton group-specific in the Ria Formosa in autumn 2015. (B) Grazing rate of phytoplankton group-specific in the Ria Formosa in autumn 2015. P.S.: positive slope. N.A.: not available.

The values of the potential instantaneous growth rate (μ_0) during spring 2016 were higher for the Other P. Nanoflagellates (Fig. 3.9A). Due to the high concentration of sediments it was not possible to analyze the Cyanobacteria *Synechococcus* and Eukaryotic Picophytoplankton of the inner station. Regarding the outer station, both organisms had a positive linear regression, so it was impossible to determine the real growth and predation rate, because there were violations of the assumptions. The grazing rate had higher amplitude on the Class Cryptophyceae and was higher at the inner station (Fig. 3.9B). The remaining data was not available, so it is not possible to take accurate conclusions.

In the inner station, the potential instantaneous growth rates for Cryptophyceae and Other P. Nanoflagellates were 0.82 d^{-1} and 1.43 d^{-1}, respectively. The predation rates were 2.16 d^{-1} and 2.30 d^{-1}, respectively. The percentage of daily phytoplankton production removed by microzooplankton was 158.29% and 119.23%, respectively.

In the outer station, the potential instantaneous growth rate for Other P. Nanoflagellates was 1.64 d^{-1}. The predation rate was 1.13 d^{-1}. The percentage of daily phytoplankton production removed by microzooplankton was 81.22%.

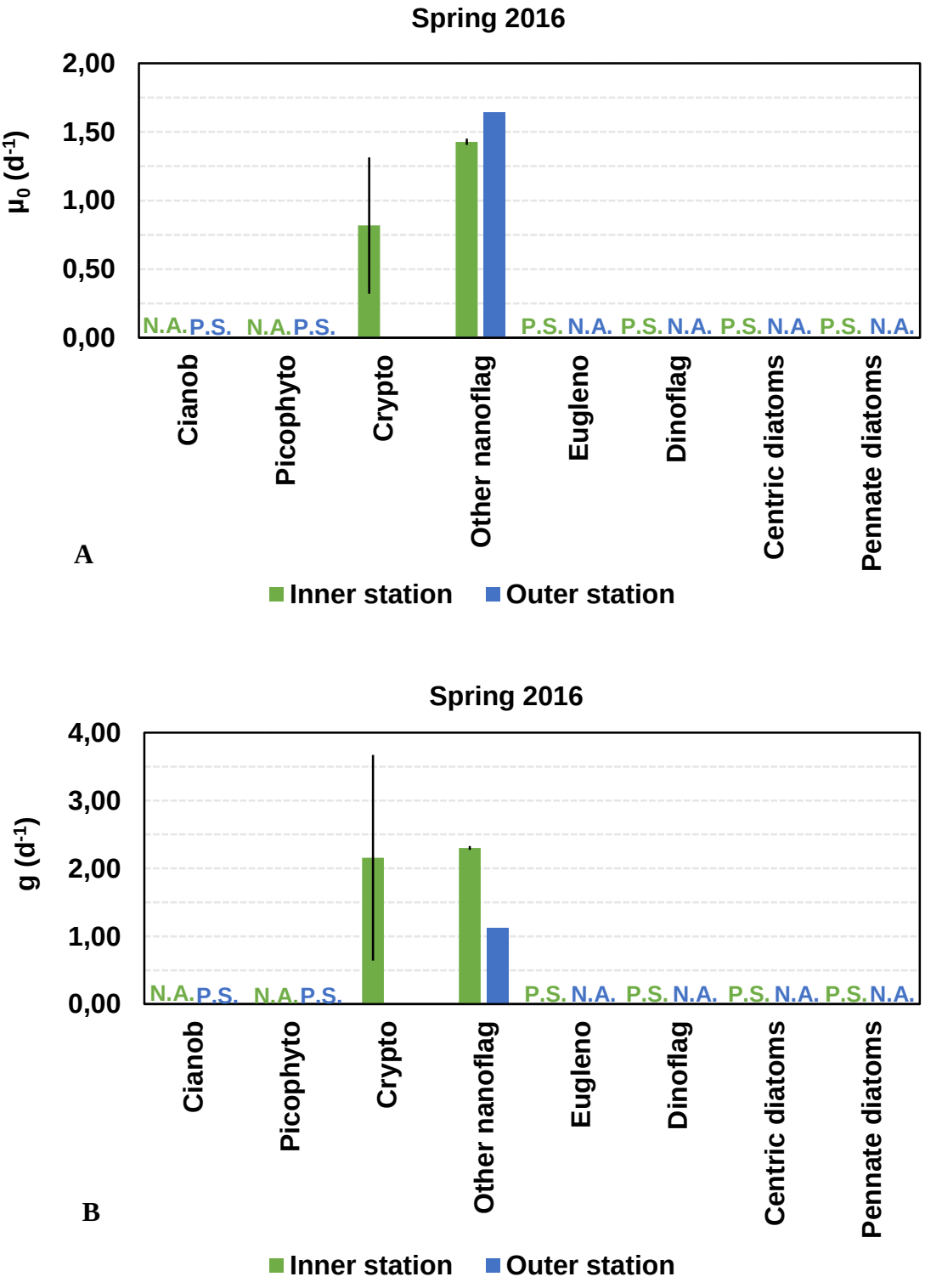
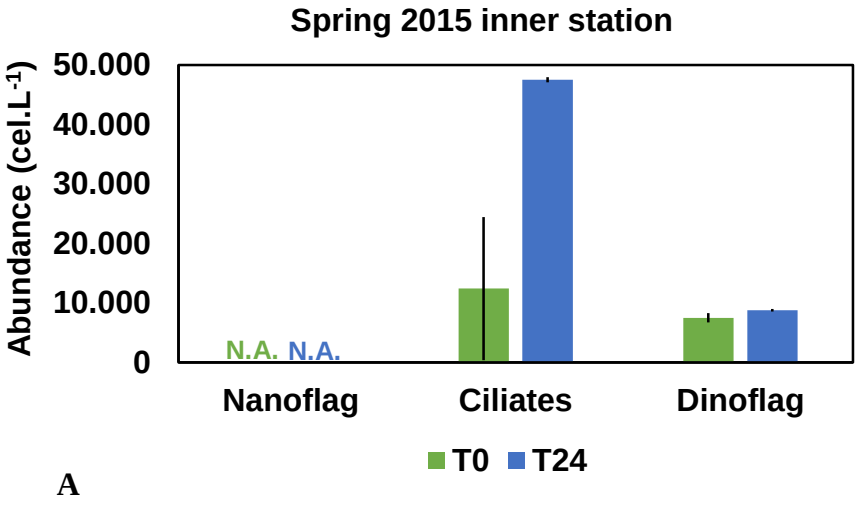


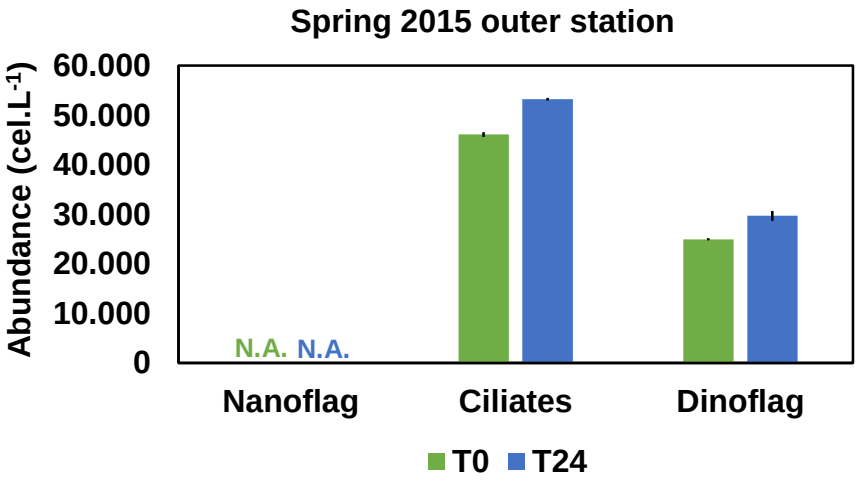
Figure 3.9 – (A) Potential instantaneous growth rate of phytoplankton group-specific in the Ria Formosa in spring 2016. (B) Grazing rate of phytoplankton group-specific in the Ria Formosa in spring 2016. P.S.: positive slope. N.A.: not available.

3.2.3. Microzooplankton growth

The phagotrophic protists with higher growth, during spring 2015, were the Ciliates and in the inner station (Fig. 3.10A). Regarding the Ap. Dinoflagellates, they had a small growth in both station, however the growth in the outer station was slightly higher (Fig. 3.10B). The data about the Ap. Nanoflagellates were not available.



A



B

Figure 3.10 – (A) Microzooplankton growth in the inner station of the Ria Formosa during spring 2015. (B) Microzooplankton growth in the outer station of the Ria Formosa during spring 2015. NA: not available.

The phagotrophic protists with higher growth were the Ap. Nanoflagellates and in the outer station (Fig. 3.11A and 3.11B). While the Ciliates and Ap. Dinoflagellates had no main role in the abundance of the microzooplankton in both stations, nevertheless in both station, Ciliates increased, while the Ap. Dinoflagellates decreased.

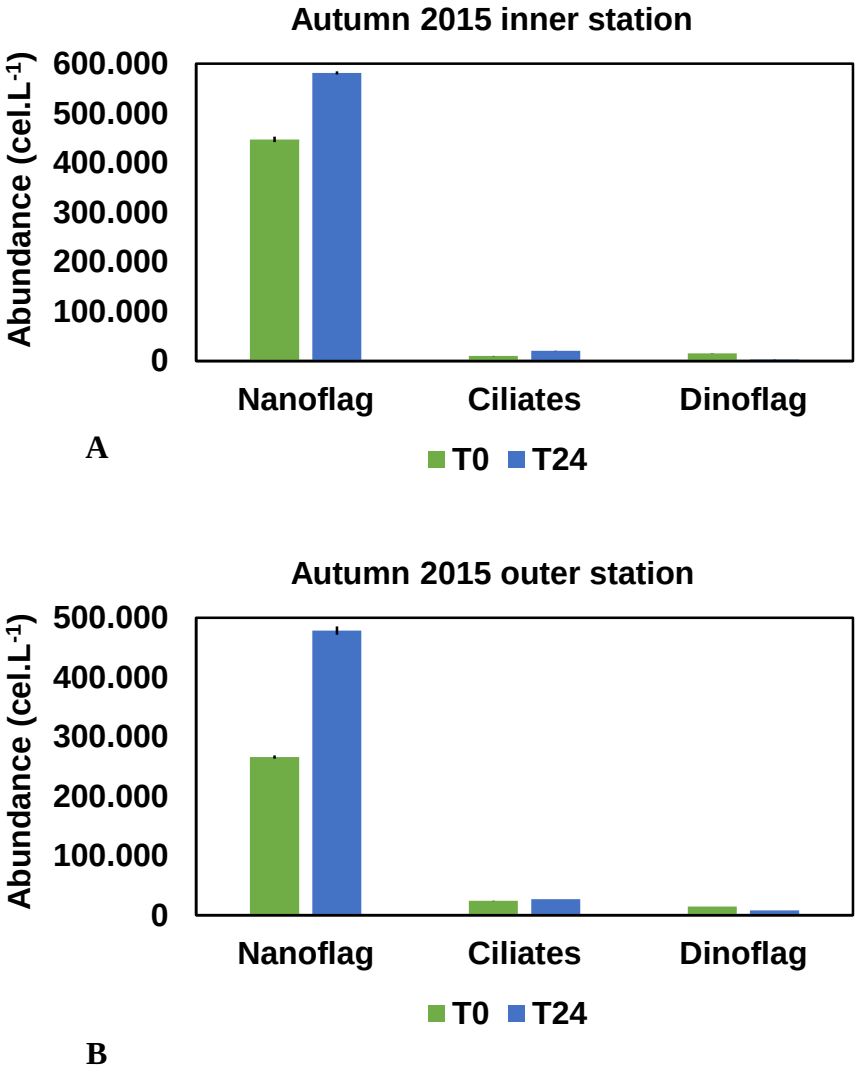
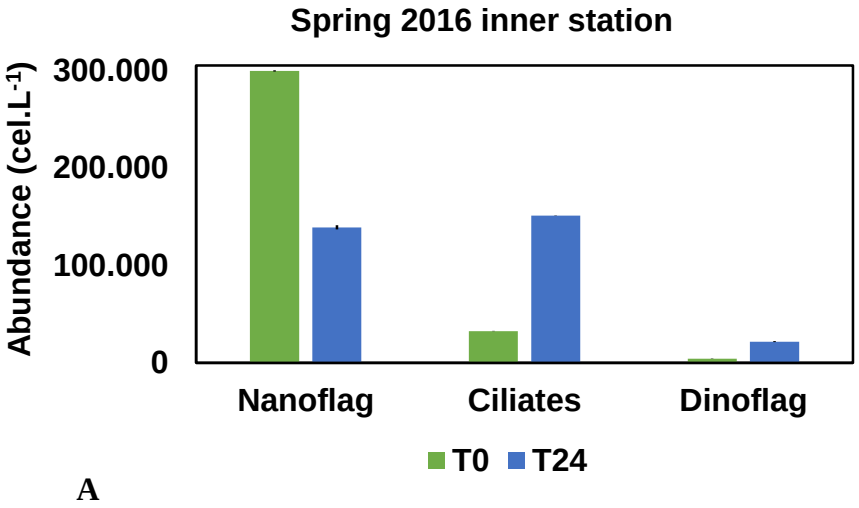
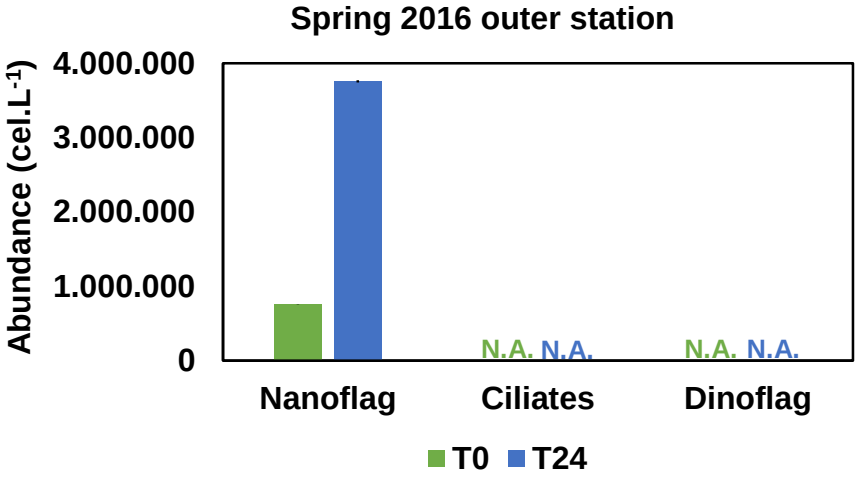


Figure 3.11 – (A) Microzooplankton growth in the inner station of the Ria Formosa during autumn 2015. (B) Microzooplankton growth in the outer station of the Ria Formosa during autumn 2015.

