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Growth and Grazing Mortality of Microbial Plankton in a Shallow Temperate Coastal Lagoon (Ria Formosa, SW Iberia)

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Abstract: Microzooplankton grazing is widely recognized as an important process of heterotrophic prokaryote and phytoplankton biomass removal. However, few studies have specifically addressed microbial mortality in the Ria Formosa coastal lagoon. This study aimed to assess the growth and mortality of heterotrophic prokaryotes and phytoplankton in this ecosystem using the dilution technique. The results revealed significant seasonal variations in the growth and grazing rates of both heterotrophic prokaryotes and phytoplankton, with mean grazing rates slightly exceeding the mean potential instantaneous growth rates. This indicates that microzooplankton consume a substantial proportion of both microbial groups in the lagoon. For specific phytoplankton taxa, the wide range of observed grazing rates suggests grazer selectivity, highlighting the need for future research to examine the dynamics of each phytoplankton group more closely.

Keywords: microbial food web; heterotrophic prokaryotes; phytoplankton; microzooplankton grazing; dilution technique

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1. Introduction

Food webs include complex ecological interactions that regulate the flow of matter and energy and are thus fundamental in understanding ecosystem functioning [1]. The microbial food web is formed by interactions that include all autotrophic and heterotrophic prokaryotic and eukaryotic unicellular microorganisms from aquatic systems [2]. The combined interactions among these various components of the microbial food web are associated with fluxes of biomass, dissolved and particulate organic matter, and inorganic nutrients [3,4]. The uptake of organic matter by heterotrophic prokaryotes is a major carbon flow pathway, and its variability can change the overall patterns of carbon fluxes to higher trophic levels [5]. Phytoplankton play a key role in the microbial food web by providing carbon to higher trophic levels and are the most important source of dissolved organic carbon in aquatic environments through exudation, losses by cell damage, or lysis [3,6]. Since a large fraction of carbon in aquatic systems flows through microbial plankton, knowledge of the factors controlling these microorganisms is highly relevant to understanding biogeochemical cycles functioning and the prediction of their evolution after perturbation.

Mortality due to microzooplankton grazing is an important process of heterotrophic prokaryote and phytoplankton biomass removal, able to affect fluxes of energy and nutrients in aquatic food webs as well as the structure of microbial communities [7,8]. Microzooplankton play an important ecological role in aquatic food webs as they are a significant cause of mortality for heterotrophic prokaryotes and phytoplankton, mediating carbon transfer to higher trophic levels [9,10]. Microzooplankton ingest, on average, 62.4% of phytoplankton daily production [11] and, specifically, heterotrophic nanoflagellates and ciliates, the most important grazers of heterotrophic prokaryotes [10,12–14], are able to remove between 40 and 95% of heterotrophic prokaryotes' daily production in different coastal systems [4].

The dilution method, introduced by Landry and Hassett [15], remains a popular technique to estimate microbial growth and mortality due to grazing. It consists of the manipulation of encounter rates between prey and their grazers through a series of different dilutions, which are prepared using particle-free water from the same source. This creates a gradient of grazer abundances and, thus, of grazing rates across the different dilutions. Nutrients are added to the dilutions to avoid nutrient limitation during incubation, which could result in incorrect estimations of grazing, ensuring one set of undiluted bottles be left without nutrients, which serve as controls for the natural growth rates of the phytoplankton. The apparent growth rate is then plotted against the dilution factor, and the estimation of potential instantaneous growth rate of prey and grazing rate exerted by grazers are taken as the coefficients of the fitted regression line, namely the y-axis intercept and the slope, respectively. The dilution method is based on three fundamental assumptions: (1) the growth rate of the prey is not affected by dilution, (2) grazing mortality is linear with respect to prey concentration, and (3) prey growth is exponential and is not affected by light or nutrients [15]. When the three fundamental assumptions are met, it is expected that the impact of grazing decreases progressively with increasing dilution, i.e., there is a negative relationship between the apparent growth rate of prey and the dilution factor. However, the linearity of this relationship is sometimes compromised, and non-linear relationships are frequently reported [16–20]. Deviations from linearity imply the violation of a basic assumption of the dilution method, namely, that grazing mortality is linear concerning prey concentration and can occur due to the complexity of plankton communities where saturated and selective feeding occurs [16–19]. This method was first introduced to estimate phytoplankton mortality due to microzooplankton grazing and it was later applied to estimate grazing on heterotrophic prokaryotes [21,22]. Despite some criticism, mostly about microzooplankton dynamics during incubation [11,16,18,19,23,24], the dilution technique is one of the most informative yet least invasive and damaging techniques available to estimate rates of phytoplankton and heterotrophic prokaryotes growth and mortality due to grazing [25]. Although this method has been used for approximately 40 years for a wide variety of ecosystems, there is still a lack of knowledge about the growth and mortality rates of the different components of microbial communities in coastal lagoons [26].

