Feeding behaviour and non-linear responses in dilution experiments in a coastal upwelling system

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RUNNING HEAD: Nonlinear feeding responses in dilution experiments

ABSTRACT: The occurrence of non-linear responses in several dilution experiments conducted in the coastal upwelling system of the Ría de Vigo was examined in relation to the possible violation of the basic assumptions of the technique. In addition to linear responses, two types of non-linear responses, saturated and saturated-increased responses, were obtained. Saturated responses are those showing constant net growth rates at lower dilutions, while saturated-increased responses depict the increase of net growth rates in low diluted bottles. Evidences relating these two non-linear responses to nutrient limitation or changes in the microzooplankton community were not definitive. In contrast, saturated and saturated-increased responses were frequent when the percentage of microzooplankton was relatively low within a very abundant and diverse plankton community. We suggest that saturated feeding responses were related to the achievement of a maximum ingestion rate by microzooplankton and that saturated-increased responses were associated with selective feeding by microzooplankton at times when microzooplankton was feeding at its maximum ingestion rate. Simulated dilution experiments incorporating these two assumptions, two consumers and three preys were able of reproducing the three types of responses. The results indicate that non-linear responses in dilution experiments must be expected in regions with high and diverse food abundance, which should allow prey selection by microzooplankton.

KEY WORDS: Non-linear feeding responses, dilution technique, microzooplankton, plankton community, upwelling systems.

INTRODUCTION

Since its introduction by Landry & Hassett (1982), the dilution technique, later adapted to accommodate conditions of nutrient limitation (e.g. Andersen et al. 1991, Landry 1993, 1994), has extensively been used to estimate the impact of nano- and microzooplankton (hereafter microzooplankton) on phytoplankton (Calbet & Landry 2004) and bacteria (e.g. Landry et al. 1984, Tremaine & Mills 1987, Worden & Binder 2003) in a wide variety of marine ecosystems. This technique, in which the seawater sample is diluted at several levels with filtered seawater from the same location to reduce the encounter rate of microzooplankton with their preys, relies on three fundamental assumptions regarding interactions among nutrients, phytoplankton and microzooplankton (Landry & Hassett 1982). First, it is assumed that phytoplankton grows exponentially. Second, the growth of a given individual phytoplankton is independent on the presence of other phytoplankton individuals and is not nutrient limited. This means that the specific phytoplankton growth rate does not differ between dilution treatments. Finally, the probability of a phytoplankton cell being grazed is directly related to its encounter rate with microzooplankton, which implies that the number of cells ingested by a given microzooplankton organism is linearly related to prey density. When these requirements are accomplished, a negative linear relationship between the net growth rate of phytoplankton (k, d^{-1}) and the fraction of unfiltered seawater (X) is obtained, with the slope representing the mortality rate of phytoplankton due to microzooplankton grazing (m, d^{-1}) and the y-axis intercept providing an estimate of the phytoplankton growth rate in the absence of predators (μ, d^{-1}):

$$k = \mu - mX \quad (1)$$

However, non-linear relationships are frequently reported (e.g. Gallegos 1989, Dolan et al. 2000, Strom et al. 2001), with saturated feeding responses, those showing constant net growth rates at low dilutions, being common in eutrophic systems. On occasions, these types of non-linear relationships have been attributed to changes in the individual feeding impact of microzooplankton in response to variations in food availability along the dilution series (Gallegos 1989, Moigis 2006). Nevertheless, changes in feeding impact can also be due to variations in the microzooplankton community (Dolan et al. 2000, Dolan & McKeon 2004, Agis et al. 2007). Thus, changes in growth and mortality of the several microzooplankton species during incubation can induce nonlinearities in their total and/or relative abundance within the dilution series and, consequently, modify their feeding impact. In fact, microzooplankton dynamics during incubation was used as a major criticism to the dilution technique, because it would cause mortality patterns of phytoplankton quite different to those occurring in natural communities (Dolan & McKeon 2004).

Here we analyse the results of 8 dilution experiments performed in a coastal upwelling system in which linear and non-linear responses were obtained. The main purpose was to investigate the causes for the occurrence of non-linear responses, taking into account the possible violation of the assumptions on which the dilution technique relies. Understanding the mechanisms behind the appearance of non-linear feeding responses in dilution experiments is crucial to accept or reject this methodology as a useful tool to estimate the microzooplankton impact in aquatic systems.