The phagotrophic protists with higher growth were the Ap. Nanoflagellates in the outer station, since in the inner station they had a decrease of abundance (Fig. 3.12A). The Ciliates had a significant growth ($p > 0.05$) in the inner station, while the Ap. Dinoflagellates did not contribute significantly to the abundance of the microzooplankton, however they had an abundance increase. Regarding the Ciliates and Ap. Dinoflagellates in the outer station it is not possible to take conclusions due to the data was not available (Fig. 3.12B).



A



B

Figure 3.12 – (A) Microzooplankton growth in the inner station of the Ria Formosa during spring 2016. (B) Microzooplankton growth in the outer station of the Ria Formosa during spring 2016. NA: not available.

Chapter 4

4. Discussion

4.1. Critical evaluation of the experimental strategy

The dilution method has been widely applied and is the only method available to estimate *in situ* microzooplankton grazing impact and phytoplankton growth rates (Strom *et al.*, 2001; Moigis & Gocke, 2003; Calbet & Landry, 2004; McManus *et al.*, 2006; Paterson *et al.*, 2007 and 2008; Stoecker *et al.*, 2015). According with Calbet and Landry (2004), through this procedure, they found that microzooplankton grazing was invariant, ranging between 59% and 74% of phytoplankton primary production across systems differing in seasonality, trophic status, latitude or salinity. Dolan and McKeon (2005) believed that these values were too high, because if 64% of the daily phytoplankton production is consumed by microzooplankton, there appears to be little left for any direct forms of carbon export from bacteria to nekton. Thus they suggested that dilution experiments are prone to providing over-estimates of grazing rates and unlikely to furnish evidence of low grazing rates. The overestimation may have occurred in this study, because the diluent was not autoclaved. This process is based on steam sterilization and is commonly used to eliminate bacteria, fungi and other transmissible agents (Merck Millipore Business). Thus without the autoclaving there was no elimination of heterotrophic bacterioplankton, another grazer of phytoplankton. So autoclaving process should have been used, because it might have reduced the overestimation of grazing rates.

Besides the possible overestimation, in studies of microzooplankton grazing, the responses to dilution are not always linear, which can make results often uninterpretable (non-significant or positive slopes), as demonstrated in this study. Some non-significant results are due to the fact that low grazing rates are difficult to detect with regression analysis using the small n values (8 to 15 bottles). Aggregated to this problem is the fact that detecting low grazing rates necessitates distinguishing slight differences on initial and final Chl a concentrations which is especially difficult in the highly dilute treatments. Another issue is that grazing pressure may not be linearly related to dilution factor (Dolan & McKeon, 2005). Furthermore, many phytoplanktons produce inhibitory metabolites that may be released during filtration of the seawater needed for dilution experiments. Under some conditions, dilution grazing experiments may underestimate phytoplankton growth coefficients and microzooplankton grazing coefficients (Stoecker

et al., 2015). Therefore, the negative slopes with values not different from zero (e.g. Fig.3.4B) should not be ignored in the dilution experiments, also more bottles and different dilutions could be used to increase the n values and to decrease the difficulty on detecting low grazing rates. The positive linear regressions cannot be used to estimate the phytoplankton growth rate and the grazing rate, because the equations can only be applied for negative linear regressions, however these unexpected responses should not be dismissed, since they can show the trophic cascades response, which will complement the expected response (negative linear regression), leading to a better and wider comprehension of the trophic dynamics.

A side the final results of the dilution method, there was, maybe, a problem with the incubation process, the incubation in tanks (used in this study) versus the incubation *in situ*. The issue is the fact that incubation in tanks has no sediment, tides, currents, and other variations that influence any ecosystem, thus this type of incubation is far from simulating *in situ* conditions. According with previous studies (Margalef, 1978; Smayda & Reynolds, 2001), the combination of sedimentation with turbulence shapes communities as the pelagic varies between extremes of a fertile-turbulent state, which promote Diatom growth, and exhausted-stratified state, which promote growth of Plastidic Flagellates and Plastidic Dinoflagellates. In this study, there was no turbulence, also in the experiment of spring 2015, Plastidic Dinoflagellates and Centric Diatoms had similar growth rates, thus if the experiment had been done *in situ*, the results could probably be different, as showed in Barbosa's study (2006), where Diatoms had a higher growth than Plastidic Flagellates. So the incubation should have been done *in situ*, however due to the lack of means it was impossible to perform the experiment *in situ*.

4.2. Initial conditions

4.2.1. Temperature, salinity and water transparency

The inner and outer stations, located at the west bay of Ria Formosa and in the border area in contact with the adjacent coastal, respectively, in the period between May 2015 and May 2016, had different thermal amplitudes, some differences in the salinity values (see Table 3.1) and there was no positive correlation between temperature and salinity. This can show the importance of the depth of the Ria Formosa in response to variations

of heat flux, the adjacent coastal zone as a water source to the lagoon system, the rainfall events as the input of fresh water and the upwelling events as the homogenizer of the water column, but also prove that there is a reduced influence of freshwater tributaries (Silva, 2001).

The inner zone had higher thermal amplitude than the outer zone, probably due to being shallower and farthest from the adjacent coastal zone, which means the inner zone has more standing water than the outer zone and heats up more easily. These results were also observed in the studies of Barbosa (2006). Comparing the spring with the autumn, the temperatures had opposite results. During the spring, the inner zone was warmer than the outer, and in the autumn, the outer zone was warmer than the inner zone, maybe because through the autumn the temperatures are lower, so the only influence comes from the adjacent coastal zone, which means that the outer zone was more susceptible to variations of temperature than the inner zone.

The water temperature values (11.6ºC to 22.0ºC) in this study had different amplitudes regarding previous studies. Nevertheless, the variations were more or less within the normal range of the Ria Formosa, e.g. between 1988 and 1989, the water temperature varied from 18.1ºC to 20.6ºC (Newton & Mudge, 2003), between 1991 and 1993, the values were 9.8ºC to 30.0ºC (Barbosa, 2006) and between 2000 and 2002, varied from 14.5ºC to 24.5ºC (Pereira *et al.*, 2007).

Relatively to the salinity, the values had low differences between the inner and outer stations, because of the reduced depth of Ria Formosa, the absence of important sources of fresh water, the precipitation rate, the upwelling events and the tides. Since the lagoon is shallow, a stratification event is very rare, as mention in studies as Newton and Mudge (2003), the lack of fresh water sources makes the salinity of the Ria Formosa similar to the adjacent coastal area, so the main fresh water input is the rainfall. This event dilute the shallow water column of the inner zone, which will lead to a decrease of salinity, while the upwelling brings brackish waters to the surface which will lead to a homogenization of the water column of the outer zone and the tides generate turbulence, which is responsible for the vertical mixing of the water column in both stations (Silva, 2001; Newton & Mudge, 2003; Barbosa, 2006).

The salinity values (31.5 to 35.4) in this study had the same amplitude as in 27 years ago (Newton & Mudge, 2003), however, in further studies, the ranges were completely different, e.g. between 1991 and 1993, the values were 15.1 to 39.0 (Barbosa, 2006) and between 2000 and 2002, varied from 35 to 37. The explanation for these differences may be in the climatological variations through the years.

The water transparency (see Table 3.1) had a big difference between the inner and outer stations, since the inner station had a higher water turbidity (K_e) and higher PAR (I_m), while the outer station had a lower water turbidity (K_e) and lower PAR (I_m). According with previous studies (Cloern, 1987; Cole *et al.*, 1992; Kocum *et al.*, 2002; Barbosa, 2006), the results were the expected, since the values of PAR radiation decrease with the increase of depth and decrease of water turbidity. This happen due to a higher concentration of detrital material probably re-suspended from the sediment, and the reduced depth in the inner station of Ria Formosa, which allowed the occurrence of PAR radiation in the mixed layer (Barbosa, 2006). Comparing the seasons, although the autumn had higher water turbidity than the spring, its PAR radiation was lower, probably because there is a limitation of light during the autumn.

4.2.2. Chlorophyll *a* concentration

Chl *a* concentration (see Fig. 3.1) had differences between stations, however they were not consistent, because in spring 2015 the inner station had a higher concentration than in the outer zone, and in spring 2016 the outer zone had a higher concentration than in the inner zone. According to many studies, the concentration of Chl *a* in the western sector of the Ria Formosa, generally increases from outer areas to the interior, since, probably, have the highest average intensity of light in the mixed layer, and less advection tide in internal areas (Barbosa, 2006), and because the intense mixing of the water column allows the continuous contact between the pelagic environmental and the main source of inorganic macronutrients, the sediment (Falcão, 1996; Falcão & Vale, 1998). Indeed, the intense contact with the sediment, the effect of the effluents only partially treated and the terrestrial surface runoff explain the occurrence of higher concentrations of inorganic macronutrients in the most confined and shallow zone of the lagoon system (inner zone) and lower in the areas that are in contact with the coastline (outer zone) (Benoliel, 1984, 1985, 1989; Cortez, 1992; Newton, 1995; Newton &

Mudge, 2005). So the spring 2015 followed the patterns to the Ria Formosa, but the spring 2016 did not.

The discrepancy between springs maybe due to the annual climatological variations and the exportation of nutrients in the lagoon, because, according with the Portuguese institute of the sea and the atmosphere (IPMA), spring 2015 was warm and dry, with an average temperature of 18.67ºC and a precipitation rate of 41.1 mm, which was lower than the normal average rate (71.2 mm), while spring 2016 was mild and wet with an average temperature of 15.78ºC and precipitation rate of 142.9 mm, which was the double of the normal average rate (71.2mm). The collecting period for spring in 2015 was on the last days of May and in 2016 was on the last days of April and middle of May. In the previous weeks to the spring 2016 sampling, it occurred a heavy precipitation rate, so there was a higher input of fresh water and sediments in the Ria Formosa, which affected the inner station, explaining the lower values in 2016. Regarding the outer zone, the sampling occurred a few weeks after the precipitation ceased, when the water column was well mixed again, so the rainfall did not affect this station as in the inner station, but actually it may contributed to the higher values of Chl *a*, because of the enrichment of nutrients that were carried out by the rainfall. Furthermore, the Ria Formosa generally exports nutrients to the adjacent coastal waters (Newton & Mudge, 2005), so the outer station in spring 2016 had an extra input of nutrients which, probably, allowed the higher growth of phytoplankton comparing with the inner station. Regarding the autumn, it was impossible to analyze and compare because not all the data were available.

The values of Chl *a* concentration (did not exceed 2 $\mu g.L^{-1}$) in this study had the same range as in 2000 to 2002 (Pereira *et al.*, 2007), but between 1991 and 1993, Chl *a* concentration had higher values in the inner station, 3.2 ± 0.3 $\mu g.L^{-1}$ (Barbosa, 2006), and in 2012 the values were 2.9 ± 0.2 $\mu g.L^{-1}$ (Barreto, 2012), then there are phytoplankton fluctuations over the years.

4.2.3. Abundance and composition of phytoplankton and microzooplankton

The outer station had a higher abundance of organisms than the inner station (see Table 3.2), however the results were underestimation due to not all the data were available, yet the difference between them was high. According with Kjerfive (1994), it should have been the opposite, because the Ria Formosa is considered a restricted or leacky coastal lagoon (large water bodies connected to the sea by two or more inlets), and in these systems, the residence time is normally higher. Though, Pereira *et al.* (2007) refuted that concept and hypothesizes that the lagoon exchange large amount of water with the ocean, which will lead to a short residence time, and consequently, occurs a substantial removal of suspended organisms from the lagoon system or dilutes the populations that growth in more inland areas.

On the subject of the composition of the phytoplankton community, the Cyanobacteria *Synechococcus* was dominant in the inner station. While in the outer station was co-dominant with the Other Plastidic Nanoflagellates. However, twenty-three years ago, *Synechococcus* was significantly abundant only in the outer zone of Ria Formosa (Barbosa, 2006), thus through the years, the Cyanobacteria could have increased their dominance in all Ria Formosa. In both stations, the dominant microzooplankton group was the Aplastidic Nanoflagellates, so in both stations, the dominant organisms were the Nanoplankton (see Fig 3.2 and 3.3). According to previous studies (Chisholm, 1992; Cloern & Dufford, 2005), phytoplankton biomass and production are dominated by micron-sized organisms, because small size provides a competitive advantage in nutrient assimilation. Later Roselli and Basset (2015) corroborated that hypothesis, saying that coastal lagoons have phytoplankton significantly smaller maybe due to mixing conditions that affect size-dependent sinking, which may drive phytoplankton size and shape distributions. These conditions are the interplay between shallow mixed layer depth, and frequent and complete mixing of transitional waters, that may likely increase the competitive advantage of small phytoplankton, limiting large cell fitness. Concerning the microzooplankton, although it can graze on large as well as small phytoplankton, including chain forming dinoflagellates (Strom *et al.,* 2007; Sherr *et al.*, 2013), their grazing rates can be influenced by phytoplankton species composition, physiological state and cell size (Olson & Strom, 2002; Strom & Fredrickson, 2008), then the size of the microzooplankton, probably, has a positive correlation with the size of the phytoplankton, explaining the dominance of micron-sized organisms.

The distribution of abundance in this study was the opposite obtained in the study of Barbosa (2006), where the inner station had a higher abundance than the outer station, however, in that study, the inverted microscopy was not used, so there was a lack of data. The author even affirms that the non-use of the inversion technique in her study may have underestimated the contribution of phytoplankton with reduced relative abundance, thus the use of epifluorescence microscopy, in conjunction with inversion microscopy, is necessary to quantitatively analyze the entire phytoplankton community. Regarding the composition of the planktonic community, there were also differences between the studies. In this study the Cyanobacteria *Synechococcus* and Other Plastidic Nanoflagellates were the dominant groups, however in Barbosa's study it was the Eukaryotic Picophytoplankton (inner station) and the Cyanobacteria *Synechococcus* (outer station). Yet concerning the microzooplankton, the dominant group was equal in both studies, the Aplastidic Nanoflagellates. A possible explanation for these results it would be variations of grazing rate, nutrient uptake and sinking rate through the years, since grazing "pushes" the community towards larger cell sizes, while nutrient uptake and sinking "pull" the community to smaller cell sizes (Acevedo-Trejos *et al.*, 2015).