The Ria Formosa coastal lagoon is located on the south coast of Portugal and is one of the most important confined marine ecosystems in Portugal [27,28]. Despite the relevance of microbial plankton for ecosystem functioning, published data on microbial plankton in the Ria Formosa coastal lagoon are scarce, particularly for heterotrophic microbes [4,27], with only a few studies addressing microbial mortality due to grazing [4,29–31]. The main goal of this study is, therefore, to evaluate the growth of heterotrophic prokaryotes and phytoplankton and their mortality due to microzooplankton grazing throughout the productive cycle (spring through autumn) in the Ria Formosa coastal lagoon using the dilution technique.

2. Materials and Methods

2.1. Study Site

The Ria Formosa is a coastal lagoon system located on the south coast of Portugal, separated from the Atlantic Ocean by five barrier islands and two peninsulas. It extends ~6 km (N–S) at its widest point and ~55 km (E–W), with a total wet area of ca. 110 km^2 [27,32]. The lagoon is shallow, with an average depth of 2 m [33], and it is connected to the ocean through seven inlets that allow for the exchange of water. Between 50 and 75% of the water in the lagoon is exchanged due to semidiurnal and mesotidal tides, with tidal amplitudes between 1.35 m during neap tides and 3 m during spring tides. Since the hydrodynamic circulation is dominated by the inflow and outflow of the coastal water mass throughout the tidal cycle, and because the flow of freshwater is relatively small, the salinity of Ria Formosa water is close to the salinity of seawater [4,32]. Most of the rainfall occurs in the winter, between November and February, and the climate is Mediterranean, with mild wet winters and hot dry summers, with a mean air temperature in the summer of 25 °C

and 12 °C in the winter. Due to the reduced depth of the system, the absence of important sources of freshwater, and the impact of tidal currents, the water column is well mixed [32]. The adjacent coastal region is located in the Gulf of Cadiz and is regularly impacted by upwelling events [27,34], most frequent from April to October and associated with local westerly winds [35]. The Ria Formosa constitutes an ecosystem of high biodiversity and is a breeding and development site for many marine species. In addition to its ecological importance, it is also a valuable national resource for tourism, fisheries, aquaculture, and salt extraction industries [27,32].

2.2. Dilution Technique

Three experiments using the dilution technique were carried out in late spring (June 2021), summer (August 2021), and autumn (October 2021). Sampling was conducted at an inner location of the Ria Formosa coastal lagoon (Figure 1). Sub-surface water samples were collected using a five-litre plastic bottle and transported under dark conditions to the laboratory. To avoid alterations in the initial phytoplankton assemblage, water samples were not pre-screened to remove larger grazers [36].