MATERIALS AND METHODS

Experimental setup. Net growth rates of plankton components ≤200 μm in the coastal upwelling system of the Ría de Vigo (NW Iberia) were estimated using the

dilution technique (Landry & Hassett 1982). A total of 8 experiments were done in February, April, July and September 2002, two times each month. All experimental containers, bottles, filters and tubing were soaked in 10% HCl and rinsed with Milli-Q water before each experiment. Sampling took place at dawn in a station situated in the main channel at the central part of the Ría de Vigo (Fig. 1) with a 30 l Niskin bottle that was dipped twice at the surface. Water from the first dip was gravity filtered through a 0.2µm Gelman Suporcap filter. Although cell breakage during filtration may increase the concentration of dissolved organic matter and inorganic nutrients, we did not observe significant differences between filtered and raw seawater either in dissolved organic carbon or dissolved inorganic nitrogen (t-test for paired samples $p = 0.94$ and 0.32, respectively) using this filtration system. Measured volumes of filtered water and unfiltered seawater obtained from the second dip were gently combined into carboys to obtain dilution levels of ~10, 20, 40, 60, 80 and 100% of unfiltered seawater. The dilution levels were checked from chlorophyll *a* (chl *a*) concentrations determined in triplicate samples. Then, two clear polycarbonate bottles of 2.3 l were completely filled from each dilution level and incubated for 24 hours at simulated *in situ* light and temperature conditions. Carbon biomass (mg C m^{-3}) of autotrophic and heterotrophic pico- (≤2 µm), nano- (2 to 20 µm) and microplankton (20 to 200 µm) were determined in the initial unfiltered seawater and in all experimental bottles after incubation. The initial concentrations for each dilution were estimated taking into account the dilution factor. Chl *a* was also determined in all bottles after incubation.

Analyses. Chl *a* was determined by fluorometry after filtering subsamples of 250 ml through 25mm Whatman GF/F filters. The filters were then stored frozen at -20º C until pigments were extracted in 90% acetone at 4º C in the dark during 24 hours.

Pico- and nanoplankton were determined in subsamples of 10 ml fixed with buffered 0.2 µm filtered formaldehyde (2% final concentration) and stained with DAPI at 0.1 µg ml^{-1} final concentration (Porter & Feig 1980). After 10 minutes in the dark, samples were filtered through 0.2 µm black Millipore-Isopore filters. The filters were then immersed in low fluorescence immersion oil and examined at x1000 magnification using an epifluorescence microscope. Autotrophic organisms were enumerated under blue light excitation and heterotrophic organisms were counted under excitation with UV light. We realize that *Prochlorococcus* cannot be accurately counted with this technique, but their abundance is not important in this coastal system (Rodriguez et al. 2003). Bacterial biomass was estimated according to Lee & Furhmann (1987). Dimensions of several individuals of the other groups were taken and cell volumes were calculated assuming spherical shape. Cell carbon was estimated following Verity et al. (1992) for pico- and nanoflagellates and Bratbak & Dundas (1984) for *Synechococcus*-type cyanobacteria.

Microplankton was determined in subsamples of 250-500 ml preserved in Lugol's iodine. Depending on chl *a* concentration, a variable volume of 10-100 ml was sedimented in composite sedimentation chambers and observed through an inverted microscope. When needed, due to low abundances, additional volumes were sedimented. The organisms were counted and identified to the species level when possible. Phototrophic and heterotrophic species of dinoflagellates were differentiated following Lessard & Swift (1986) and also using epifluorescence microscopy. Dimensions were taken to calculate cell biovolumes after approximation to the nearest geometrical shape (Hillebrand et al. 1999) and cell carbon was calculated following Strathmann (1967) for diatoms and dinoflagellates, Verity et al. (1992) for flagellates and Putt & Stoecker (1989) for ciliates.

Net growth rates k (d^{-1}) were estimated as:

$$k = \frac{1}{t} \cdot \ln\left(\frac{C_t}{C_0}\right) \quad (2)$$

where t is the duration of the experiment (1 day) and C_0 and C_t are the initial and final chl *a* concentration or carbon biomass, respectively.

RESULTS AND DISCUSSION

Feeding responses

Three types of feeding responses were found for total autotrophic (AC), total heterotrophic (HC) plankton ≤200 µm, chl *a* and the several plankton groups considered (Figs. 2 & 3, Tables 1 & 2). In addition to linear (Figs. 2a-c) and saturated feeding responses (Figs. 2e-f), a third type of response (Figs. 2g-i) in which the net growth rates increased in the low diluted bottles (hereafter named saturated-increased response) was also obtained. To our knowledge, this third type of response was only reported by Gallegos (1989) and Elser & Frees (1995). Linear relationships were common in February and April, and saturated or saturated-increased responses were more frequent in July (Fig. 3). September did not show a clear dominance of any of the three types of responses.