4.3. Final conditions

4.3.1. Phytoplankton community growth rate and microzooplankton grazing

The experiments carried out in this study had 3 types of responses, a positive slope, a negative slope and an insignificant slope. The experiments with expected results (negative linear regression) in the relationship between dilution factors and the apparent growth rate of phytoplankton community were in the outer station of autumn and in the inner station of spring 2016. The unexpected responses were many (four in six experiments), and were a positive linear regression in the inner station (see Fig. 3.2A) and an insignificant in the outer station (see Fig. 3.2B) of spring 2015, a positive linear regression in the inner station (see Fig. 3.3A) of autumn 2015, a positive linear regression in the outer station (see Fig. 3.4B) of spring 2016. However, these results were not unforeseen, because the dilution method is not free of problems, since it has been reported unexpected results related to the apparent growth rate of phytoplankton versus the dilution factors. Many studies have reported cases of non-interpretable results, i.e., the relationship between the dilution factor and the growth rate of phytoplankton do not have a significant linear regression (Kamiyama, 1994; Gifford *et*

al., 1995; Landry *et al.*, 1995b; Reckermann & Veldhuis, 1997; Lessard & Murrell, 1998; Murrell & Hollibaugh, 1998; Caron & Dennett, 1999; Gaul *et al.*, 1999; Kuipers & Witte, 1999; Caron *et al.*, 2000; Dix & Hanisak, 2015). Dix and Hanisak (2015) organized these results in 5 types of response: insignificant (A), negative linear (B), negative saturated (C), saturated increasing (D) and positive linear (E) (see Fig.4.1).

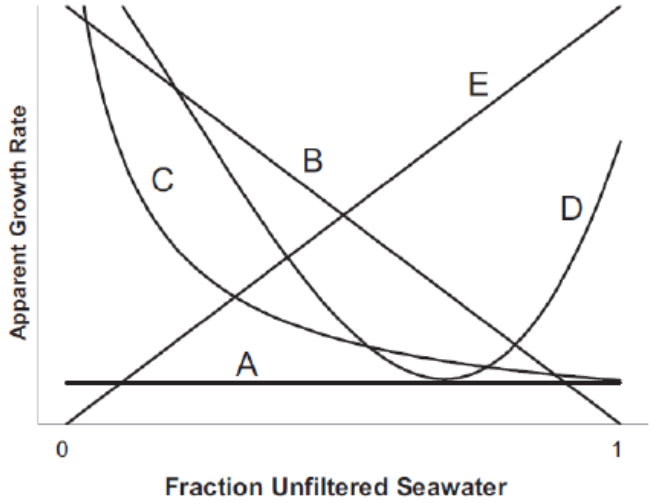


Figure 4.1 – Five types of responses between the apparent growth of phytoplankton and dilution factors: (A) insignificant, (B) negative linear, (C) negative saturated, (D) saturated increasing and (E) positive linear (from Dix & Hanisak, 2015).

This non-expected responses (A, C, D and E) are due to violations of the assumptions, like the fact that sometimes the grazer abundance relative to dilution levels change over the incubation period (Dolan *et al.*, 2000; Berninger & Wickham, 2005; Agis *et al.*, 2007; Teixeira & Figueiras, 2009; Calbet *et al.*, 2011), because grazer community have differences in growth and mortality among dilutions levels (Dolan *et al.*, 2000; First *et al.*, 2007; First *et al.*, 2009; Modigh & Franzè, 2009; Calbet *et al.*, 2011).

The cases of positive linear response (E) in this study occurred in both seasons, then the difference of water temperature did not affect the apparent growth rate of phytoplankton, as presented in the study of Obayashi and Tanoue (2002), where no significant difference was found between the growth rate of phytoplankton community at colder and warmer waters. Although, Barbosa (2006) observed, in the inner station, a positive and significant correlation between the water temperature and the phytoplankton growth rate. However, it has to be taken into account that Barbosa has

analyzed all the seasons of the year, while this study only focused on two seasons, thus that difference may explain the different results obtained in the two studies.

Positive linear responses are outcomes of changes in phytoplankton intrinsic growth rates among dilutions levels, i.e., from toxic contaminants in the particle-free seawater used for dilutions (Landry, 1993; Landry *et al.*, 1995b) or from elevated nutrients in less-dilute samples via grazing-induced nutrient regeneration (Modigh & Franzè, 2009). However, no nutrients effect occurred, so probably, the cause was the presence of high biomass of phytoplankton, because a preparation of filtered seawater from water containing high biomass of phytoplankton results in release of allelochemicals that inhibit phytoplankton growth, lowering the net growth of phytoplankton in the more diluted treatments (Stoecker *et al.*, 2015). A proof of this hypothesis could be the experiment of spring 2016, where the outer station had a higher initial Chl *a* concentration than the inner station and as result had a positive linear response, while the inner station had a negative linear response.

Another hypothesis is that positive linear responses may also result from complexities in trophic relationships such as mixotrophy (Calbet, 2008; Calbet *et al.*, 2011 and 2012) and trophic cascades (Calbet *et al.*, 2008 and 2011; Calbet & Saiz, 2013), for example, if the main grazers contain chlorophyll it could result in a positive Chl-based slope, or if grazers are prey for others grazers, phytoplankton could be released from grazing pressure. Thus, the presence of mixothrophy and/or trophic cascades during incubation may alter the linearity of the relationship between dilution levels and phytoplankton net growth rates, leading to incorrect interpretations of the microzooplankton-phytoplankton relationship (Calbet & Saiz, 2013). So the positive slope suggest that the dilution can affect negatively on phytoplankton physiology and can compromise their growth rates (Stoecker *et al.*, 2014), since phytoplankton not always increase with the decreasing of predators and grazing actually may stimulate prey growth (Dix & Hanisak, 2015), an example of that is the study of Tijdens *et al.* (2008), where the apparent growth rate of filamentous Cyanobacteria *Synechococcus* have a significant decrease with the dilution of microzooplankton.

The case of insignificant response (A) tend to be assume as the grazing impact by microzooplankton was nil, because the slope of the dilution equation was not

significantly different from zero, though it can be certain that microbial grazers (nano and microplankton) were probably plentiful and must have fed. This artificial slope has minor consequences for true estimates of phytoplankton mortality rates when the dilution level is sufficiently high (10%: dil 0.10), but it may result in significant underestimation of the microzooplankton grazing impact when using typical maximum dilution levels (25% and 50%; dil 0.25 and 0.50) (Calbet & Saiz, 2013).

Therefore the dilution affected the predation exerted by microzooplankton, so the growth and mortality of predators can result in uncertain predation rates. One example is the data obtained by Dolan *et al.* (2000), in which there was a decline in the abundance of tintinnids of small dimensions with increased dilution, however tintinnids with higher biomass increase their abundance in the more dilute samples and the rotifers did not have a consistent effect. Other studies show that the dilution experiments are likely to provide over-estimates of the grazing rate (Dolan & McKeon, 2005). This overestimation may be due to the fact that the virus are not removed from the samples, and as such, may influence the mortality rate of phytoplankton (Calbet *et al.*, 2011).

Comparing the stations, it was possible to observe that the inner station had a positive linear response, except in spring 2016, when an uncommon high precipitation rate occurred. Hence this event changed the normal standards of the inner station, since it diluted the phytoplankton community, as was observed in the initial Chl *a* concentration (Fig.3.1). The outer station had a negative linear response, except, again, during spring 2016. Regarding the potential instantaneous growth rate (μ_0), the predation rate (g) and the percentage of daily phytoplankton production removed by microzooplankton (I) it was only possible to compare between seasons, since between stations was not possible to compare positive and negative linear regressions. These values were higher during autumn, so maybe in that season the environmental conditions were better than in spring.

4.3.2. Phytoplankton group-specific growth rates and microzooplankton grazing

The potential instantaneous growth (μ_0) and grazing (g) rates of phytoplankton group-specific had differences from the type of responses to the effect of nutrients, through the year (May 2015 to May 2016) and between stations. The type of responses obtained

were positive or negative linear regressions, and the nutrients did not affect in general the community, however it did occurred effects in group-specific. Thus, this plankton community was shaped by nutrient supply and species interactions across trophic levels (e.g. selective grazing).

Cyanobacteria were dominated by rounded or short rod-shaped cells, usually solitary or in groups of two cells, similar to the characteristics of the genus *Synechococcus* (Waterbury *et al.*, 1986; Waterbury & Ripka, 1989). The Eukaryotic Picophytoplankton was composed by cells of taxonomic identity not evaluable based on the direct observation in epifluorescence microscopy. The observation of non-picoplankton plastidic flagellates, with the exclusion of dinoflagellates, in epifluorescence microscopy allowed the taxonomic recognition of a small number of taxa including Cryptophyceae, Eugenophyceae and some genera with different morphology (Other Nanoflagellates). The epifluorescence microscopy analysis, assisted by the inversion microscopy, allowed the classification of Plastidic Dinoflagellates in many taxa, some of them with different morphotypes (*Dynophysis*, Gymnodinoide, *Peridiniella catenata* and *Prorocentrum*). Both microscopy analyses allowed the classification of Diatoms in different groups such as Centric Diatoms (*Thalassiosira* and *Guinardia*) and Pennate Diatoms (*Navicula* and *Nitzschia*).

The Aplastidic Nanoflagellates was composed by cells of taxonomic identity not evaluable based on the direct observation in epifluorescence microscopy. The inversion microscopy analysis allowed the classification of Ciliates in many taxa, *Cyclidium*, *Strombidium conicum* and *Tintinnopsis*. The observation of Aplastidic Dinoflagellates, in inversion microscopy allowed the taxonomic recognition of a small number of taxa including *Gyrodinium* and *Protoperidinium*.

The inner station, during spring 2015, had a higher growth rate of Pennate diatoms and Euglenophyceae, however the Euglenophyceae were also the group-specific with higher predation rate, and the Centric Diatoms were the group-specific with higher percentage of daily phytoplankton production removed by microzooplankton. During autumn 2015, occurred a change, the Cyanobacteria *Synechococcus* and Eukaryotic Picophytoplankton had positive linear regression, the Centric Diatoms and Other Plastidic Nanoflagellates were the ones with higher growth rate, but the Centric

Diatoms were also the group-specific with higher predation rate, and the Cryptophyceae were the group-specific with higher percentage of daily phytoplankton production removed by microzooplankton. In spring 2016, again a change occurred, since the larger cells (Euglenophyceae, Dinoflagellates, Centric and Pennate Diatoms) had a positive linear regression, the group-specific with higher growth rate was Other Plastidic Nanoflagellates and also these organisms were the ones with higher predation rate, while the Cryptophyceae were the group-specific with higher percentage of daily phytoplankton production removed by microzooplankton.

The outer station, in spring 2015, had a higher growth rate of both Diatoms (Centric and Pennate), and also these phytoplanktonic organisms were the most grazed, while the organisms with higher percentage of daily phytoplankton production removed by microzooplankton were the Dinoflagellates. During autumn 2015, again the Diatoms (Centric and Pennate) were the group-specific with higher growth rate, but just one of them were the most grazed, the Centric Diatoms, and the Euglenophyceae had the higher percentage of daily phytoplankton production removed by microzooplankton. In this period of time occurred a positive linear regression on the Other Nanoflagellates. In spring 2016, two positive linear regressions occurred, with Cyanobacteria *Synechococcus* and Eukaryotic Picophytoplankton, and the rest of the data were not available, so the only existing data was about Other Nanofalgellates, which had a higher growth rate than grazing rate.

Comparing both stations through the year, one event was common, the phytoplankton with higher growth rate was also the one with higher grazing rate, so probably, the microzooplankton, despite being selective (Obayashi & Tanoue, 2002; Cloern & Dufford, 2005), had an opportunistic feeding strategy. Nevertheless, there were differences in the growth and grazing rates between stations and seasons. Comparing stations, the outer station had a higher growth, probably due to a lower encounter rate between prey and grazer but also a lower or absent competition for nutrients between phytoplankton. While the inner station had a higher grazing rate, maybe correlated with the water temperature, because a previous study has showed that the mortality rate tend to be higher at warmer water stations (Obayashi & Tanoue, 2002). Regarding the seasons, during 2015, the autumn had a higher grazing rate but also a higher growth rate than the spring, so as mentioned before, phytoplankton not always increase with the

decrease of grazers and grazing could stimulate prey growth (Dix & Hanisak, 2015). From the few data available from spring 2016 it is impossible to take comparisons and conclusions.

4.3.2.1.Cyanobacteria *Synechococcus*

Although it was not possible to estimate the accurate value of the final abundance of this group-specific (represented 49.31% of the abundance of phytoplankton in the inner station and 58.06% in the outer station), due to the lack of data, it was evident that they were abundant and played a main role in the phytoplankton community, because when they had a positive response, the slope between dilution factors and the apparent growth rate of phytoplankton community was also positive (e.g. inner station in autumn 2015 and outer station in spring 2016). Also, when Cyanobacteria *Synechococcus* had a negative slope, the relationship between dilution factors and the apparent growth rate of phytoplankton community had too a negative slope (e.g. outer station in autumn 2015). In aquatic environments, Cyanobacteria are important primary producers (Sivonen, 2009) and since phytoplankton biomass and production are dominated by micron-sized cells (Cloern and Dufford, 2005), these results were within the normal standards.

The addition of macronutrients only was beneficial in the inner station during the autumn 2015, though the response was a positive linear regression, so it was not take into account to estimate the growth and grazing rates. Analyzing the outer station in autumn 2015 it was possible to verify that the macronutrient enrichment did not affect the growth rate of the Cyanobacteria, which could mean that there was no macronutrient limitation.

The abundance of Cyanobacteria *Synechococcus* in the inner station was higher than in the outer station, as in the initial conditions. The answer could be a difference in salinity between stations or a higher increase of grazers in the outer station than in the inner station. Regarding the salinity, that hypothesis was rejected since the salinity was lower than 36 in both zones, because according with previous studies (Wood *et al.*, 1985; Ray *et al.*, 1989; Putland & Rivkin, 1999), this group-specific is generally representative only in areas of reduced salinity. The hypothesis of an increase of grazers can be true

taking into account that the increase of Aplastidic Nanoflagellates was higher in the outer station than in the inner station (see Fig.3.11A and B).

The abundance between seasons, was not possible to compare, however it was observed a positive linear regression in both seasons but in different stations (e.g. autumn 2015 inner station and spring 2016 outer station), thus through seasons the growth rate of Cyanobacteria *Synechococcus* varied. The periods of growth rate with a positive linear regression coincided with high abundance of Cyanobacteria *Synechococcus* and with temperature increase. The water temperature *in situ* was lower than the water in the tank, thus the water sample had a temperature increase. So the abundance correlated positively with the water temperature. In Barbosa's study (2006), the abundance correlated negatively and significantly with the temperature, although the response was a negative linear regression, an expected response. The pattern obtained in this study regarding the positive correlation between abundance and temperature was resembling with the generally associated with the protected coastal environments, which shows a significant and positive relationship between the abundance of Cyanobacteria *Synechococcus* and the temperature (Malone *et al.*, 1991; Affronti & Marshall, 1994; Iriarte & Purdie, 1994; Pinckney *et al.*, 1998; Murrell & Lores, 2004; Sorokin *et al.*, 2004). Besides the temperature, the I_m seems to have been positively related with the abundance of Cyanobacteria *Synechococcus*, since the abundance during autumn 2015 was higher with a higher I_m.