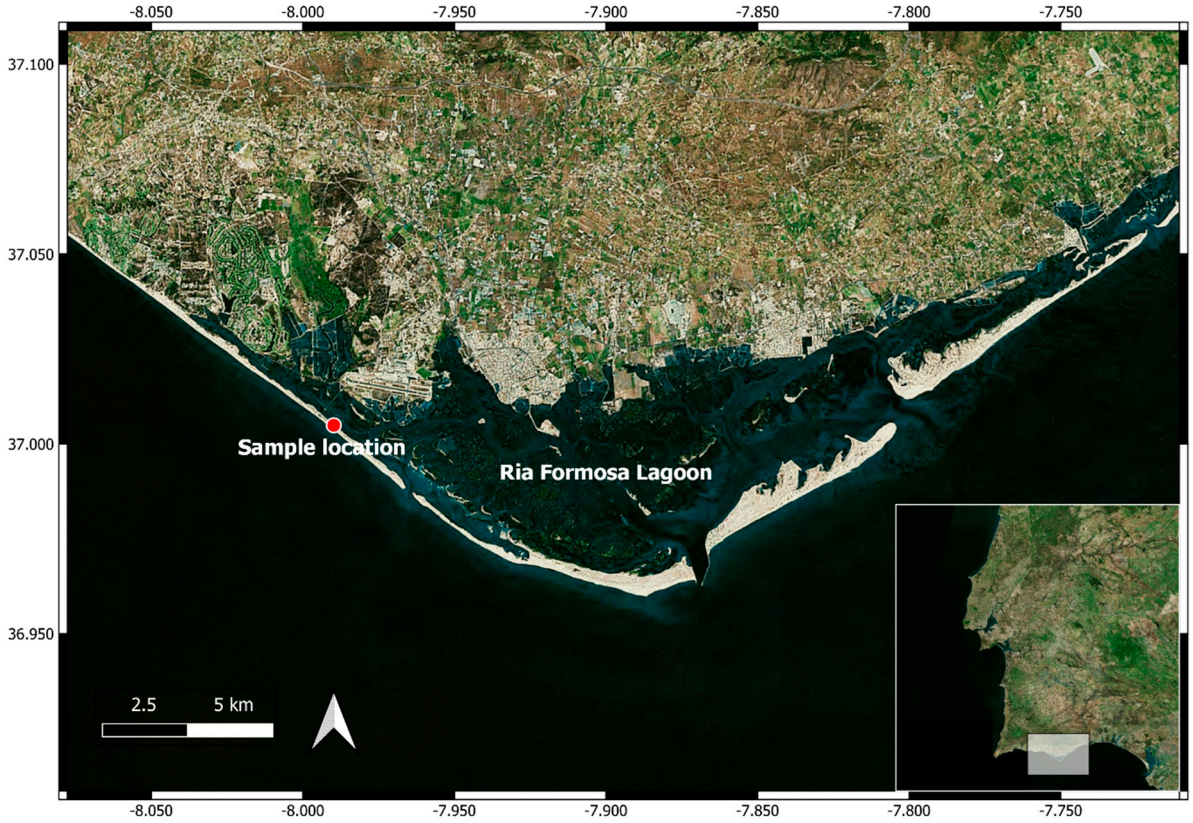

Figure 1. Location of the Ria Formosa coastal lagoon system and sampling site (red circle) (made using QGIS 3.18. Base map: Kosmosnimski.ru satellite).

Dilutions were prepared in 10 L Thermo Scientific Nalgene (Rochester, NY, USA) bottles using grazer-free diluent obtained by the filtration of seawater through a Pall cartridge (<0.1 µm). Five dilutions were prepared, combining the grazer-free diluent with the following relative amounts of seawater: DIL0.125+, DIL0.25+, DIL0.50+, DIL0.70+, and DIL1+. These dilutions were enriched with inorganic macronutrients at saturating concentrations (+40 µM of nitrate as potassium nitrate, +10 µM of ammonium as ammonium chloride, +4 µM of phosphorus as potassium dihydrogen phosphate, and +50 µM of silicon as sodium hexafluorosilicate) to avoid potential nutrient limitation. An additional dilution (DIL1-) was prepared without the addition of nutrients to evaluate the potential effects of nutrients. After homogenization, water from each dilution was transferred in triplicate to

the experimental units (2 L polycarbonate Nalgene bottles). The bottles were incubated for 24 h in the laboratory under in situ temperature and in ambient light–dark cycle and were exposed to photosynthetically active radiation (PAR) intensity of 90–120 μmol photons m^{-2} s^{-1} (depending on the season). Bottles were gently shaken to avoid the settling of cells. Aliquots were collected from all experimental treatments at the beginning and end of incubation for quantification of heterotrophic prokaryotes and phytoplankton.

Apparent (net) prey growth rates (r, d^{-1}) were calculated for each experimental treatment as the change in abundance during the incubation period, assuming exponential growth, according to Equation (1):

$$r = (\ln ABU_f - \ln ABU_i)/t \quad (1)$$

where ABU_i and ABU_f are the abundance at the beginning and end of the experiment, respectively, and *t* is the incubation period (24 h).

Linear regression analysis was then used to analyze the relationship between the dilution factor and the apparent growth rate in the nutrient-enriched dilutions. The potential instantaneous growth rates of prey (μ_0) and grazing rates (g) were obtained as the y-intercept (equivalent to growth in 100% dilution, i.e., in the absence of grazers) and the slope of the linear regression line, respectively [15]. Analysis of variance (ANOVA) was used to test the significance of the slopes. In the case of statistically non-significant slopes (slopes not significantly different from zero), the grazing rate was assumed to be zero and the instantaneous growth rate of prey was considered the mean of the apparent growth rate in all nutrient-enriched bottles [18,31,37–39]. When V-shaped, L-shaped, or inverted V-shaped responses were observed, the potential instantaneous growth rate was estimated as the regression intercept of the linear and negative portion of the slope, and the grazing rate was calculated as the difference between the intercept and net growth rate in DIL1+ [40]. In the case of significant non-negative slopes (e.g., positive slopes), the mortality rates were undeterminable, since negative grazing rates are theoretically impossible [18,38,41].