According to the architecture of the dilution technique (Landry & Hassett 1982), negative linear relationships between the net growth rate and the fraction of unfiltered seawater occur when the specific growth rate keeps constant at all dilution levels and the mortality increases proportionally with the increase in food abundance (represented

by the fraction of unfiltered seawater in eq. 1). Therefore, deviations from linearity mean that any of these two conditions are not accomplished: i.e., (1) the specific growth rate differs between dilution treatments and/or (2) mortality rate is not linearly related to prey concentration. In the following sections, the occurrence of non-linear responses will be examined in relation to possible variations in these 2 rates.

Changes in the specific growth rate

As all bottles were incubated under the same conditions during each particular experiment, the influence of physical variables prone to affect growth rates, as light and temperature, should have been similar in all incubation bottles. In contrast, changes in concentrations of some chemical components within the dilution bottles could occur. Among all chemicals, nutrients are of main concern in these experiments, since it has been shown that differences in nutrient concentrations along the dilution series can induce changes in phytoplankton growth rates, whenever phytoplankton is nutrient-limited (Andersen et al. 1991, Ayukai 1996, Gaul et al. 1999). Thus, high concentrations of regenerated nutrients in low diluted bottles can result from increased microzooplankton activity due to high food abundance, and this can enhance phytoplankton specific growth rates. In contrast, highest diluted bottles would content lower concentrations of regenerated nutrients, since microzooplankton consumption should be less important. This raise in the specific growth rates in the low diluted bottles would result in the increment of net growth rates and so lead to the appearance of non-linear feeding responses.

To avoid the effect of nutrient limitation, it has been proposed to add nutrients to all incubation bottles well in excess for phytoplankton growth (Landry & Hassett 1982, Andersen et al. 1991, Landry 1994). However, the consequences of this experimental

step are controversial, because it can cause losses of oligotrichous ciliates (Gifford 1988) and also affect phytoplankton growth negatively (Lessard & Murrell 1998, Worden & Binder 2003). Moreover, changes in microzooplankton behaviour and in the shape of the functional response have also been reported when nutrients were added (Worden & Binder 2003). Owing to these uncertainties and with the aim of maintaining the plankton community as close as possible to *in situ* conditions, we did not add nutrients to our incubation bottles. Consequently, this could have caused the appearance of non-linear responses in our dilution experiments.

Initial nutrient concentrations (Table 3) were in some cases extremely low. Total inorganic nitrogen was significantly lower ($p < 0.01$, t-test for two samples) in the two experiments of April (<1 μmol l^{-1}) and in the experiment of July 18 (1.38 μmol l^{-1}). Initial concentrations of phosphate were also significantly lower ($p < 0.05$) in both experiments of April and in the experiment of September 19. If nutrient limitation was responsible for the appearance of non-linear responses, a higher frequency of saturated and saturated-increased responses should be expected under the most limiting conditions of April. However, responses in April were linear whereas saturated and saturated-increased responses were more frequent in July (Fig. 3). On September 19 the three responses occurred (Fig. 3). Besides, saturated and saturated-increased responses were also obtained for heterotrophic plankton organisms in July (Fig. 3), and although heterotrophs can compete with phytoplankton for inorganic nutrients (e.g. Wheeler & Kirchman 1986), their specific growth rates should be less affected. Therefore, we can cautiously conclude that nutrient limitation was not the main factor causing saturated and saturated-increased response in our experiments, despite nutrient influence was not specifically assessed.

Changes in the mortality rate

The mortality rate at each dilution level (m_X, d^{-1}) is a function of microzooplankton clearance rate (c, µl pg C^{-1} d^{-1}) and microzooplankton abundance (Z_X, pg C μl^{-1}) (Landry & Hasset 1982, Gallegos 1989):

$$m_X = cZ_X \quad (3)$$

For the case of linear relationships, it is assumed that microzooplankton ingests preys at its maximum clearance rate. Therefore, the mortality rate along a dilution series should increase according to the increase in microzooplankton abundance, which in turn is proportional to the fraction of unfiltered seawater. Consequently, any change in the proportionality of the mortality rate (m_X) in a dilution experiment must be related to (a) changes in microzooplankton abundance and/or (b) changes in the clearance rate, during the incubation and along the dilution series.