Aside the high abundance, the growth rate was low (autumn 2015 outer station), which could mean a growth limitation due to micronutrients. Some micronutrients as iron stimulate Cyanobacteria *Synechococcus* growth (Timmermans *et al.*, 2005), while others as cadmium and copper inhibit (Brand *et al.*, 1986; Payne & Price, 1999). Indeed, in previous studies, the limitation of Cyanobacteria *Synechococcus* by iron bioavailability has been demonstrated experimentally in exposed and protected marine environments (Nakamura *et al.*, 1993; Kawaguchi *et al.*, 1997; Stal *et al.*, 1999; Wells, 1999; Lewitus *et al.*, 2004). While copper was used to explain the reduced abundance of Cyanobacteria *Synechococcus* within a shallow coastal lagoon (Vaquer *et al.*, 1996), its spatial distribution in confined coastal environments (Moffett *et al.*, 1997) or the vertical distribution of species of cyanobacteria in ocean environments (Mann *et al.*, 2002). To confirm this hypothesis it would obviously be necessary to study the

speciation of the micronutrients in question and the growth rate of Cyanobacteria *Synechococcus* before and after the experimental removal of micronutrients.

According with Agawin and Agusti (1997), when Cyanobacteria *Synechococcus* have a negative correlation between abundance and growth rate, it points to losses and not growth rate. This hypothesis coincided with the results obtained in this study, because Cyanobacteria, during autumn 2015 in the outer zone, had a negative linear regression and the grazing rate was higher than the growth rate. However, the grazing rate of these small organisms was lower than the grazing rate for larger organisms (e.g. Diatoms), and usually microzooplankton grazed more on smaller phytoplankton than on diatoms (Yang *et al.*, 2015). Thus this group-specific was clearly a non-preferential prey for phagotrophic protists (Verity & Villareal, 1986; Verity, 1988; Caron *et al.*, 1991). The result was also demonstrated in Barbosa's study (2006) for the inner station, however in the outer station occurred the opposite. So once the predation could not explained completely the fact that the grazing rate was higher than the growth rate, maybe the answer could be viral lysis and/or natural mortality (Veldhuis *et al.*, 2005).

4.3.2.2.Eukaryotic Picophytoplankton

As the Cyanobacteria, the Eukaryotic Picophytoplankton had lack of data, so it was not possible to estimate the accurate value of the final abundance of this group-specific (represented 21.18% of the abundance of phytoplankton in the inner station and 24.79% in the outer station). However, it was evident that they were also abundant and played a main role in the phytoplankton community, because their positive slope coincided with the positive slope between dilution factors and the apparent growth rate of phytoplankton community (e.g. inner station in autumn 2015 and outer station in spring 2016), likewise when Eukaryotic Picophytoplankton had a negative slope, the relationship between dilution factors and the apparent growth rate of phytoplankton community had too a negative slope (e.g. outer station in autumn 2015). Since phytoplankton biomass and production are dominated by micron-sized cells (Cloern and Dufford, 2005), these results were within the normal standards.

The addition of macronutrients only was beneficial in the inner station during the autumn 2015, though the response was a positive linear regression, so it was not take

into account to estimate the growth and grazing rates. Analyzing the outer station in autumn 2015 it was possible to verify that the macronutrient enrichment did not affect the growth rate of the Eukaryotic Picophytoplankton, which could mean that there was no macronutrient limitation.

The abundance in the inner station was higher than the outer station during autumn 2015, in both initial (T0) and final stage (T24) of the experiment. These results could be explained by phytoplankton saturation in the inner station (grazing by microzooplankton becomes irrelevant; Redden *et al.*, 2002), by the fact that grazers in the inner station preferred other preys, or by a higher predation in the outer station. The Aplastidic Nanoflagellates and Ciliates are considered the main predators of picophytoplankton (Verity & Vernet, 1992; Bernard & Rassoulzadegan, 1993; Reckerman & Veldhuis, 1997; Samuelsson & Andersson, 2003; Caron *et al.*, 2004), so the supposition that grazers in the inner station preferred other preys it is not very plausible. However, it has been shown that for high concentrations of prey, its assimilation seems to be less efficient (Jumars *et al.*, 1989; Nagata & Kirchman, 1991) and the maximum growth efficiency of grazers seems to be associated with intermediate values of food availability (Straile, 1997). In the outer station the grazing rate was higher than the growth rate (see Fig.3.8), which means that in the outer station, the Eukaryotic Picophytoplankton was one of the preferred prey.

The growth rate between seasons had a high difference, since the outer station during autumn 2015 had a negative linear regression while the same station in spring 2016 had a positive linear regression. Also the abundance in the outer station of Eukaryotic Picophytoplankton in spring 2016 was almost twice the abundance during autumn 2015. The explanation to these results could be the input of nutrients through rainfall that occurred days before the sampling period in spring 2016, because according with previous studies (Joint *et al.*, 2001; Shalapyonok *et al.*, 2001; Sherr *et al.*, 2005), the abundance of Eukaryotic Picophytoplankton increase after an input of nutrients, so it was normal that during spring 2015 the abundance was higher than in autumn 2015. The *in situ* growth rate of Eukaryotic Picophytoplankton in the outer station during autumn 2015 was 0.46 d^{-1}, which was within the standards of the maximum growth rates reported for several Eukaryotic picoplanktons (0.23 to 1.20 d^{-1}; Iriarte & Purdie, 1993;

Buskey *et al.*, 1998; Jacquet *et al.*, 2001; MacIntyre *et al.* 2004; Timmermans *et al.*, 2005).

Comparing the abundance of Cyanobacteria and Eukaryotic Picophytoplankton, there was a dominance of Cyanobacteria relative to Eukaryotic Picophytoplankton, in both stations and seasons. This may be related to lower maintenance metabolic costs (Weisse, 1993), lower diffusion limitation and lower impact of predation due to reduced biovolume (Tamigneaux *et al.*, 1995), buoyancy regulation capacity (Phlips *et al.*, 1999) and fixation of atmospheric nitrogen (Zehr *et al.*, 2001), particularly important characteristics that promote selective growth of Cyanobacteria. Also this difference may reflect the fact that Eukaryotic Picophytoplankton present higher saturation light intensities than *Synechococcus* (Veldhuis *et al.*, 2005). However, the growth rate of these two group-specific was equal, then the lower impact of microzooplankton on *Synechococcus* may have allowed its relative dominance (Barbosa, 2006).

4.3.2.3.Cryptophyceae

As in the previous group-species, it was not possible to estimate the accurate value of abundance of Cryptophyceae (represented 1.05% of the abundance of phytoplankton in the inner station and 0.40% in the outer station), because of the lack of data and many uninterpretable data (e.g. Cryptophyceae only grown in dil. 0.50). Analyzing the inner station in autumn 2015 and spring 2016 it was possible to verify that the macronutrient enrichment did not affect the growth rate of the Cryptophyceae, which could mean that there was no macronutrient limitation. The abundance in the inner station was higher than in the outer station. Nevertheless, the abundance in both stations, when compared with the Other Plastidic Nanoflagellates, was much reduced. In the inner station the Other P. Nanoflagellates were almost two times more abundant than the Cryptophyceae, and in the outer zone were nineteen times more abundant.

In the inner station of the Ria Formosa, the Cryptophyceae had a high seasonal variability of growth rate, however they presented a relatively low growth rate (average of 0.43 d^{-1}), lower than the other group-specifics. The maximum growth rate of this group-specific is generally reduced (0.24 to 1.0 d^{-1}; Ojala, 1993; MacIntyre *et al.*, 2004). Still, the growth rate obtained in the present study was in line with the growth

rate referred for Cryptophyceae (Braunwarth & Sommer, 1985; Burkill *et al.*, 1987; Sommer, 2000). However, they were lower than the values reported for some coastal environments (Ferrier-Pagès & Rassoulzadegan, 1994; Fahnenstiel *et al.*, 1995; Gaul & Antia, 2001) which indicate average growth rate up to 1.2 d^{-1}. While in the outer station, it was not possible to estimate the growth rate, because the dilutions did not show any growth rate. Comparing the seasons, the growth rate was higher during spring 2016, which is common, since Cryptophyceae are generally considered a group well adapted to reduced light intensities, typical of winter-spring, and able to form small and brief blooms (Klaveness, 1988; Litaker *et al.*, 2002b; Nuccio *et al.*, 2003). However, the higher growth rate in spring 2016 coincided with a higher water temperature and light intensity, so the growth rate correlated positively with the temperature and light intensity. This positive correlation has already been demonstrated in previous studies (Bruno *et al.*, 1983; Verity, 1986a; Cole *et al.*, 1986).

The grazing rate of Cryptophyceae was higher in spring than in autumn, regarding the comparison of grazing rates between stations was not possible due to lack of data, since the dilutions did not show any grazing rate. The impact of microzooplankton predation on Cryptophyceae production was constantly higher than 100% $.d^{-1}$, through the experimental period, obviously indicating that only this removal process can prevent the occurrence of blooms and justify the decrease of the observed abundance in this period. In previous studies (Burkill, 1982; Admiraal & Venekamp, 1986; Kamiyama, 1994; Gallegos *et al.*, 1996; Lewitus *et al.*, 1998; Strom *et al.*, 2001), similar results were also reported for coastal environments.

4.3.2.4.Other Plastidic Nanoflagellates

As in the previous group-species, it was not possible to estimate the accurate value of abundance of Other Plastidic Nanoflagellates (represented 19.67% of the abundance of phytoplankton in the inner station and 13.73% in the outer station), because of the lack of data. Analyzing autumn 2015, it was possible to verify that the macronutrient enrichment did affect the growth rate of this group-specific, however during spring 2016, did not affect the growth rate, which could mean that there was a macronutrient limitation during autumn 2015, although during spring 2016 there was none. This possible limitation only occurred in the inner station, since in the outer station during

autumn 2015, the macronutrient enrichment did not affect the growth rate however the final abundance was still higher in the outer station than in the inner station. Then perhaps the inner station during autumn did not have all the essential requirements for the growth rate of the Other Plastidic Nanoflagellates.

Comparing stations, an inconsistency occurred since the abundance was not higher in only one station during all the experiment (e.g. the abundance of Plastidic Dinoflagellates was higher in the outer station in both seasons). During autumn 2015, the abundance of Other Plastidic Nanoflagellates was higher in the outer station, while during spring 2016, it was in the inner station, which coincided with the higher water temperatures, thus the abundance correlated positively with the temperature. Comparing seasons, there was a high difference between autumn 2015 and spring 2016, since this group-specific was more abundant during spring 2016 than in autumn 2015 (three times more abundant), thus there was seasonal variability. Some studies report a relatively high seasonal variability of nanophytoplankton (Cole *et al.*, 1986; Abreu *et al.*, 1994; Lonsdale *et al.*, 1996; Lewitus *et al.*, 1998; Murrell & Lores, 2004), although sometimes this includes nanoplankton diatoms responsible for the occurrence of blooms (Cole *et al.*, 1986; Abreu *et al.*, 1994; Murrell & Lores, 2004). However, the occurrence of maximum abundances of plastidic flagellates during spring was reported for several coastal environments in previous studies (Furnas, 1983; Cadeé & Hegeman, 1991; Thompson, 1998; Tolomio *et al.*, 1999; Druzhkov & Druzhkova, 2000; Rodriguez *et al.*, 2000; Gilabert, 2001; Marty *et al.*, 2002; Rodriguez *et al.*, 2003). This occurrence may reflect the growth response of plastidic flagellates to the increase of water temperature and light intensity (Andersson *et al.*, 1994; Tamigneaux *et al.*, 1995).

In the inner station of the Ria Formosa, the Other Plastidic Nanoflagellates had a high seasonal variability of the growth rate, however in the outer station, it was not possible to estimate all the growth rates, because in the outer station during autumn 2015, the type of response was a positive linear regression. Comparing the stations during spring 2016, the growth rate was slightly higher in the outer station. Regarding the growth rate between seasons, it was higher during spring, which coincided with the higher abundance of these organisms, thus indeed there was an increase of these nanoflagellates. Of the flagellate group (Cryptophyceae, Other Plastidic Nanoflagellates, Euglenophyceae and Plastidic Dinoflagellates), the Other Plastidic

Nanoflagellates were those with the highest growth rate, however when compared with the Diatoms, they had the lowest growth rate. Despite some exceptions (Burkill *et al.*, 1987; Gieskes & Kraay, 1989; McManus & Ederington-Cantrell, 1992; Fahnenstiel *et al.*, 1995; Gaul & Antia, 2001; Suzuki *et al.*, 2002), the occurrence of *in situ* growth rates of Plastidic Nanoflagellates lower than Diatoms growth is often reported for several marine systems (Furnas, 1982a,b; Landry *et al.*, 1984; Furuya *et al.*, 1986; Furnas, 1991; Strom & Welschmeyer, 1991; Welschmeyer *et al.*, 1991; Goericke & Welschmeyer, 1993; Verity *et al.*, 1996; Latasa *et al.*, 1997; Landry *et al.*, 2000; Crosbie & Furnas, 2001; Brown *et al.*, 2002; Goericke, 2002; Obayashi & Tanoue, 2002). However, these results are sometimes associated with underestimation of Plastidic Flagellate growth caused by increased sensitivity to handling and confinement (Sommer, 1985; Furnas, 1990; Fahnenstiel *et al.*, 1995) or by increased probability of predation by phagotrophic protists (Vyhnalek & Budejovice, 1989; Balode *et al.*, 1998; Granéli & Turner, 2002).

In this study, the hypothesis of underestimation by sensitivity to handling and confinement cannot obviously be excluded, because although the experiment was carried out with extreme care, the negative effect of handling (filtration) and confinement on the growth of the Plastidic Flagellates may have occurred. The underestimation by increased predation by phagotrophic protists seems unlikely given that of the flagellate group (Cryptophyceae, Other Plastidic Nanoflagellates, Euglenophyceae and Plastidic Dinoflagellates), the Other Plastidic Nanoflagellates were the ones that had the lowest grazing rate in the inner station during autumn 2015, so they were a non-preferential prey for phagotrophic protists during this period of time.