As the apparent growth rates were derived from nutrient-enriched experimental treatments, the potential instantaneous growth rate of prey (μ_0) is an estimation of prey growth when nutrients are not limiting and, thus, can be overestimated. To obtain a growth rate representative of in situ conditions and to control for possible nutrient stimulation of growth in the experiments, the in situ instantaneous growth rate of prey (μ_{is}) (growth in the absence of grazers and with no nutrient enrichment) was estimated as the difference between μ_0 and the difference in apparent prey growth rates between undiluted samples with added nutrients (r(DIL1+)) and without added nutrients (r(DIL1−)), according to Equation (2):

$$\mu_{is} = \mu_0 - [r(DIL1+) - r(DIL1-)] \quad (2)$$

Standard errors (SEs) for μ_{is} were estimated using error propagation equations (http://julianibus.de/index.html, accessed on 14 May 2024).

The net production of heterotrophic prokaryotes (NBP) (μg C L^{-1} d^{-1}) and particulate net phytoplankton production (pNPP) (μg C L^{-1} d^{-1}) were obtained according to Equation (3)

$$NBP \text{ or } pNPP = B_i\ (e^{\mu is^{*}t} - 1) \quad (3)$$

where Bi (μg C L^{-1}) is the initial prokaryote or phytoplankton biomass in experimental treatments with unfiltered seawater and *t* is the duration of the incubation. The impact of grazing (I) on both heterotrophic prokaryotes and phytoplankton was estimated as the percentage of the daily production of prey removed by grazers, which is considered a reasonable proxy for the percentage of prey production consumed by microzooplankton [9], according to Equation (4):

$$I = g/\mu_{is} * 100 \quad (4)$$

2.3. Quantification of Heterotrophic Prokaryotes and Phytoplankton

Epifluorescence microscopy was used for the quantification of heterotrophic prokaryotes and pico- (<2 µm) and nanophytoplankton (2–20 µm) following the methods of [42] and [43], respectively. Samples were collected at the beginning and end of incubation, fixed with glutaraldehyde (final concentration 0.2%), and kept at 4 °C until slide preparation (within 24 h). Slides for the quantification of heterotrophic prokaryotes were obtained by filtering 1 mL of fixed sample onto a 0.2 µm black polycarbonate membrane (Nucleopore) and staining with acridine orange. Slides for phytoplankton quantification and identification were obtained by filtering 1–5 mL of fixed sample onto a 0.4 µm black polycarbonate membrane and staining with proflavine. Slide preparation was made using glass slides and non-fluorescent Cargille Type A immersion oil. Slide observation was made using a Leica DM LB epifluorescence microscope (Wetzlar, Germany) at 1250× magnification and a Zeiss AxioImager A1 epifluorescence microscope (Oberkochen, Germany) at 1000× magnification, respectively.

Samples were also taken for the identification and quantification of microphytoplankton (20–200 µm) following the method of [44]. These were fixed with acid Lugol's solution and settled in 5–50 mL sedimentation chambers over a 24 h period. Observation was made using a Zeiss AxioObserver S1 inverted microscope (Oberkochen, Germany), at 400× magnification.

For both methods, at least 50 random visual fields, 400 cells in total, and 100 cells of the most abundant species/taxon were counted. The counting precision was ±10%, assuming the cells were randomly distributed [45].

The biomass of heterotrophic prokaryotes was estimated based on the abundance of heterotrophic prokaryotes and the mean carbon content per cell. Cells were categorized into spheres and rods, measured with a New Porton G12 graticule [46], and biovolume was obtained using geometric formulas [47]. Carbon content was then estimated using a non-linear function of cell volume [48] and multiplied by abundance to obtain biomass.

Chlorophyll *a* (Chl*a*) concentration was determined spectrophotometrically for experimental treatments with unfiltered seawater (DIL1− and DIL1+) after filtration through glass fibre filters (GF/F, Whatman, particle retention > 0.7 µm) and overnight extraction with acetone 90% [49]. CHla was converted into carbon biomass using a carbon-to-chlorophyll ratio of 49 mg C (mg Chl*a*)$^{-1}$, obtained empirically for the Ria Formosa system [4].