Changes in microzooplankton abundance

Changes in microzooplankton growth and mortality within dilution series, which resulted in changes in microzooplankton abundance, have been reported (Dolan et al. 2000, Dolan & McKeon 2004, Agis et al. 2007). Thus, Dolan et al. (2000) observed that net growth rates of oligotrichs and tintinnids increased in the high diluted bottles following the increase in the abundance of potential nanoplankton preys. In contrast, net growth rates remained constant at the high prey abundance in the low diluted bottles, which coincided with saturation in the chl *a* response. Interestingly, Gallegos (1989) simulated a saturated-increased response assuming higher predation on microzooplankton in low diluted bottles, and concluded that predation on

microzooplankton would release preys from microzooplankton impact allowing them to increase their net growth rates.

Changes in the microzooplankton community also occurred in some of our experiments (Table 4). However, these changes were linear, with microzooplankton increasing (slope > 1) or decreasing (slope < 1) during incubations. For the cases with not significant regressions ($0.37 \leq r^2 \leq 0.66$) between initial and final microzooplankton abundance, changes were also linear. There was no case showing a regular decrease at lower dilutions that could be related to the appearance of saturated-increased responses. Moreover, most of the not significant regressions occurred on February 28 and in the two experiments of April, when the responses in dilution experiments were linear (Fig. 3).

According to equation (3) linear changes in the microzooplankton abundance without variations in the clearance rate during the incubation can cause modifications in the slope defining mortality rates, but not in the shape of the response. This can be appreciated comparing the real responses obtained in the experiments with the expected responses assuming that microzooplankton abundance varied linearly while clearance rate was maintained constant. The results show that real and simulated responses were different (Fig. 4). Saturated-increased responses occurred when microzooplankton biomass did not change during the experiment (Fig. 4a, Table 4), as well as when there was an increase (Fig. 4b) or decrease (Fig. 4c) in the microzooplankton biomass. Consequently, we can conclude that the non-linear responses that we found in our dilution experiments were not apparently related to changes in the microzooplankton community. Instead, they would be attributed to changes in the clearance rate.

Changes in feeding behaviour

The carbon specific ingestion rate of a given microzooplankton organism (I, pg Cprey pg $Cmicrozoo^{-1}$ d^{-1}) is a function of the specific clearance rate (c, µl pgC^{-1} d^{-1}) and prey concentration (P, pgC μl^{-1}) (e.g. Frost 1972, Landry & Hasset 1982):

$$I = cP \quad (4)$$

In linear responses it is supposed that microzooplankton ingests preys at its maximum clearance rate, and so ingestion rates increase in proportion to the increase in prey abundance, represented by the fraction of unfiltered seawater. However, it has been observed that microzooplankton may reach a maximum ingestion rate at high food concentration. For example, Heinbokel (1978) showed that the ingestion rate of tintinnids increased up to a maximum value following the availability of preys. Then, the maximum ingestion rate remained constant in spite of further increases in prey abundance. In these cases, as food availability increases there is a proportional decrease in the microzooplankton clearance rate to maintain the ingestion at its maximum constant value.

$$Imax = cte = cP \quad (5)$$

This feeding behaviour is an argument often used to explain the occurrence of saturated feeding responses in dilution experiments (e.g. Gallegos 1989, Moigis 2006). However, the occurrence of saturated-increased responses necessarily implies the decrease in the ingestion rate when food increases, which means that the decrease in the

clearance rate should be more abrupt and not proportional to the increase in food availability.

Total carbon biomass in our experiments (Table 3) was significantly higher in July and September than in February and April ($p < 0.05$, t-test for two samples). In July, when only two responses were linear (Fig. 2), initial chl *a* concentrations were also higher ($p < 0.05$) and percentages of microzooplankton biomass lower ($p < 0.001$) than in the other experiments (Table 2). From this, we can infer that saturated and saturated-increased responses were more frequent in the experiments where there was a low percentage of microzooplankton within a very abundant plankton community. The excess of food can saturate the ingestion of microzooplankton and therefore cause saturated feeding responses. The reasons for the decrease in the ingestion rate that lead to the occurrence of saturated-increased responses are not clear, but we hypothesize they could result from active or passive food selection by microzooplankton when prey availability is high. Active prey selection is a well documented process and it has been attributed to several factors, as morphological characteristics, presence of metabolites or nutritive value of preys (e.g. Verity 1991, Wolfe et al. 1997, John & Davidson 2001). This selectivity is more important in situations with high food availability (Heinbokel 1978, Jürgens & DeMott 1995, John & Davidson 2001), when the concentration of preferred preys is sufficient to satisfy the nutritional needs of the predator. Passive selection could be due to different encounter rates between microzooplankton and its preys, which can release the less abundant preys from predation in situations of high food availability, because the maximum ingestion rate of microzooplankton is satisfied with the more abundant preys. Both types of selection would explain why saturated-increased responses in our dilution experiments were more frequent in July, when food was abundant (Table 3).