This group-specific was more grazed in the inner station when comparing stations, and in spring 2016 when comparing seasons, which coincided with the higher growth rate. In a previous study (Dix & Hanisak, 2015) it was showed that phytoplankton not always increase with the decreasing of predators and grazing actually may stimulate prey growth, thus these results were not uncommon. Also the abundance of Other Plastidic Nanoflagellates was positively and significantly related to the abundance of Ciliates, and more strongly with the abundance of *Tintinnopsis*. In fact, the occurrence of positive correlations between *Tintinnopsis* or Ciliates and plastidic flagellates, in particular nanoplankton, has also been reported for several coastal environments

(Burkill, 1982; Capriulo & Carpenter, 1983; Verity, 1987; Admiraal & Venekamp, 1986; Sanders, 1987; Dolan & Coats, 1990; Kamiyama, 1994; Verity *et al.*, 1999; Rodriguez *et al.*, 2000; Strom *et al.*, 2001). In Barbosa's study (2006), this positive association occurred with the Cryptophyceae and only in the inner station.

4.3.2.5.Euglenophyceae

As in the previous group-species, it was not possible to estimate the accurate value of abundance of Euglenophyceae (represented 0.52% of the abundance of phytoplankton in the inner station and 0.0% in the outer station), because of the lack of data and many uninterpretable data (e.g. Euglenophyceae had abundance of zero in dil 1.0). Analyzing the spring 2015 and autumn 2015 it was possible to verify that the macronutrient enrichment did not affect the growth rate of this group-specific, which could mean that there was no macronutrient limitation.

The abundance may have been high in the inner station however it was unknown in the outer station due to the inexistence of the same in the dilutions, so it was impossible to compare and be certain. Between seasons, the abundance was higher during spring, as in Barbosa's study (2006**)**. The occurrence of maximum abundances of plastidic flagellates in spring (Furnas, 1983; Andersen & Sorensen, 1986; Cadeé & Hegeman, 1991; Haigh *et al.*, 1992; Thompson, 1998; Tolomio *et al.*, 1999; Verity *et al.*, 1999; Druzhkov & Druzhkova, 2000; Rodriguez *et al.*, 2000; Gilabert, 2001; Marty *et al.*, 2002; Rodriguez *et al.*, 2003) was previously reported for several coastal environments. This occurrence may reflect the growth response of plastidic flagellates to the increase of water temperature and light intensity (Andersson *et al.*, 1994; Tamigneaux *et al.*, 1995). Also the abundance of Euglenophyceae correlated significantly and positively with the water temperature and light intensity, which was demonstrated in previous studies (Levasseur *et al.*, 1984; Tremblay *et al.*, 1997; Facca *et al.*, 2002).

In the inner station of the Ria Formosa, the Euglenophyceae had a high seasonal variability of the growth rate, however in the outer station, it was not possible to estimate all the growth rates, because the data from springs (2015 and 2016) were not available. However, comparing the stations of autumn 2015, it was possible to verify that the inner station had a higher growth rate. Between seasons, the growth rate was

higher during spring. During autumn 2015, the Euglenophyceae presented a relatively low growth rate and lower than the other group-specifics, except the Cryptophyceae, which had a lower growth rate than the Euglenophyceae. According with Tang (1995), there is an inverse relationship between the cell size and the maximum growth rate, so the fact that Euglenophyceae had a growth rate lower than the Other Plastidic Nanoflagellates it was normal. However, the Diatoms had a higher growth rate than the Euglenophyceae, which goes against the hypothesis of Tang (2015). Nevertheless, the occurrence of higher growth rates in the larger phytoplankton (Furuya *et al.*, 1986; Neuer & Cowles, 1994; Strom & Strom, 1996; Cermeno *et al.*, 2003) were reported for several coastal environments.

The grazing rate was higher in the outer station, when comparing stations, and between seasons was higher in autumn 2015. The impact of microzooplankton predation on Euglenophyceae production was constantly higher than 100% $.d^{-1}$, in autumn 2015, obviously indicating that only this removal process can prevent the occurrence of blooms and justify the decrease of the observed abundance in autumn 2015. In previous studies (Burkill, 1982; Admiraal & Venekamp, 1986; Kamiyama, 1994; Gallegos *et al.*, 1996; Lewitus *et al.*, 1998; Strom *et al.*, 2001), similar results were also reported for coastal environments. Besides the microzooplankton, other removal factors, such as the impact of tidal advection and benthic predation, may have controlled the dynamics of Euglenophyceae, since in the spring 2015 the removal was 70%. However, in this study, no studies have been performed in this matter, thus it will not be possible to confirm such hypothesis. In a previous study (Barbosa, 2006), these impacts were roughly estimated, only for the Spring-Summer period, and were equivalent to the average removal of 16-35% of daily production.

4.3.2.6.Plastidic Dinoflagellates

As in the previous group-species, it was not possible to estimate the accurate value of abundance of Plastidic Dinoflagellates (represented 1.97% of the abundance of phytoplankton in the inner station and 1.69% in the outer station), due to the lack of data. Analyzing the spring 2015 and autumn 2015 it was possible to verify that the macronutrient enrichment did not affect the growth rate of this group-specific, which could mean that there was no macronutrient limitation.

The abundance of Plastidic Dinoflagellates was dominated by an unidentified microplanktonic gymnodinoid, which was probably toxic, since the bivalve catch was partially interdicted in that time period, according with the Portuguese institute of the sea and the atmosphere (IPMA). These results were also obtained in Barbosa's study (2006). Furthermore the abundance of the toxic dinoflagellate was higher in the outer station. The occurrence of microplanktonic gimnodinoid blooms, sometimes toxic, has been reported for several exposed coastal systems (Mallin *et al.*, 1991; Blasco *et al.*, 1996; Rodriguez *et al.*, 2000; Aubry & Acri, 2004; Millie *et al.*, 2004), including the Portuguese continental shelf (Palma, 1996; Moita, 2001), and its advection to the interior of the adjacent protected coastal systems was also documented (Figueiras *et al.*, 1998; Bennouna *et al.*, 2002).

The higher abundance of Plastidic Dinoflagellates occurred in the outer station, when comparing stations, which can be explain by the fact that this group-specific have high mobility and storage capacity of nutrients (Andersson *et al.*, 1994; Badylak & Phlips, 2004), and are mixotrophic (Stoecker *et al.*, 1997; Coats, 2002), particularly advantageous characteristics that give them advantage over the other phytoplankton groups. Therefore, in the outer station, the Plastidic Dinoflagellates, probably, did not have to compete for resources. Comparing seasons, the abundance was higher in spring 2015, which coincided with higher temperature and light intensity. In previous studies (Langdon, 1988; Garcés *et al.*, 1999; Badylak & Phlips, 2004), this positive effect of temperature and light intensity were demonstrated. In Barbosa's study (2006), this group-specific also had maximum values in spring. This pattern was distinct from that classically reported for temperate coastal systems where dinoflagellates exhibit maximum abundances and generally dominate the microphytoplankton community in summer (Revelante & Gilmartin, 1976; Holligan & Harbour, 1977; Roden 1984; Smetacek, 1985; Haigh *et al.*, 1992; Rodriguez *et al.*, 2000).

The Plastidic Dinoflagellates correlated negatively with the Diatoms. In the inner station it were the Diatoms that dominated, while in the outer station it were the Plastidic Dinoflagellates. These results were not uncommon, because the dinoflagellates abundance is generally higher in periods of relaxation, stratification and, in particular, in periods of coastal convergence (Moita, 2001; Nogueira & Figueiras, 2005), characteristics events of the outer station. Also the coastal convergence passively

eliminates diatoms and creates a suitable ecological niche for highly mobile species, such as dinoflagellates, which are capable of minimizing the effects of sinking (Figueiras *et al.*, 1996; Nogueira *et al.*, 2000).

This group-specific had a high seasonal variability of the growth rate. The growth rate was higher in the inner station, when comparing stations, however the higher abundance was in the outer station, then a difference of removal processes between stations and the mixotrophy could explain the results. The impact of microzooplankton predation on Plastidic Dinoflagellates production was constantly higher in the outer station, thus other removal processes, e.g. viruses, should have occurred at the inner station to justify a lower abundance. At least 20 marine phytoplankton species are infected with host-specific viruses (Zingone *et al.*, 1999), suggesting that pathogens play an important role in regulating phytoplankton biomass and species composition (Short & Suttle, 2003). Some Plastidic Dinoflagellates are mixotrophic (Stoecker *et al.*, 1997; Coats, 2002), thus perhaps, in the inner station this group-specific used the two resources available, other algae and macronutrients, which improved their growth in the inner station. The direct ingestion of its competitors by mixotrophy (Skovgaard, 1996) was previous used to explain a higher abundance of dinoflagellates. Comparing seasons, the growth rate was higher in autumn 2015. The higher growth rate in autumn 2015 was probably due to the fact that this group-specific is well adapted to reduced light intensity (Litaker *et al.*, 2002a).

The grazing rate between stations had variations through the year, because during spring 2015, it was higher in the outer station, while during autumn 2015, the grazing rate was higher in the inner station, and of the flagellates, the Plastidic Dinoflagellates were the most grazed. Therefore it seems that, in the inner station during autumn 2015, this group-specific was a preferential prey for phagotrophic protists, regarding only the plastidic flagellates. These variations of the grazing rate could be the result of toxins produced by the dinoflagellates, of a variation on Euglenophyceae abundance, and/or a decrease of predation. The reduced intake of some dinoflagellates by phagotrophic protists due to the production of toxic substances has been previously explained (Stoecker *et al.*, 1981 and 1986). Through the experiment period, the growth rates between Plastidic Dinoflagellates and Euglenophyceae were inversely proportional, and the grazing rates were also inversely proportional. Then, it appears that between these

groups-specific, the microzooplankton prefers the most abundant, so the grazer was opportunistic. The avoidance or minimization of dinoflagellates predation has also been reported in previous studies (Granéli *et al.*, 1993; Smayda, 1997; Badylak & Phlips, 2004). Comparing seasons, it was higher in the autumn 2015. The higher grazing rate, in stations and seasons, coincided with the lower water temperatures, however according with Litaker *et al.* (2002b), with a decrease of temperature, occurs a decrease of predation, thus the results in this study was unexpected.

4.3.2.7.Centric Diatoms

As in the previous group-species, it was not possible to estimate the accurate value of abundance of Centric Diatoms (represented 4.74% of the abundance of phytoplankton in the inner station and 0.90% in the outer station), due to the lack of data. Analyzing autumn 2015, it was possible to verify that the macronutrient enrichment did affect the growth rate of this group-specific, however during spring 2015, did not affect the growth rate, which could mean that there was a macronutrient limitation during autumn 2015, although during spring 2015 there was none. This possible limitation only occurred in the inner station, since in the outer station during autumn 2015, the macronutrient enrichment did not affect the growth rate. Also, the final abundance was higher in the inner station than in the outer station, thus there was a fast-responding of Centric Diatoms to the macronutrients which promoted their growth rate during autumn 2015 in the inner station. In Cloern and Dufford (2005) study this fast-responding to nutrient pulses also occurred. The abundance of Centric Diatoms was higher in the inner station, when comparing stations, and between seasons was higher in spring 2015. According with Ansotegui *et al.* (2003), Diatoms have an exponential growth in spring as a response to the improvement of light and/or nutritional environment.

This group-specific had a high seasonal variability of the growth rate, and was the highest of the whole phytoplankton group, in the outer station. The growth rate of the Centric Diatoms were within the values mentioned for many marine systems (Furnas, 1982a,b; Landry *et al.*, 1984; Furuya *et al.*, 1986; Gieskes & Kraay, 1989; Furnas, 1991; McManus & Ederington-Cantrell, 1992; Fahnenstiel *et al.*, 1995; Verity *et al.*, 1996; Latasa *et al.*, 1997; Gaul & Antia, 2001; Crosbie & Furnas, 2001; Brown *et al.*, 2002).

The growth rate was higher in the outer station, when comparing stations, however the higher abundance and the effect of nutrients occurred in the inner station, then the explanation could be the higher grazing rate that occurred in the inner station, and/or the fact that the limitation of growth by nutrients did not seem to be important for the growth of diatoms. In fact, Furnas (1982b; 1991) reported the occurrence of high growth rate of diatoms simultaneously with extremely variable nutrient concentrations. According to Andersson *et al.* (1994) this may reflect diatoms ability to consume and store nutrients in excess during pulses of nutrient production. This capacity could explain the difference between the growth rates of the Centric Diatoms and Plastid Flagellates. Regarding the seasons, it was higher in autumn 2015, which coincided with a decrease of Aplastidic Dinoflagellates, showing that these predators are essential for controlling the centric diatom. According to previous studies (Paranjape, 1990; Hansen, 1992; Sime-Ngando *et al.*, 1995; Strom & Strom, 1996; Latasa *et al.*, 1997; Uitto *et al.*, 1997; Schluter, 1998; Hall *et al.*, 1999; Levinsen *et al.*, 1999; Strom *et al.*, 2001; Saito *et al.*, 2005), heterotrophic dinoflagellates are generally associated with intense predation of diatoms.

The Centric Diatoms had a higher growth rate than the flagellate group (Cryptophyceae, Other Plastidic Nanoflagellates, Euglenophyceae and Plastidic Dinoflagellates). Although of the inverse relationship between the cell size and the maximum growth rate (Tang, 1995), the lack of differences between the growth of several dimensional classes (Cole *et al.*, 1986; Kamiyama, 1994; Gallegos *et al.*, 1996; Strom *et al.*, 2001) and the occurrence of higher growth rates in the larger phytoplankton (Furuya *et al.*, 1986; Neuer & Cowles, 1994; Strom & Strom, 1996; Cermeno *et al.*, 2003) were reported for several coastal environments. Indeed, diatoms divide faster than other taxa, either because they have inherently high growth rates (Smayda, 1997), accelerated N assimilation (Dugdale & Wilkerson, 1992), or high growth efficiency at low light (Goldman & McGillicuddy, 2003). Also these Diatoms were more abundant than the Euglenophyceae and the Plastidic Dinoflagellates, however they were not more abundant than the Cryptophyceae and the Other Plastidic Nanoflagellates, thus the diatom dominance may represent the result of a growth and removal differential (Riegman *et al.*, 1993; Juhl & Murrell, 2005), since they had a higher growth rate but also a higher grazing rate. In Barbosa's study (2006) only the growth differential happened.