2.4. Statistical Analysis

To test nutrient limitation in dilution experiments, the significance of the difference between the apparent growth rates in undiluted treatments with and without nutrients was determined using a one-tailed *t*-test; significant differences indicated potential nutrient limitation. A comparison between the regression lines obtained in each experiment was made using analysis of covariance (ANCOVA) to check for statistical differences in potential instantaneous growth rates and grazing rates among experiments, groups of phytoplankton, and between rates of heterotrophic prokaryotes and total phytoplankton community in each experiment. Thereafter, one-tailed t-tests and confidence interval comparison were used to identify which groups were significantly different from the others [50]. A significance level of 0.05 was set for all data analyses. All statistical analyses were performed using IBM SPSS Statistics v.28 software.

3. Results

3.1. Initial Environmental Conditions and Microbial Assemblages

The initial abundance and biomass of planktonic heterotrophic prokaryotes in the Ria Formosa coastal lagoon varied between experiments, with abundance and biomass values of 1.03 × 10^9 cell L^{-1} and 24.27 µg C/L in the spring, 4.27 × 10^9 cell L^{-1} and 64.25 µg C/L in the summer, and 2.57 × 10^9 cell L^{-1} and 35.26 µg C/L in the autumn. Initial Chla concentrations presented mean values of 0.46 µg L^{-1} in the spring, 1.59 µg L^{-1} in the summer, and 0.93 µg L^{-1} in the autumn. For the same period, the initial abundance

and biomass of the phytoplankton assemblage was 9.29×10^6 cell L^{-1} and 39.68 µg C L^{-1} in June, 5.45×10^6 cell L^{-1} and 68.2 µg C L^{-1} in August, and 3.82×10^6 cell L^{-1} and 37.17 µg C L^{-1} in October. In terms of phytoplankton community structure, cyanobacteria were the dominant group in terms of abundance, with a relative contribution ranging between 63% (3.44×10^6 cell L^{-1}) in the autumn and 87% (8.08×10^6 cell L^{-1}) in the summer. Eukaryotic picophytoplankton were the second most abundant group, with abundances ranging between 6.91×10^5 cell L^{-1} in the spring and 1.38×10^6 cell L^{-1} in the summer. Other phytoplankton groups were present but showed lower contributions to the community abundance; these were plastidic nanoflagellates (4–5%), cryptophytes (1–4%), diatoms (0–4%), dinoflagellates (<1%), and euglenophytes (<1%).

3.2. Growth and Grazing Rates of Heterotrophic Prokaryotes

Significant grazing rates of heterotrophic prokaryotes (i.e., significant negative slopes in the dilution experiments) were observed in all experiments. Grazing rates varied between 1.16 ± 0.11 d^{-1} in the summer and 2.17 ± 0.26 d^{-1} in the autumn (Table 1). Potential instantaneous growth rates of heterotrophic prokaryotes varied between 1.02 ± 0.10 d^{-1} and 1.99 ± 0.11 d^{-1} for the summer and spring experiments, respectively. In situ instantaneous growth rates showed the same trend, with higher values in the spring (2.59 ± 0.20 d^{-1}) and lower in the summer (1.17 ± 0.33 d^{-1}). The daily production of heterotrophic prokaryotic varied between 132.18 and 291.95 µg C L^{-1} d^{-1}, with a higher value in the spring and a lower value in the autumn. Grazing by phagotrophic protists removed 63.59% of daily production of heterotrophic prokaryotes in the spring, 98.58% in the summer, and 143.28% in the autumn (Table 1).

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

	μ_0	g	μ_{is}	NBP	I	R^2	*p*-Value	n
Spring	1.99 ± 0.11	1.64 ± 0.17	2.59 ± 0.20	291.95	63	0.92	<0.001	11 (a)
Summer	1.02 ± 0.10	1.16 ± 0.16	1.17 ± 0.33	152.76	99	0.81	<0.001	15
Autumn	1.70 ± 0.15	2.17 ± 0.26	1.52 ± 0.15	132.18	143	0.86	<0.001	14 (a)

3.3. Growth and Grazing Rates of Phytoplankton

Phytoplankton grazing and potential instantaneous growth rates varied between 0.55 ± 0.16 d^{-1} and 0.90 ± 0.20 d^{-1} and between -0.40 ± 0.110 d^{-1} and 0.33 ± 0.05 d^{-1}, respectively (Table 2). No significant differences in grazing rates were observed between experiments ($p = 0.267$). There were significant differences in the potential instantaneous growth rates of phytoplankton between experiments ($p < 0.001$), which were more apparent between the summer and the other two seasons. In situ instantaneous growth rates of the phytoplankton community ranged between -0.27 ± 0.15 d^{-1} in summer (corresponding to a decrease in abundance during incubation) and 0.88 ± 0.21 d^{-1} in autumn. Particulate net phytoplankton production results showed great variability in total phytoplankton community daily production between experiments, with values of 12.89 µg C L^{-1} d^{-1} in the spring, −20.29 µg C L^{-1} d^{-1} in the summer, and 87.28 µg C L^{-1} d^{-1} in the autumn. Grazing impact was higher in the spring, when 232% of total phytoplankton community daily production was removed per day.