This hypothesis on prey selectivity was assessed simulating 2 dilution experiments. The 2 simulations contained 3 preys (A, B and C) and two microzooplankton species (M1 and M2) with initial biomasses (pg C μl^{-1}) proportional to the dilution levels (prey A = 30X, prey B = 100X, prey C = 80X, M1 = 20X and M2 = 20X, X is the fraction of unfiltered seawater). In the first simulation, the maximum ingestion rate of both M1 and M2 was set up at 5 pg C pg C^{-1} d^{-1}. M1 ingests prey A and B but prefers prey A, and this preference establishes when its maximum ingestion rate is satisfied. Note that this preference of M1 for the less abundant prey A can be ascribed to active selection. M2 only ingests prey C. With these assumptions the ingestion rates of each prey at each dilution level were calculated with equation (4) assuming a maximum clearance rate of 0.11μl pg C^{-1} d^{-1} for both consumers. Then, once the available food (prey A + prey B) in the low diluted bottles allows M1 to ingest $\geq$5 pg C pg C^{-1} d^{-1}, the ingestion of prey B was calculated as the difference between this maximum ingestion rate and the ingestion calculated for prey A. This implies that the clearance rate of M1 for prey B diminishes. With these ingestions calculated for each prey and dilution level, the corresponding clearance rates for each prey and dilution were estimated with equation (4). Clearance rates were then used in equation (3) to estimate mortality rates at each dilution level, which were used to estimate the net growth rates of each prey as the difference between the specific growth rate (μ = 0.7 d^{-1} for the 3 preys) and the mortality rates. In the second simulation, which corresponded to passive selection of the more abundant prey, the maximum ingestion rate of M1 was set at 10 pg C pg C^{-1} d^{-1} with no prey preference. All the other parameters and rates were equal to those used in the first simulation.

The outputs of the 2 simulations (Fig. 5) show the 3 types of responses. Feeding on prey C was in the two cases saturated, because consumption of this prey remained

constant after M2 reached its maximum ingestion rate, despite further increases in food concentration. In contrast, the responses of prey A and B changed according to the restriction imposed. For the case of active selection (Fig. 5a), the release of pressure on the more abundant prey B allows it to grow, whereas for the case of passive selection (Fig. 5b) the release of pressure occurs on the less abundant prey A. The biomass of the other preys (A and B respectively) was not enough to saturate the ingestion rate of M1, and this resulted in linear responses for both.

Some considerations on viral influence

It must be noted that in the present analysis the virus influence was not specifically assessed. Regarding the dilution technique, two basic concerns can be distinguished. The first refers to the use of filtered water through 0.2 μm pore size, which is grazer-free but not virus-free. Thus, virus concentration should be equal at all dilution levels, and if viral infection is density dependent, mortality due to virus should be constant and hence lead to the sub-estimation of specific growth rates (Evans et al. 2003, Baudoux et al. 2006). Although an accurate estimate of rates is the objective of dilution experiments, a constant mortality factor due to virus at all dilution levels should not induce the appearance of non-linear responses. The second concern relates to enhanced viral infection at high grazing activity (Šimek et al. 2001, Sime-Ngando & Pradeep Ram 2005, Weinbauer et al. 2007), which would lead to higher viral infection in low diluted bottles. This can be relevant concerning non-linear responses, because the enhanced virus infection would cause a significant increase in lysis mortality during the incubation time, enhancing nutrient and DOM concentrations in those bottles and so affect specific growth rates. To what extent viruses are responsible for the appearance of non-linear responses in dilution experiments is a topic that needs further assessment.