The grazing rate between stations had a variation through the year, because during spring 2015, it was higher in the outer station, which coincided with a higher abundance of Ciliates, in fact the Centric Diatoms can be efficiently ingested by Ciliates (Capriulo & Carpenter, 1980; Verity & Villareal, 1986; Paranjape, 1990; Nielsen & Hansen, 1995; Nejstgaard *et al.*, 1997; Urrutxurtu *et al.*, 2003). While during autumn 2015, the grazing rate was higher in the inner station, which coincided with an increase of Ciliates. Thus the grazing rate between stations had a positive and significant correlation with the abundance of Ciliates, showing that these were the main grazers of the Centric Diatoms. Comparing seasons, the grazing rate was higher during autumn 2015. Usually, grazers seem to prefer smaller phytoplankton (Obayashi & Tanoue, 2002; Yang *et al.*, 2015), although they can graze on large as well as small cells (Strom *et al.*, 2007; Sherr *et al.*, 2013), however in this study the Centric Diatoms were more grazed than the plastidic flagellates (Cryptophyceae, Other Plastidic Nanoflagellates, Euglenophyceae and Plastidic Dinoflagellates), maybe due to the intensification of diatom predation by phagotrophic protists (Paranjape, 1990; Strom & Strom, 1996; Strom *et al.*, 2001) and/or because the flagellates have evolved defenses against predation. According to previous studies (Carlsson *et al.*, 1995; Teegarden, 1999; Calbet *et al.*, 2002), some Plastidic Dinoflagellates, such as *Gymnodinium* and *Dinophysis*, synthesize metabolites that inhibit feeding and growth of grazers.

4.3.2.8.Pennate Diatoms

As in the previous group-species, it was not possible to estimate the accurate value of abundance of Pennate Diatoms (represented 1.55% of the abundance of phytoplankton in the inner station and 0.44% in the outer station), due to the lack of data. Analyzing the spring 2015 and autumn 2015 it was possible to verify that the macronutrient enrichment did not affect the growth rate of this group-specific, which could mean that there was no macronutrient limitation. Comparing stations, an inconsistency occurred since the abundance was not higher in only one station during all the experiment (e.g. the abundance of Plastidic Dinoflagellates was higher in the outer station in both seasons). During spring 2015, the abundance of Pennate Diatoms was higher in the inner station, while during autumn 2015, it was in the outer station, which coincided with the higher water temperatures, therefore the abundance correlated positively with

the temperature. Between seasons, it was higher during spring 2015. According with Ansotegui *et al.* (2003), Diatoms have an exponential growth in spring as a response to the improvement of light and/or nutritional environment.

In this study occurred a predominance of plastid flagellates on diatoms (Centric plus Pennate) and it cannot be explained by a growth differential, for example associated with their better adaptation to reduced light intensities (Levasseur *et al.*, 1984; Madariaga & Orive, 1989; Riegman *et al.*, 1993), since diatoms had higher growth rates. Thus, in the case of the Ria Formosa, this dominance reflected the smaller relative impact of the removal processes on the plastid nanoflagellates.

The Pennate Diatoms had a high seasonal variability of the growth rate, and was the highest of the whole phytoplankton group, in both stations. The growth rate of this group-specific was within the values mentioned for many marine systems (Furnas, 1982a, b; Landry *et al.*, 1984; Furuya *et al.*, 1986; Gieskes & Kraay, 1989; Furnas, 1991; McManus & Ederington-Cantrell, 1992; Fahnenstiel *et al.*, 1995; Verity *et al.*, 1996; Latasa *et al.*, 1997; Gaul & Antia, 2001; Crosbie & Furnas, 2001; Brown *et al.*, 2002).

The growth rate was not higher in only one station through the experimental period, because during spring 2015, was higher in the inner station, and in autumn 2015, was in the outer station, which coincided with the higher water temperatures. Then the growth rate correlated positively and significantly with temperature, as in Barbosa's study (2006). Several studies have reported this positive relationship between temperature and microphytoplankton growth (Malone, 1977; Bruno *et al.*, 1983; Andersson *et al.*, 1994). Between seasons, it was higher in autumn 2015, which coincided with a decrease of Aplastidic Dinoflagellates. According to previous studies (Paranjape, 1990; Hansen, 1992; Sime-Ngando *et al.*, 1995; Strom & Strom, 1996; Latasa *et al.*, 1997; Uitto *et al.*, 1997; Schluter, 1998; Hall *et al.*, 1999; Levinsen *et al.*, 1999; Strom *et al.*, 2001; Saito *et al.*, 2005), heterotrophic dinoflagellates are generally associated with intense predation of diatoms.

Comparing the growth rates of both Diatoms, between stations, it was verified a negative correlation (inversely proportional) in the inner station, and a similarity

(statistically they were not different) in the outer station. Then in the inner station, an intraspecific competition may have occurred. Between seasons, the Pennate Diatoms had a higher growth rate than the Centric Diatoms however the abundance of Centric Diatoms was higher than the Pennate Diatoms. The predation could explained this difference, however the impact of microzooplankton predation on Pennate Diatoms production was constantly lower than the impact on Centric Diatoms, so the Pennate Diatoms had perhaps other types of removal processes.

The grazing rate was higher in the outer station, when comparing stations, which coincided with a higher abundance of Aplastidic Dinoflagellates. According to previous studies (Paranjape, 1990; Hansen, 1992; Sime-Ngando *et al.*, 1995; Strom & Strom, 1996; Latasa *et al.*, 1997; Uitto *et al.*, 1997; Schluter, 1998; Hall *et al.*, 1999; Levinsen *et al.*, 1999; Strom *et al.*, 2001; Saito *et al.*, 2005), heterotrophic dinoflagellates are generally associated with intense predation of diatoms. And between seasons was higher in autumn 2015, which coincided with a decrease of Aplastidic Dinoflagellates, therefore other removal processes should have occurred, e.g. virus. At least 20 marine phytoplankton species are infected with host-specific viruses (Zingone *et al.*, 1999), suggesting that pathogens play an important role in regulating phytoplankton biomass and species composition (Short & Suttle, 2003).

The Pennate and Centric Diatoms had grazing rates statistically similar during spring 2015 however during autumn 2015, the Centric Diatoms were more grazed than the Pennate Diatoms. This difference between seasons coincided with an increase and decrease of Aplastidic Dinoflagellates, respectively. Then during autumn, the heterotrophic dinoflagellates seem to have preferred the Centric Diatoms or the Pennate Diatoms had better defenses against predation than the Centric Diatoms.

Usually, grazers seem to prefer smaller phytoplankton (Obayashi & Tanoue, 2002; Yang *et al.*, 2015), although they can graze on large as well as small cells (Strom *et al.*, 2007; Sherr *et al.*, 2013), however in this study the Pennate Diatoms were more grazed than the plastidic flagellates (Cryptophyceae, Other Plastidic Nanoflagellates, Euglenophyceae and Plastidic Dinoflagellates), maybe because these flagellates have evolved defenses against predation. According to previous studies (Carlsson *et al.*, 1995; Teegarden, 1999; Calbet *et al.*, 2002), some Plastidic Dinoflagellates, such as

Gymnodinium and *Dinophysis*, synthesize metabolites that inhibit feeding and growth of grazers.

4.3.3. Microzooplankton growth

Stoecker *et al.* (2014) observed that the dilution had a negative effect on phytoplankton physiology and could have compromised their growth rate, which could have also resulted in an underestimation of microzooplankton grazing. Nevertheless it was clear that microzooplankton was an important link in food webs. Beside this negative effect on phytoplankton, the response of the grazer community to dilution in terms of apparent growth and mortality was also recognized as a possible problem (Landry *et al.*, 1995a). Dolan *et al.* (2000) observed that the grazer growth in undiluted waters and grazer mortality in dilute water may be common and result in uncertainty in measured grazing rates. Then the values obtained in this study should be considered as an estimative.

4.3.3.1. Aplastidic Nanoflagellates

As in the previous group-species, it was not possible to estimate the accurate value of abundance of Aplastidic Nanoflagellates (represented 74.07% of the abundance of microzooplankton in the inner station and 97.30% in the outer station), due to the lack of data. The abundance had a poorly defined seasonal cycle through the experiment period, since it had high values during autumn 2015 in the inner station, and a maximum value during spring 2016 in the outer station. Seasonal cycles of Aplastidic Nanoflagellates in several protected temperate coastal systems, generally show maximum values at the end of spring (Dolan & Coats, 1990; Tobiensen, 1991; Wikner & Hagstrom, 1991; Vaqué *et al.*, 1992; Coffin & Connolly, 1997). However, some studies have shown that this group-specific may have poorly defined seasonal cycles (McManus & Fuhrman, 1990; Galvão & Fritz, 1991). In Barbosa's study (2006), the Aplastidic Nanoflagellates abundance also exhibited a seasonal variation with maximum values, but quite variable, in the spring.

Significant relationships were detected between the abundance of Aplasidic Nanoflagellates and the temperature in several protected coastal systems (Coffin & Sharp, 1987; Wright *et al.*, 1987), however in this study there was no such significant

relationship. Also, the seasonal variation of this group is often associated with the variation of biomass or activity of heterotrophic bacterioplankton (Davis *et al.*, 1985; Coffin & Sharp, 1987; Wright *et al.*, 1987; Galvão, 1990; Tobiensen, 1991; Wikner & Hagstrom, 1991; Solic & Krstulovic, 1994), however in this study it was impossible to affirm or reject such hypothesis since there was no data regarding this subject.

The increase of Plastidic Nanoflagellates, between stations, was higher in the outer station, however it was not possible to compare between seasons, because during spring 2016, occurred a decrease and a high increase of this group-specific, while during autumn 2015 it only occurred an increase of abundance. The decrease of this group-specific coincided with a high increase of Ciliates and with a high temperature. In previous studies (Andersen & Sorensen, 1986; Kuosa & Kivi, 1989; Dolan & Coats, 1990; McManus & Fuhrman, 1990; Tobiensen, 1991; Vaqué *et al.*, 1992; Solic & Krstulovic, 1995; Solic *et al.*, 1998) has been show that the action of predators, particularly Ciliates, can control these protists. Also the decrease in the growth efficiency of several phagotrophic protists associated with temperature increase (Rassoulzadegan, 1982) or at high and low temperature values (Sherr *et al.*, 1983) was reported for several marine phagotrophic protists. Nevertheless, the absence of a seasonal variation in the growth rate of the Aplastidic Nanoflagellates was not uncommon, since Galvão (1990), Galvão & Fritz (1991) and Ferrier-Pagès & Rassoulzadegan (1994) did not detect a clear seasonal variation in the growth rate of these protists.

Regardless of their origin, the reduction of the growth efficiency of the Aplastidic Nanoflagellates observed in the late spring experiments implies that the potential for remineralization of organic matter was probably higher during this period (Caron & Goldman, 1990). As this period of the year coincided with minimum concentrations of inorganic macronutrients (Falcão, 1996; Newton & Mudge, 2005), DON and DOP (Falcão, 1996), the action of Aplastidic Nanoflagellates may eventually stimulate phytoplankton communities.

The Aplastidic Nanoflagellates were always more abundant than the Ciliates and the Aplastidic Dinoflagellates, probably because this group-specific did not compete for preys. According to previous studies (Solic & Krstulovic, 1994, 1995; Christaki *et al.*,

2001), the main prey of the Aplastidic Nanoflagellates is the heterotrophic bacterioplankton. In Barbosa's study (2006), the diet of this group was based essentially on the intake of heterotrophic bacterioplankton. Nevertheless, because there was no data about that subject it was impossible to affirm that hypothesis. Aside the heterotrophic bacterioplankton, the Picophytoplankton was also a prey of the Aplastidic Nanoflagellates, although this prey was probably also disputed by the Ciliates (see chapter IV, section 4.3.2.2). Another aspect for the higher abundance of Aplastidic Nanoflagellates was the lack of grazers of this group-specific, because in the Ria Formosa, they are grazed by benthic phagotrophic protists (Capriulo, 1990) and benthic metazoans, including bivalves (Kreeger & Newell, 1996; Findlay *et al.*, 1998).

4.3.3.2.Ciliates

As in the previous group-species, it was not possible to estimate the accurate value of abundance of Ciliates (represented 22.49% of the abundance of microzooplankton in the inner station and 1.84% in the outer station), due to the lack of data. Comparing stations, the abundance was higher in the outer station, which coincided with a high grazing rate of diatoms. In fact the Centric Diatoms can be efficiently ingested by Ciliates (Capriulo & Carpenter, 1980; Verity & Villareal, 1986; Paranjape, 1990; Nielsen & Hansen, 1995; Nejstgaard *et al.*, 1997; Urrutxurtu *et al.*, 2003). Comparing seasons, the abundance was higher during spring 2015. The abundance of ciliates exhibited a clear seasonal variation with maximum values in spring, and relatively low values in autumn. The observation of maximum abundances of ciliates in spring was previously reported for the interior of the western sector of the Ria Formosa by Thiele-Gliesche (1992).

Some species of ciliates feed on the sediment (Fenchel & Jonsson, 1988). In some cases, this observation is associated with the formation of resting cysts (Jonsson, 1994), whereas in the case of tintinids it was associated with the use of detrital material to form loric (Revelante & Gilmartin, 1990). In the Ria Formosa, for example, Thiele-Gleische (1992) observed a pattern of nocturnal migration to the bottom in some types of ciliates. Verity (1987) was the only author to suggest a relationship between the association of planktonic ciliates to sediment and food activity, after observing the aggregation of tintinids at the bottom in periods of reduced Chl *a* concentration. Thus the absence of

sediment could explain the lower abundance of Ciliates during autumn 2015, since during this period of time the abundance of some preys was also lower.

The Ciliates were dominated by Tintinnopsis, probably because these microciliates, beside the phytoplankton, also consumed other microciliates. The ingestion of ciliates by ciliates of distinct functional groups (Dolan, 1991; Leakey *et al.*, 1992) or identical (Robertson, 1983; Stoecker *et al.*, 1983; Gifford, 1985; Verity, 1986b) was documented in several coastal systems. Occasionally, tintinids may even ingest particles close to the oral diameter of the loric (Capriulo, 1982).

As mention before, the abundance of Other Plastidic Nanoflagellates was positively and significantly related to the abundance of Ciliates, and more strongly with the abundance of *Tintinnopsis*, as in Barbosa's study (2006). In fact, the occurrence of positive correlations between *Tintinnopsis* or Ciliates and phytoplankton, in particular nanoplankton, has also been reported for several coastal environments (Burkill, 1982; Capriulo & Carpenter, 1983; Verity, 1987; Admiraal & Venekamp, 1986; Sanders, 1987; Dolan & Coats, 1990; Rodriguez *et al.*, 2000). In fact, Plastidic Nanoflagellates are generally considered the main food of microplanktonic ciliates (Jonsson, 1986; Rassoulzadegan *et al.*, 1988; Bernard & Rassoulzadegan, 1990). This type of association is generally used to explain the global distribution of ciliates in the marine environment (Lynn & Montagnes, 1991; Suzuki *et al.*, 1998; Suzuki & Taniguchi, 1998) and the seasonal cycle of ciliates in several protected coastal systems, and implies bottom-up regulation of growth (Andersen & Sorensen, 1986; Revelante & Gilmartin, 1987; Dolan & Coats, 1990; Figueiras & Pazos, 1991; Leakey *et al.*, 1992; Sime-Ngando *et al.*, 1995).