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

	μ_0	g	μ_{is}	pNPP	I	R^2	*p*-Value	n
Spring	0.33 ± 0.05	0.65 ± 0.08	0.28 ± 0.08	12.89	232	0.83	<0.001	15
Summer	−0.40 ± 0.11	0.55 ± 0.16	−0.27 ± 0.15	−20.29	−203	0.53	0.007	12 (a)
Autumn	0.31 ± 0.12	0.90 ± 0.20	0.88 ± 0.21	87.28	102	0.62	<0.001	15

Regarding specific phytoplankton groups, values varied across seasons and specific phytoplankton groups (Table 3, Supplementary Materials. In general, mean values for potential instantaneous growth rate, grazing rate, in situ instantaneous growth rate, and grazing impact ranged between −2.49–1.78 d^{-1}, 0.55–3.08 d^{-1}, −2.88–2.06 d^{-1}, and 59–536%, respectively. Eukaryotic picophytoplankton and cryptophytes showed higher average grazing rates, whereas eukaryotic picophytoplankton showed higher average potential instantaneous growth rates. Regarding the different seasons, cryptophytes had the highest potential instantaneous growth rate in the spring, while euglenophytes had the highest values in the summer and eukaryotic picophytoplankton in the autumn. Cryptophytes showed the highest grazing rate during spring, and euglenophytes and eukaryotic picophytoplankton had the highest grazing rate during summer and autumn, respectively. The grazing rates of the different groups of phytoplankton were generally higher than their potential instantaneous growth rates. The mean grazing impact on cyanobacteria and cryptophytes was higher, with mean values of 291.50% and 250.44%, respectively. The highest grazing impact value was observed for cyanobacteria in the summer.

Table 3. Summary of results (±SE) of potential instantaneous growth rates (μ_0, d^{-1}), grazing rates (g, d^{-1}), in situ instantaneous growth rate (μ_{is}, d^{-1}), and grazing impact (I, %) for the different specific groups of phytoplankton in nutrient-enriched experimental treatments. The regression coefficient of determination (R^2), number of values (n), and significance level of the regression slope (*p*-value) are also provided. (a) Outlier(s) removed for compliance with dilution assumptions; (b) V-shaped or L-shaped saturated response curves; (c) inverted V-shaped responses curves; (**) grazing rate values marked by asterisks are not significantly different from zero (i.e., statistically non-significant slopes) and are assumed to be zero; (nd) grazing rate not determined because of positive slope.

		μ_0	g	μ_{is}	I	R^2	*p*-Value	n
Cyanobacteria	**Spring**	0.40 ± 0.05	0.63 ± 0.08	0.28 ± 0.08	225	0.82	<0.001	15
	Summer	0.13 ± 0.05	1.02 ± 0.08	0.19 ± 0.09	536	0.93	<0.001	15
	Autumn	0.24 ± 0.03	0.76 ± 0.05	0.68 ± 0.05	112	0.97	<0.001	10 (a)
Eukaryotic picophytoplankton	**Spring**	0.96 ± 0.26	2.33 ± 0.34	1.37 ± 0.30	170	0.87	<0.001	9 (c)
	Summer	1.74 ± 0.17	0.36 ± 0.25 **	−1.44 ± 0.19	-	0.20	0.197	10 (a)
	Autumn	1.34 ± 0.23	1.82 ± 0.30	2.06 ± 0.29	88	0.84	<0.001	9 (c)
Plastidic nanoflagellates	**Spring**	0.72 ± 0.16	1.63 ± 0.21	1.33 ± 0.21	123	0.90	<0.001	9 (c)
	Summer	0.48 ± 0.20	0.73 ± 0.27	1.24 ± 0.29	59	0.59	0.043	7 (c)
	Autumn	1.21 ± 0.58	1.21 ± 0.45 **	1.39 ± 0.56	-	0.42	0.084	8 (c)