Feeding behaviour in the Ría de Vigo

Whilst definitive conclusions on the occurrence of food selection cannot be attained from the output of dilution experiments, because incubations are done with all populations interacting at the same time, the different responses obtained (Fig. 3a, Tables 1 & 2) point at its occurrence in the Ría de Vigo. Non-linear responses were more frequent in summer (July) and at the beginning of autumn (September), when typically upwelling brings nutrients to the system and plankton diversity is high (Figueiras et al. 2002). At that time, several heterotrophic (ciliates, *Protoperidinium* spp.) and mixotrophic species (*Ceratium* spp., *Dinophysis* spp.) of different size coexist with a very abundant and diverse autotrophic community composed of several diatom species and ANF. This situation would be suitable for the appearance of different trophic relationships in which passive or active selection of food could be possible. Winter blooms of diatoms, like that recorded in February (Álvarez-Salgado et al. 2005), may also be appropriate for this type of relationships (Fig. 3a), because grazing on diatoms can release of predation to other species. In situations with relatively low abundance of food, such as that in April, selection of food should be less feasible (Fig. 3a). In general, HB and HPF appear to be a suitable food for microzooplankton, because responses (with the exception of HB on February 21) were linear or saturated (Fig. 3a). In contrast, *Synechococcus* frequently showed saturated-increased responses, mainly in summer and autumn (Fig. 3a). This suggests that *Synechococcus* was not a preferred prey, which agrees with previous results reported by other authors. Thus, Caron et al. (1991) observed one case of active selection against *Synechococcus*, which was not a good food source for protozoa when compared with bacteria. The results obtained by Fonda Umani & Beran (2003) also suggest this type of negative selection on *Synechococcus*.

Chl *a* and AC not always showed the same type of response and rates (Fig. 3b, Table 1), which might be attributed to the different carbon:chla ratios of the several phytoplankton species and then result in different feeding responses for these two bulk properties.

Concluding remarks

Although not clearly observed, there is evidence that non-linear feeding responses in this work occurred due to a non-linear feeding behaviour of microzooplankton. Furthermore, we suggest that the non-linear feeding behaviour of microzooplankton may be consequence of the complexity of the microbial food web, where a diverse plankton community can lead to the occurrence of numerous trophic interactions. Microzooplankton can feed on a wide variety of preys, from phytoplankton to bacteria and other heterotrophic organisms (e.g. Rassoulzadegan & Sheldon 1986, Jeong 1999). Consumers may hence modify their food preference depending on the abundance and quality of preys and so release or hold pressure on some plankton components, producers or consumers, which would cause an increase or decrease in their populations. Moreover, mixotrophic organisms are relatively abundant in plankton communities (Bockstahler & Coasts 1993, Stoecker 1999, Zubkov & Tarran 2008), and they often regulate their nutrition mode according to food and nutrient accessibility, changing to heterotrophy under conditions of enough food or nutrient limitation (Sanders et al. 1990, Arenovski et al. 1995). Therefore, feeding behaviour should be especially relevant in aquatic systems with high availability and diversity of preys.

Non-linear feeding behaviour of microzooplankton implies the violation of the assumption that the consumption rate is linearly related to the dilution factor. However, this must not invalidate the use of the dilution technique. Non-linear feeding behaviour

of microzooplankton is an intrinsic property of these organisms and it should also occur in the environment. The main concern under the occurrence of non-linear feeding responses in dilution experiments is the accurate calculation of the rates. If we assume that the net growth rate in the undiluted bottle integrates all growth and mortality processes operating in the environment, the critical point is to obtain accurate estimates of specific growth rates in the absence of predators. In our experiments the specific growth rates calculated as the y-axis intercept of the regression of the linear part of the non-linear responses (Strom et al. 2001, Moigis 2006) do not differ from the specific growth rates estimated from simulated responses as in figure 4, that is, using final microzooplankton biomasses and assuming maximum clearance rates (Fig. 6). This suggests that estimates of specific growth rates using the linear part of the responses are robust. However, this may not be the rule and therefore it seems appropriate to increase the number of highly diluted bottles to ensure that these regressions are obtained where processes are more similar to those occurring in a sample without predators (Gallegos 1989). This would avoid the use of the intermediate dilutions which carry higher uncertainties in the accomplishment of the requirements of the method. The specific growth rates thus calculated can be used to estimate the mortality due to microzooplankton in the natural sample (Gallegos 1989, Strom et al. 2001).

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Table 1. Growth (μ, d^{-1}) and mortality (*m*, d^{-1}) rates obtained from dilution experiments for autotrophic plankton. APF, autotrophic picoflagellates; ANF, autotrophic nanoflagellates; AC, total autotrophic carbon; chl *a*, chlorophyll *a*; ns, not significant; [a]saturated response; [b]saturated-increased response. For saturated and saturated-increased responses r^2 and μ were obtained by regression of the linear part of the responses and *m* was estimated as the difference between μ and the net growth rate (*k*) in the undiluted sample. See text (concluding remarks) and Fig. 6 for more details. $p < 0.05$ for linear responses. The significance of saturated and saturated-increased responses was in some occasions > 0.05 owing to the few points included in the regression.