The increase of abundance was higher in the inner station, when comparing stations, and during spring 2015, when comparing seasons, which coincided with high temperatures. The positive and significant relationships between community or group-specifics growth of ciliates and temperature were previously reported for several natural aquatic systems (Verity, 1986b; Dolan, 1991; Nielsen & Kiorboe, 1994; Lonsdale *et al.*, 1996; Weisse & Muller, 1998) and for laboratory cultures (Stoecker *et al.*, 1983; Aelion & Chisholm, 1985). The relationship between the growth rate of the ciliates and the total production of potentially available food was not significant. This result was also

reported by Nielsen & Kiorboe (1994) and Lonsdale *et al.* (1996). The independence between growth rate of Ciliates and food availability is considered an indicator of the non-limitation of ciliate growth by the food availability (Barbosa, 2006).

4.3.3.3.Aplastidic Dinoflagellates

As in the previous group-species, it was not possible to estimate the accurate value of abundance of Aplastidic Dinoflagellates (represented 3.44% of the abundance of microzooplankton in the inner station and 0.87% in the outer station), due to the lack of data. The abundance was higher in the outer station, when comparing stations, which was expected, since the dinoflagellates abundance is generally higher in periods of relaxation, stratification and, in particular, in periods of coastal convergence (Moita, 2001; Nogueira & Figueiras, 2005), characteristics events of the outer station. The abundance was higher during spring 2015, when comparing seasons, with a strong short-term variability, which was also observed in other coastal systems (Davis *et al.*, 1985; Andersen & Sorensen, 1986; Coffin & Sharp, 1987; Wright *et al.*, 1987; Kuosa & Kivi, 1989; Galvão, 1990; Wikner & Hagstrom, 1991; Brussard *et al.*, 1995; Tanaka & Taniguchi, 1999). Barbosa (2006) also had these results.

The increase of abundance between stations was higher in the outer station, which coincided with a lower increase of Ciliates, and between seasons, was during spring. Actually, during autumn occurred a decrease of Aplastidic Dinoflagellates, which coincided with a high decrease of Diatoms. In fact, due to their capture and food intake characteristics (Strom & Buskey, 1993; Hansen *et al.*, 1994; Jeong, 1999; Hansen & Calado, 1999), heterotrophic dinoflagellates are generally associated with intense predation of microphytoplankton and/or diatoms (Paranjape, 1990; Hansen, 1992; Sime-Ngando *et al.*, 1995; Strom & Strom, 1996; Latasa *et al.*, 1997; Uitto *et al.*, 1997; Schluter, 1998; Hall *et al.*, 1999; Levinsen *et al.*, 1999; Strom *et al.*, 2001; Saito *et al.*, 2005). The higher increase of Aplastidic Dinoflagellates coincided with high abundances of Other Plastidic Nanoflagellates and Plastidic Dinoflagellates, so these organisms may have been preferential preys for the Aplastidic Dinoflagellates.

The abundance and increase of Aplastidic Dinoflagellates were always inferior to the Aplastidic Nanoflagellates and Ciliates, because heterotrophic dinoflagellates have

relatively low maximum growth rates compared to Aplastidic Nanoflagellates and Ciliates, so they may be a relatively starvation-resistant group (Dolan & McKeon, 2005).

Chapter 5

5. Conclusion

5.1. Initial conditions: temperature, salinity, water transparency and Chl *a*

The inner and outer stations, located at the west bay of Ria Formosa and in the border area in contact with the adjacent coastal, respectively, in the period between May 2015 and May 2016, had different thermal amplitudes, some differences in the salinity values, but there was no stratification, and there was no positive correlation between temperature and salinity. The water temperature values in this study had different amplitudes regarding previous studies. Nevertheless, the variations were more or less within the normal range of the Ria Formosa.

The water transparency had a big difference between the inner and outer stations, since the inner station had a higher water turbidity (K_e) and higher PAR (I_m), while the outer station had a lower water turbidity (K_e) and lower PAR (I_m). Comparing the seasons, although the autumn had higher water turbidity than the spring, its PAR radiation was lower.

Chl *a* concentration had differences between stations, however they were not consistent. During spring 2015, the values of Chl *a* concentration followed the patterns to the Ria Formosa, but during spring 2016, the values did not followed the patterns.

5.2. Final conditions: phytoplankton growth and grazing rates

The experiments carried out in this study had 3 types of responses, a positive slope, a negative slope and an insignificant slope. Thus the dilution had, sometimes, a negative effect on phytoplankton physiology. The Cyanobacteria dominated the phytoplankton community.

The potential instantaneous growth (μ_0) and grazing (g) rates of phytoplankton group-specific had differences from the type of responses to the effect of nutrients, through the year (May 2015 to May 2016) and between stations. Thus the plankton community was shaped by nutrient supply and species interactions across trophic levels (e.g. selective grazing).

The Cyanobacteria was abundant and played a main role in the phytoplankton community and its abundance correlated positively with the water temperature. The growth rate was low, which could mean a growth limitation due to micronutrients. Regarding the grazing rate, it was lower than the grazing rate for larger organisms. Therefore this group-specific was clearly a non-preferential prey for phagotrophic protists.

The Eukaryotic Picophytoplankton was also abundant and played a main role in the phytoplankton community. Its growth rate between seasons had a high difference, since the outer station during autumn 2015 had a negative linear regression while the same station in spring 2016 had a positive linear regression. Comparing the abundance of Cyanobacteria *Synechococcus* and Eukaryotic Picophytoplankton, there was a dominance of Cyanobacteria, in both stations and seasons. However, the growth rate of these two groups-specific was equal, then the lower impact of microzooplankton on *Synechococcus* may have allowed its relative dominance.

The Cryptophyceae abundance was lower than the Other Plastidic Nanoflagellates. In the inner station of the Ria Formosa, the Cryptophyceae had a high seasonal variability of growth rate, however they presented a relatively low growth rate, lower than the other groups-specific. While in the outer station, it was not possible to estimate the growth rate, because the dilutions did not show any growth rate. Thus the dilution had a negative effect on phytoplankton physiology. The growth rate correlated positively with the temperature and light intensity. The grazing rate of Cryptophyceae was higher in spring than in autumn. The impact of microzooplankton predation on Cryptophyceae production was constantly higher than 100% d^{-1}, through the experimental period, obviously indicating that only this removal process can justify the decrease of the observed abundance in this period.

The macronutrient enrichment did affect the growth rate of Other Plastidic Nanoflagellates. Its abundance and growth rate correlated positively with the temperature. Comparing seasons, there was a high difference between autumn 2015 and spring 2016, since this group-specific was more abundant during spring 2016 than in autumn 2015 (three times more abundant), thus there was seasonal variability. Of the flagellate group, the Other Plastidic Nanoflagellates were those with the highest growth

rate, however when compared with the Diatoms, they had the lowest growth rate. The abundance of Other Plastidic Nanoflagellates was positively and significantly related to the abundance of Ciliates, and more strongly with the abundance of *Tintinnopsis.* Proving that phytoplankton not always increases with the decreasing of predators and grazing actually may stimulate prey growth.

The abundance of Euglenophyceae was higher during spring and correlated significantly and positively with the water temperature and light intensity. Its growth rate was higher during spring. While during autumn 2015, the Euglenophyceae presented a relatively low growth rate and lower than the other group-specifics, except the Cryptophyceae. The impact of microzooplankton predation on Euglenophyceae production was constantly higher than 100% $.d^{-1}$, in autumn 2015, obviously indicating that only this removal process can justify the decrease of the observed abundance in autumn 2015. Besides the microzooplankton, other removal factors, such as the impact of tidal advection and benthic predation, may have controlled the dynamics of Euglenophyceae, since in the spring 2015 the removal was 70%.

The abundance of Plastidic Dinoflagellates was dominated by an unidentified microplanktonic gymnodinoid, which was probably toxic. The temperature and light intensity had a positive effect in the abundance of Plastidic Dinoflagellates. This group-specific correlated negatively with the Diatoms. The growth rate was higher in the inner station however the higher abundance was in the outer station. Also the impact of microzooplankton predation on Plastidic Dinoflagellates production was constantly higher in the outer station, thus other removal processes, e.g. viruses, should have occurred at the inner station to justify a lower abundance. Of the flagellates, the Plastidic Dinoflagellates were the most grazed, during autumn. The growth rates between Plastidic Dinoflagellates and Euglenophyceae were inversely proportional, and the grazing rates were also inversely proportional. Then, it appears that between these groups-specific, the microzooplankton prefers the most abundant, so the grazer was opportunistic. The higher grazing rate, in stations and seasons, coincided with the lower water temperatures.

The macronutrient enrichment did affect the growth rate of the Centric Diatoms, during autumn. The abundance of Centric Diatoms was higher in the inner station, when

comparing stations, and between seasons was higher in spring 2015. This group-specific had a high seasonal variability of the growth rate, and was the highest of the whole phytoplankton group, in the outer station. The growth rate was higher in the outer station, when comparing stations, however the higher abundance and the effect of nutrients occurred in the inner station, then the limitation of growth by nutrients did not seem to be important for the growth of diatoms. Regarding the seasons, it was higher in autumn 2015, which coincided with a decrease of Aplastidic Dinoflagellates, showing that these predators are essential for controlling the centric diatom. Also these Diatoms were more abundant than the Euglenophyceae and the Plastidic Dinoflagellates, however they were not more abundant than the Cryptophyceae and the Other Plastidic Nanoflagellates, thus the diatom dominance may represent the result of a growth and removal differential. Its grazing rate had a positive and significant correlation with the abundance of Ciliates, showing that these were the main grazers of the Centric Diatoms. Usually, grazers seem to prefer smaller phytoplankton, however in this study the Centric Diatoms were more grazed than the plastidic flagellates.

The abundance and growth rate of Pennate Diatoms correlated positively with the temperature. In this study occurred a predominance of plastid flagellates over the diatoms, which reflected the smaller relative impact of the removal processes on the plastid nanoflagellates. The Pennate Diatoms had a high seasonal variability of the growth rate, and was the highest of the whole phytoplankton group, in both stations. Between seasons, it was higher during autumn 2015, which coincided with a decrease of Aplastidic Dinoflagellates. Comparing the growth rates of both Diatoms, between stations, it was verified a negative correlation (inversely proportional) in the inner station, and a similarity (statistically they were not different) in the outer station. Then in the inner station, an intraspecific competition may have occurred. Between seasons, the Pennate Diatoms had a higher growth rate than the Centric Diatoms however the abundance of Centric Diatoms was higher than the Pennate Diatoms. The predation could explained this difference, however the impact of microzooplankton predation on Pennate Diatoms production was constantly lower than the impact on Centric Diatoms, so the Pennate Diatoms had perhaps other types of removal processes. Also the heterotrophic dinoflagellates seem to have preferred the Centric Diatoms. The grazing rate was higher in the outer station, when comparing stations, which coincided with a higher abundance of Aplastidic Dinoflagellates. And between seasons was higher in

autumn 2015, which coincided with a decrease of Aplastidic Dinoflagellates, therefore other removal processes should have occurred. Usually, grazers seem to prefer smaller phytoplankton however in this study the Pennate Diatoms were more grazed than the plastidic flagellates.

5.3. Final conditions: microzooplankton growth

Microzooplankton removed, daily, between 44.83% and more than 100% of phytoplankton production.

The abundance of Aplastidic Nanoflagellates had a poorly defined seasonal cycle through the experiment period. During spring 2016, occurred a decrease and a high increase of this group-specific. The decrease of this group-specific coincided with a high increase of Ciliates and with a high temperature. The Aplastidic Nanoflagellates were always more abundant than the Ciliates and the Aplastidic Dinoflagellates.

The Ciliates were dominated by Tintinnopsis. The abundance of Ciliates was higher in the outer station, which coincided with a high grazing rate of diatoms. The abundance of ciliates exhibited a clear seasonal variation with maximum values in spring, and relatively low values in autumn. Some species of ciliates feed on the sediment. Thus the absence of sediment could explain the lower abundance of Ciliates during autumn 2015, since during this period of time the abundance of some preys was also lower. The abundance of Other Plastidic Nanoflagellates was positively and significantly related to the abundance of Ciliates, and more strongly with the abundance of *Tintinnopsis.* The growth rate of Ciliates had a positive and significant relationship with the temperature. The relationship between the growth rate of the ciliates and the total production of potentially available food was not significant. This independence between growth rate of Ciliates and food availability was considered an indicator of the non-limitation of ciliate growth by the food availability.

The abundance of Aplastidic Dinoflagellates was higher in the outer station and during spring 2015. The increase of abundance between stations was higher in the outer station, which coincided with a lower increase of Ciliates. During autumn occurred a decrease of Aplastidic Dinoflagellates, which coincided with a high decrease of Diatoms. The higher increase of Aplastidic Dinoflagellates coincided with high abundances of Other

Plastidic Nanoflagellates and Plastidic Dinoflagellates, so these organisms may have been preferential preys. The abundance and increase of Aplastidic Dinoflagellates were always inferior to the Aplastidic Nanoflagellates and Ciliates, because heterotrophic dinoflagellates have relatively low maximum growth rates compared to Aplastidic Nanoflagellates and Ciliates, so they may be a relatively starvation-resistant group.