Table 3. *Cont.*

		μ_0	g	μ_{is}	I	R^2	*p*-Value	n
Cryptophytes	**Spring**	1.47 ± 0.35	1.95 ± 0.35	0.95 ± 0.49	205	0.69	<0.001	12 (b)
	Summer	0.34 ± 0.33	2.03 ± 0.46	0.63 ± 0.39	322	0.71	0.002	10 (a)
	Autumn	0.77 ± 0.23	1.41 ± 0.37	1.59 ± 0.42	89	0.57	<0.001	13 (a)
Diatoms	**Spring**	1.20 ± 0.23	0.57 ± 0.30	0.89 ± 0.34	64	0.60	0.014	9 (b)
	Summer	−2.49 ± 0.06	−0.36 ± 0.18 **	−2.88 ± 0.20	-	0.24	0.061	15
	Autumn	0.36 ± 0.07	0.55 ± 0.10	0.32 ± 0.15	171	0.76	<0.001	11 (a)
Dinoflagellates	**Spring**	−1.77 ± 0.38	nd	−2.80 ± 0.46	-	0.59	0.014	15
	Summer	−0.14 ± 0.13	nd	−0.03 ± 0.23	-	0.40	0.012	15
	Autumn	−0.15 ± 0.13	0.09 ± 0.28 **	−0.39 ± 0.21	-	0.01	0.755	12 (a)
Euglenophytes	**Spring**	0.15 ± 0.26	0.96 ± 0.36 **	−1.23 ± 0.27	-	0.64	0.056	6 (a)
	Summer	1.78 ± 0.22	3.08 ± 0.35	1.42 ± 0.51	217	0.87	<0.001	14 (a)
	Autumn	−0.36 ± 0.99	6.62 ± 3.18 **	1.34 ± 1.28	-	0.52	0.106	6 (b)

Relationships between apparent phytoplankton growth rates and the dilution factor in enriched dilutions were not always negative and linear, with some non-linear or positive responses observed; this occurred more frequently for plastidic nanoflagellates and eukaryotic picophytoplankton. Positive slopes were observed for dinoflagellates during spring and summer experiments; therefore, no grazing rates were determined for these two specific cases.

4. Discussion

The dilution experiments conducted in the Ria Formosa coastal lagoon revealed significantly higher growth rates (μ_0) in the spring and a higher grazing rate (g) in the autumn, for both heterotrophic prokaryotes and phytoplankton. Our values are within the range reported for heterotrophic prokaryotes in a Mediterranean coastal lagoon [26] and for the phytoplankton community in the Ria Formosa coastal lagoon during winter [31], as well as for other temperate coastal lagoons [51–54]. The estimates are also within the range of values reported for other temperate coastal ecosystems [55–57]. Growth rates of some specific phytoplankton groups higher than those of the whole phytoplankton community indicate that specific phytoplankton groups experienced more rapid growth compared to the overall phytoplankton community.

Overall, the rates of microzooplankton grazing on heterotrophic prokaryotes and phytoplankton were lower than their mean growth rates. Since microzooplankton grazing is just one of many top–down processes (e.g., metazooplankton grazing, sinking, lysis, advection), it is uncommon for microzooplankton grazing to exceed prey growth. This suggests a decoupling of these processes, typical of temperate coastal waters [58] like the Ria Formosa coastal lagoon. A g:μ ratio greater than 1, i.e., grazing exceeding growth, suggests that microzooplankton consume a significant proportion of heterotrophic prokaryotes and phytoplankton in this ecosystem. In estuarine or productive coastal environments, the grazing response may be stronger due to the higher standing stocks of microzooplankton, driven by overall greater ecosystem productivity and biomass [9].

Moreover, microzooplankton consumed more than 100% of the daily production of heterotrophic prokaryote and phytoplankton in the Ria Formosa. These values exceed the mean values obtained by previous studies (~60%) [9,11], further highlighting the importance of microzooplankton grazing in this ecosystem. The imbalance between growth and grazing rates, which results in the frequent removal of over 100% of daily heterotrophic prokaryote and phytoplankton production, suggests that other top–down control mecha-

nisms, such as tidal advection and metazooplankton grazing, may contribute to microbial mortality less than microzooplankton grazing.

The impact of grazing varied depending on the phytoplankton group, with the most significant losses occurring in the smaller phytoplankton groups. Smaller phytoplankton generally have advantages in functional traits related to growth and resource use, while larger phytoplankton are more resistant to grazing [59]. Large phytoplankton tend to have lower nutritional quality (with the exception of diatoms) and are more difficult for microzooplankton to consume, leading to selective grazing on smaller, more nutritious phytoplankton [60]. In Thau Lagoon, for instance, grazing rates were higher on smaller prey like cyanobacteria compared to larger phytoplankton groups [26].