Date	*Synechococcus*			APF			ANF			Diatoms			AC			chl *a*		
	m	μ	r^2	*m*	μ	r^2	*m*	μ	r^2	*m*	μ	r^2	*m*	μ	r^2	*m*	μ	r^2
21 Feb	0.11	0.56	0.95[b]	0.70	1.47	0.96[b]	ns	ns	ns	0.36	1.32	0.68	0.35	1.27	0.77	0.35	1.36	0.87
28 Feb	ns	ns	Ns	ns	ns	ns	0.75	1.03	0.78[b]	0.10	0.70	0.93	0.21	0.73	0.81	0.23	0.72	0.69
11 Apr	1.18	2.08	0.81	1.58	0.73	0.97	0.85	0.37	0.89	ns	ns	ns	0.77	0.42	0.93	0.61	0.07	0.81
18 Apr	0.39	0.31	0.95	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.39	0.31	0.95	0.56	0.09	0.99[a]
18 Jul	0.88	1.13	0.94	1.39	1.39	0.98[b]	0.33	1.46	0.99[b]	1.13	1.33	0.92[a]	1.10	1.33	0.92[a]	0.58	1.57	0.98[b]
26 Jul	0.28	1.63	0.89[b]	2.55	2.86	0.99[a]	0.0	0.85	0.97[b]	0.73	1.33	0.99[b]	0.74	1.35	0.99[b]	2.22	2.15	0.99[a]
19 Sep	0.24	0.75	0.92[b]	1.27	3.14	0.97[b]	0.79	0.86	0.99[a]	ns	ns	ns	0.49	0.87	0.77	0.24	0.37	0.87
26 Sep	1.09	1.78	0.99[b]	1.76	3.04	0.91	0.32	1.49	0.89	ns	ns	ns	0.58	1.75	0.70	0.25	1.31	0.73

Table 2. Growth (μ, d^{-1}) and mortality (*m*, d^{-1}) rates obtained from dilution experiments for heterotrophic plankton. HB, heterotrophic bacteria; HPF, heterotrophic picoflagellates; HNF, heterotrophic nanoflagellates; HC, total heterotrophic carbon; ns, not significant; [a]saturated response; [b]saturated-increased response. For saturated and saturated-increased responses r^2 and μ were obtained by regression of the linear part of the responses and *m* was estimated as the difference between μ and the net growth rate (*k*) in the undiluted sample. See text (concluding remarks) and Fig. 6 for more details. $p < 0.05$ for linear responses. The significance of saturated and saturated-increased responses was in some occasions > 0.05 owing to the few points included in the regression.

Date	HB			HPF			HNF			HC		
	m	μ	r^2	*m*	μ	r^2	*m*	μ	r^2	*m*	μ	r^2
21 Feb	0.94	1.42	0.97[b]	2.66	2.24	0.97	ns	ns	ns	0.88	0.73	0.74
28 Feb	1.31	2.24	0.92	1.41	1.25	0.66	ns	ns	ns	1.05	1.57	0.87
11 Apr	1.54	2.44	0.90	1.27	1.97	0.87	0.87	0.74	0.86	0.79	1.06	0.99
18 Apr	1.48	1.24	0.96	2.00	1.78	0.88	0.63	0.11	0.98	0.99	0.48	0.95
18 Jul	1.92	1.56	0.98[a]	1.99	1.98	0.98[a]	0.22	1.58	0.68[b]	0.81	1.40	0.96[b]
26 Jul	1.68	2.25	0.96[a]	1.70	2.70	0.96[a]	1.12	1.81	0.74	1.27	1.82	0.90[a]
19 Sep	1.82	2.01	0.99	1.88	1.39	0.90	ns	ns	ns	0.63	0.62	0.93
26 Sep	0.97	1.14	0.92[a]	2.15	1.95	0.91	ns	ns	ns	0.46	0.80	0.81

Table 3. Initial conditions for each experiment. DIN, dissolved inorganic nitrogen; TC, total carbon of plankton community, Microzoo %, percentage of microzooplankton biomass in the plankton community.