Chapter 6

6. References

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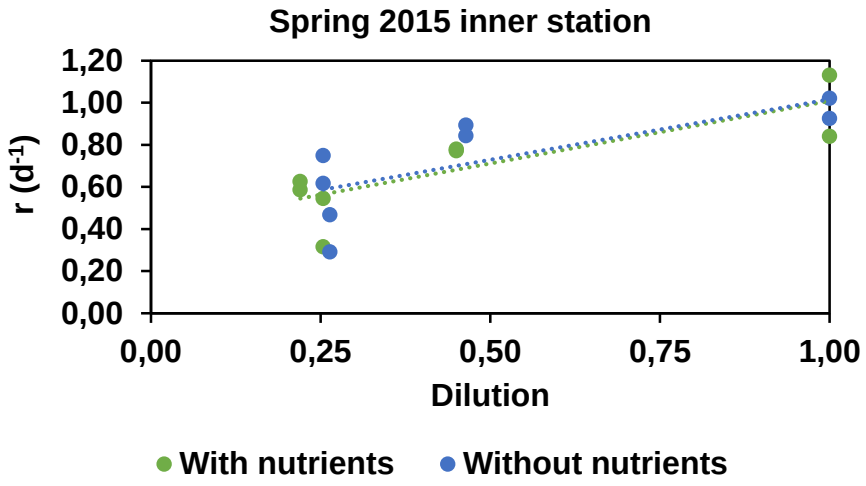
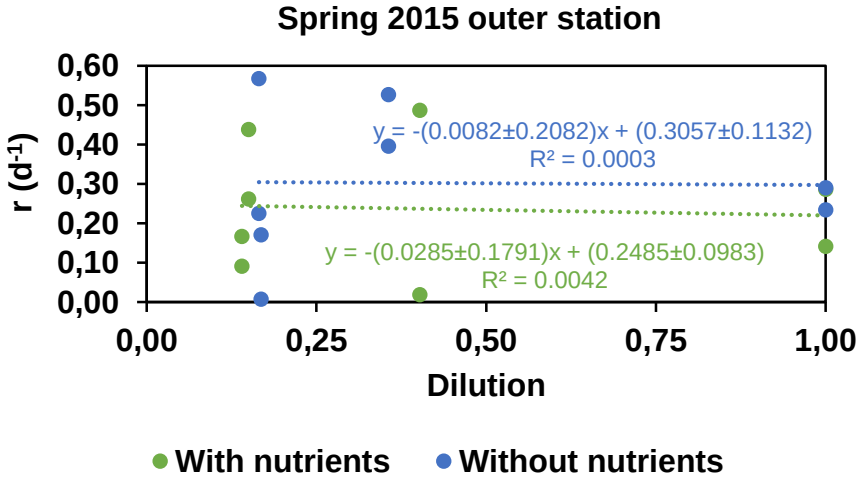
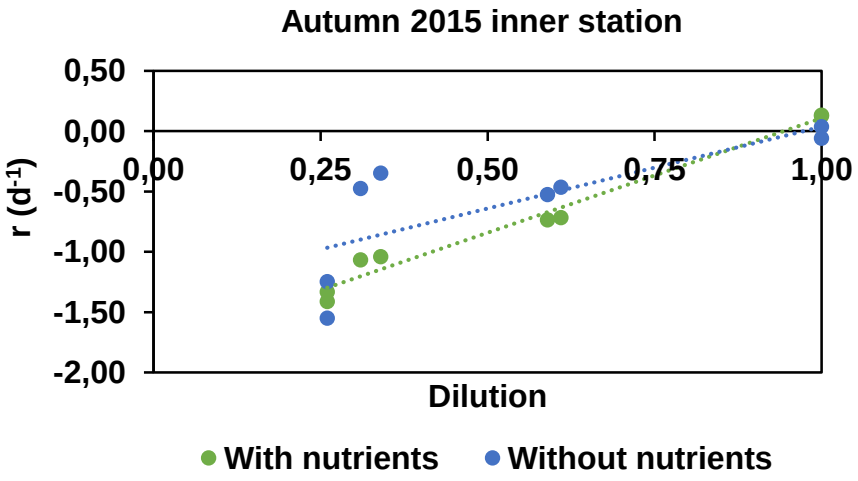
Annex



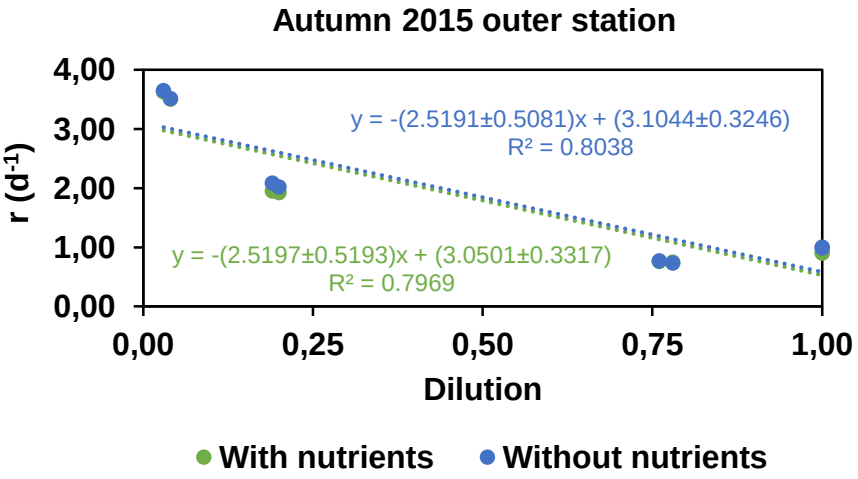
Plot of the apparent growth rate of phytoplankton community and dilution factors, with and without the addition of macronutrients, in the inner zone of the Ria Formosa in spring 2015.



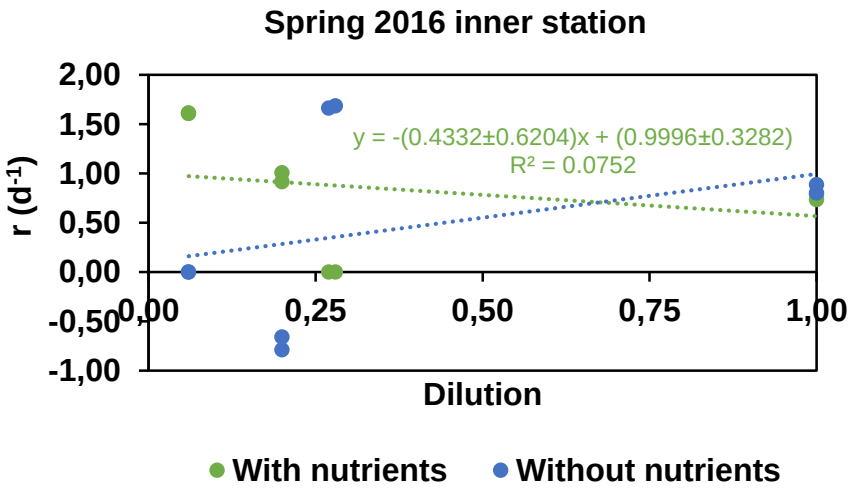
Plot of the apparent growth rate of phytoplankton community and dilution factors, with and without the addition of macronutrients, in the outer zone of the Ria Formosa in spring 2015.



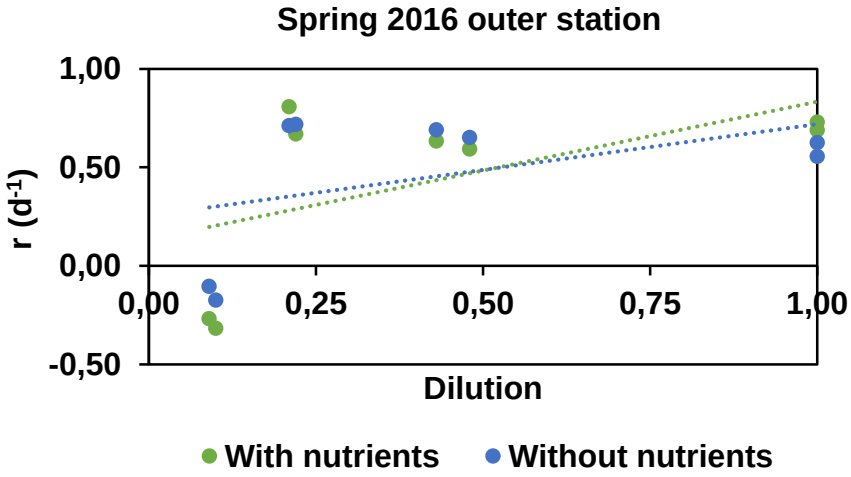
Plot of the apparent growth rate of phytoplankton community and dilution factors, with and without the addition of macronutrients, in the inner zone of the Ria Formosa in autumn 2015.



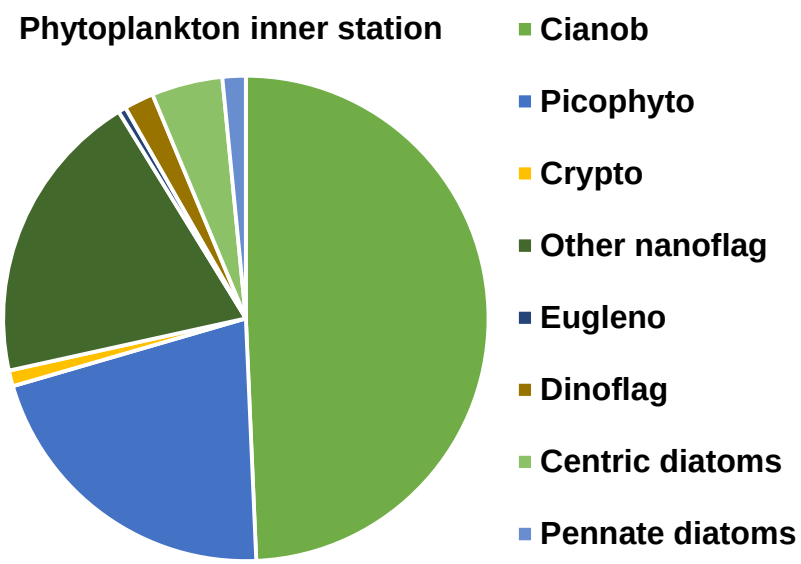
Plot of the apparent growth rate of phytoplankton community and dilution factors, with and without the addition of macronutrients, in the outer zone of the Ria Formosa in autumn 2015.



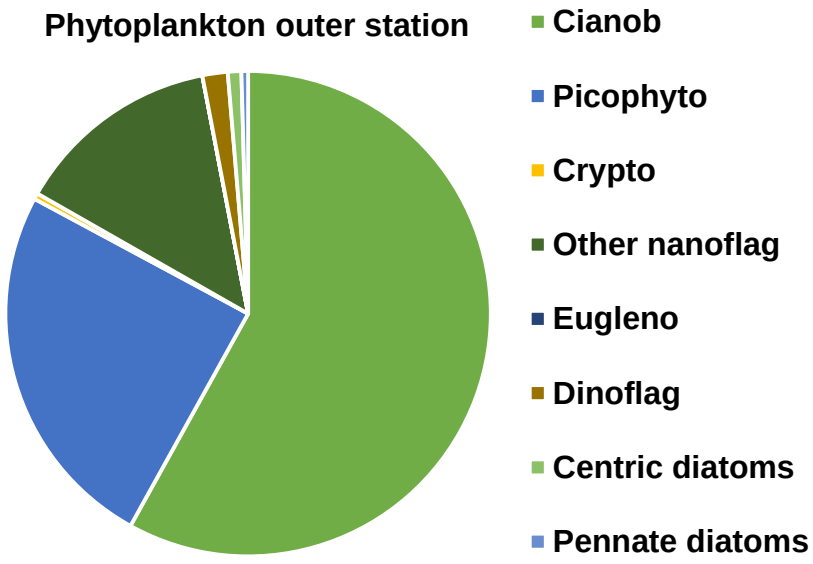
Plot of the apparent growth rate of phytoplankton community and dilution factors, with and without the addition of macronutrients, in the inner zone of the Ria Formosa in spring 2016.



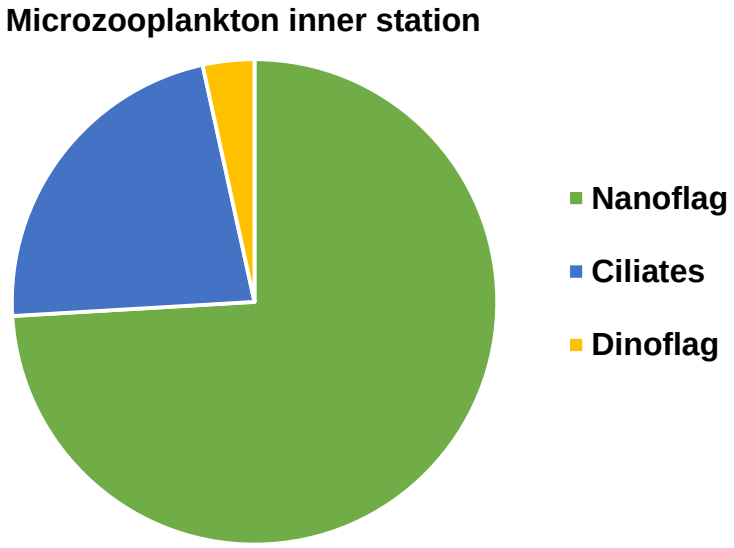
Plot of the apparent growth rate of phytoplankton community and dilution factors, with and without the addition of macronutrients, in the outer zone of the Ria Formosa in spring 2016.



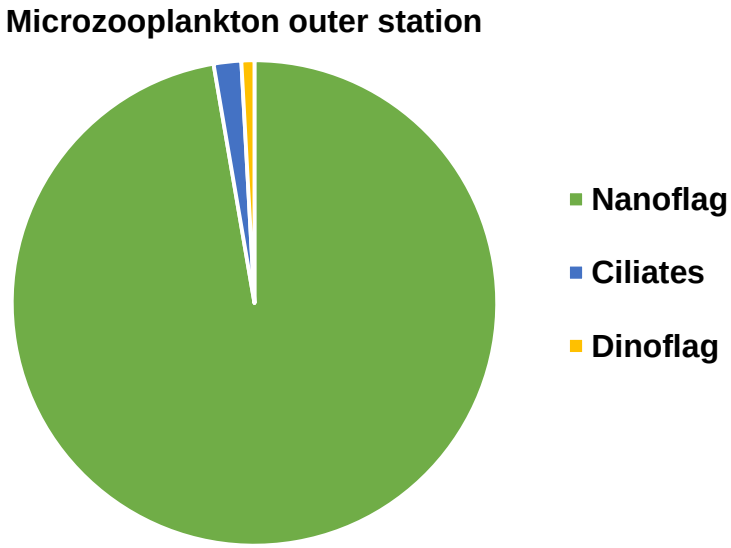
Final abundance of the group-specifics of phytoplankton in the inner station through the experimental time. Cyanobacteria *Synechococcus*: 49.31%; Eukaryotic Picophytoplankton: 21.18%; Cryptophyceae: 1.05%; Other Plastidic Nanoflagellates: 19.67%; Euglenophyceae: 0.52%; Plastidic Dinoflagellates: 1.97%; Centric Diatoms: 4.74% and Pennate Diatoms: 1.55%.



Final abundance of the group-specifics of phytoplankton in the outer station through the experimental time. Cyanobacteria *Synechococcus*: 58.06%; Eukaryotic Picophytoplankton: 24.79%; Cryptophyceae: 0.40%; Other Plastidic Nanoflagellates: 13.73%; Euglenophyceae: 0.00%; Plastidic Dinoflagellates: 1.69%; Centric Diatoms: 0.90% and Pennate Diatoms: 0.44%.



Final abundance of the group-specifics of microzooplankton in the inner station through the experimental time. Aplastidic Nanoflagellates: 74.07%; Ciliates: 22.49% and Aplastidic Dinoflagellates: 3.44%.



Final abundance of the group-specifics of microzooplankton in the outer station through the experimental time. Aplastidic Nanoflagellates: 97.30%; Ciliates: 1.84% and Aplastidic Dinoflagellates: 0.87%.