The mean grazing rates for eukaryotic picophytoplankton and cryptophytes were significantly higher, with grazing removing an average of 130% and 264% of primary production per day, respectively. Earlier dilution studies in the Ria Formosa reported higher grazing rates on diatoms during winter [31]. Size is a critical factor in determining grazer–prey dynamics, as reflected in early microbial loop models [61]. However, overlaps in the size ranges of prey consumed by different microzooplankton groups, such as some aplastidic flagellates and ciliates that consume phytoplankton almost as large as themselves [59], complicate the planktonic food web, making it difficult to establish clear cause-effect relationships.

Selective feeding by microzooplankton is common and widespread [62–65], effectively explaining the higher grazing rates on eukaryotic picophytoplankton and cryptophytes compared to other phytoplankton groups, including the most abundant group, cyanobacteria. Ciliates and nanoflagellates preferentially feed on cells smaller than 20 µm (i.e., picoplankton and nanoplankton) [66–69], making picophytoplankton a vital food source for microzooplankton due to their small size [70]. Cryptophytes, in particular, are known to be optimal prey for many protists [71], such as aplastidic and mixotrophic dinoflagellates [72,73] and ciliates [74,75], which selectively feed on cryptophytes. Selective feeding on eukaryotic picophytoplankton has also been reported in several coastal ecosystems [51,76,77]. Given that aplastidic nanoflagellates were the most abundant microzooplankton group in the Ria Formosa during the study period, the higher grazing rates on eukaryotic picophytoplankton can be linked to the abundance and ecological interactions between these groups, as eukaryotic picophytoplankton fall within the prey size range of aplastidic nanoflagellates [78]. Even plastidic nanoflagellates could have been grazing on eukaryotic picophytoplankton, as most are mixotrophs [79].

Euglenophytes and eukaryotic picophytoplankton exhibited the highest growth rates among the phytoplankton groups. Previous studies conducted in the Ria Formosa [4,31] also found that diatoms had the highest growth rates among various functional groups of phytoplankton. Euglenophytes, which are mixotrophic [80,81], can feed on picophytoplankton, a trait that likely contributes to their higher growth rates compared to other groups. The higher growth rates of eukaryotic picophytoplankton in the absence of grazers may reflect their efficient resource utilization, aided by their larger surface area-to-volume ratio. This trait provides them with an advantage in nutrient acquisition and light absorption when competing with larger cells [82].

Diatoms may be more frequently avoided by microzooplankton compared to other phytoplankton groups, likely due to their larger size and grazing defenses, such as spines, which deter microzooplankton from consuming them [83,84]. However, aplastidic and mixotrophic dinoflagellates, with their varied feeding strategies (e.g., direct engulfment, pallium feeding, tube feeding), may be capable of consuming large diatoms [85–87]. Additionally, diatoms tend to sink faster than other phytoplankton groups, which, even with bottle mixing during incubation, may have indirectly contributed to the observed lower grazing rates.

5. Conclusions

Given the essential ecological roles of microbial plankton, quantifying their mortality rates is crucial for understanding the dynamics of aquatic ecosystems. As shown in this study, microzooplankton grazing is a significant biomass removal process for both phytoplankton and heterotrophic prokaryotes, with microzooplankton consuming a substantial proportion of their daily production.

The high growth and grazing rates of heterotrophic prokaryotes suggest that the microbial loop plays a major role in carbon flow within the lagoon, with heterotrophic prokaryotes acting as a vital link between phytoplankton and higher trophic levels. Prey losses to grazers were most pronounced among smaller phytoplankton groups, highlighting the importance of size in determining predator–prey dynamics, although larger phytoplankton cells were also heavily consumed. This study highlights the significance of all microbial components in the overall functioning of the microbial food web and reaffirms the key role of microzooplankton in the Ria Formosa coastal lagoon.

Given the importance of this ecosystem, future research should explore various mortality factors, such as viral lysis, under different conditions and across temporal and spatial scales, to better assess their relative impact and refine carbon flow models for the Ria Formosa coastal lagoon. Additionally, such studies can contribute to improving models and predictions related to climate change, ocean acidification, and other global environmental processes.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/w16233401/s1, Figure S1: Dilution plots for heterotrophic prokaryotes. Figure S2: Dilution plots for phytoplankton in the spring. Figure S3: Dilution plots for phytoplankton in the summer. Figure S4: Dilution plots for phytoplankton in the autumn.

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