Date	Salinity psu	Temperature ºC	DIN µmol l^{-1}	HPO_4^{2-} µmol l^{-1}	Chl *a* mg m^{-3}	TC mg m^{-3}	Microzoo %
21 Feb	35.5	13.2	5.41	0.41	3.3	200	25
28 Feb	35.4	13.2	4.38	0.34	2.7	84	19
11 Apr	34.6	13.4	0.20	0.17	4.2	143	27
18 Apr	35.3	13.8	0.68	0.18	6.2	341	37
18 Jul	35.2	15.3	1.38	0.37	6.7	819	7
26 Jul	35.3	16.6	3.51	0.51	5.8	333	10
19 Sep	34.8	16.9	2.13	0.31	5.3	407	29
26 Sep	35.0	17.8	3.66	0.41	3.4	460	19

Table 4. Slopes ± standard error of the initial *versus* final carbon biomass of microzooplankton in the eight dilution experiments. Microzoo, total microzooplankton; HNF, heterotrophic nanoflagellates; HDF, heterotrophic dinoflagellates. For all significant relationships, the y-axis intercept was not significantly different from zero. *p < 0.05; **p < 0.01; ns, not significant.

Date	Microzoo	HNF	HDF	Ciliates
21 Feb	0.93 ± 0.19**	0.89 ± 0.17**	ns	4.15 ± 0.86**
28 Feb	Ns	ns	2.10 ± 0.60*	5.09 ± 1.48*
11 Apr	Ns	ns	1.05 ± 0.17**	1.02 ± 0.29*
18 Apr	Ns	ns	0.18 ± 0.03**	1.11 ± 0.34*
18 Jul	2.06 ± 0.61*	2.99 ± 0.91*	1.13 ± 0.26*	ns
26 Jul	1.19 ± 0.19**	1.50 ± 0.49*	0.54 ± 0.06**	ns
19 Sep	0.99 ± 0.11**	1.63 ± 0.49*	0.86 ± 0.10**	0.73 ± 0.23*
26 Sep	1.41 ± 0.30**	2.35 ± 0.56*	0.56 ± 0.15*	ns

Figure legends

Fig. 1. Map of the Ría de Vigo with the location of the sampled station.

Fig. 2. Examples of the 3 types of responses obtained in the 8 dilution experiments made in the Ría de Vigo. (a to c) linear, (d to f) saturated, (g to i) saturated-increased responses (see text for more details). HB, heterotrophic bacteria; ANF, autotrophic nanoflagellates.

Fig. 3. Distribution of the 3 types of responses found in the 8 dilution experiments for (a) plankton groups and (b) bulk plankton properties. ANF, autotrophic nanoflagellates; APF, autotrophic picoflagellates; HNF, heterotrophic nanoflagellates; HPF, heterotrophic picoflagellates; HB, heterotrophic bacteria; AC, total autotrophic carbon; HC, total heterotrophic carbon. Y-axis is dimensionless.

Fig. 4. Real (black circles) and simulated (open circles) responses of 3 dilution experiments. Simulated responses were made with the microzooplankton biomass at the end of the experiments and assuming a constant maximum clearance rate. These maximum clearance rates were estimated, using the equation (3) in the text, from the mortality rates observed at highest dilution levels in real responses. In (a) and (b) simulated responses are for total microzooplankton; in (c) for heterotrophic dinoflagellates. Note that the impact of changes in microzooplankton biomass is minimal at the highest dilutions.

Fig. 5. Output of the simulations made with dilution experiments considering (a) active and (b) passive selection of preys. See text for details.

Fig. 6. Relationship between the specific growth rates estimated from the regressions of the linear part in non-linear responses of dilution experiments (μ) and the specific growth rates estimated considering the final concentration of microzooplankton (μ') (see also Fig. 4 and Table 4).

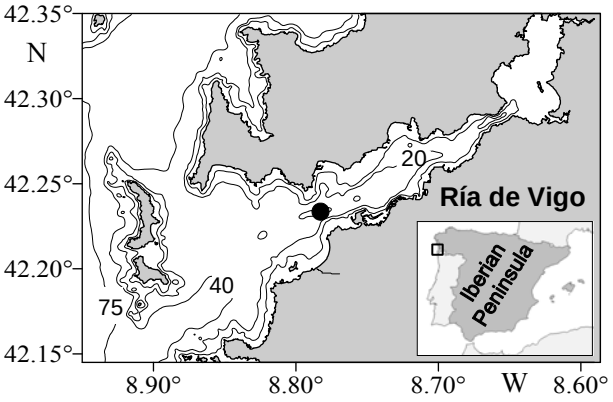

Fig. 1
Teixeira & Figueiras

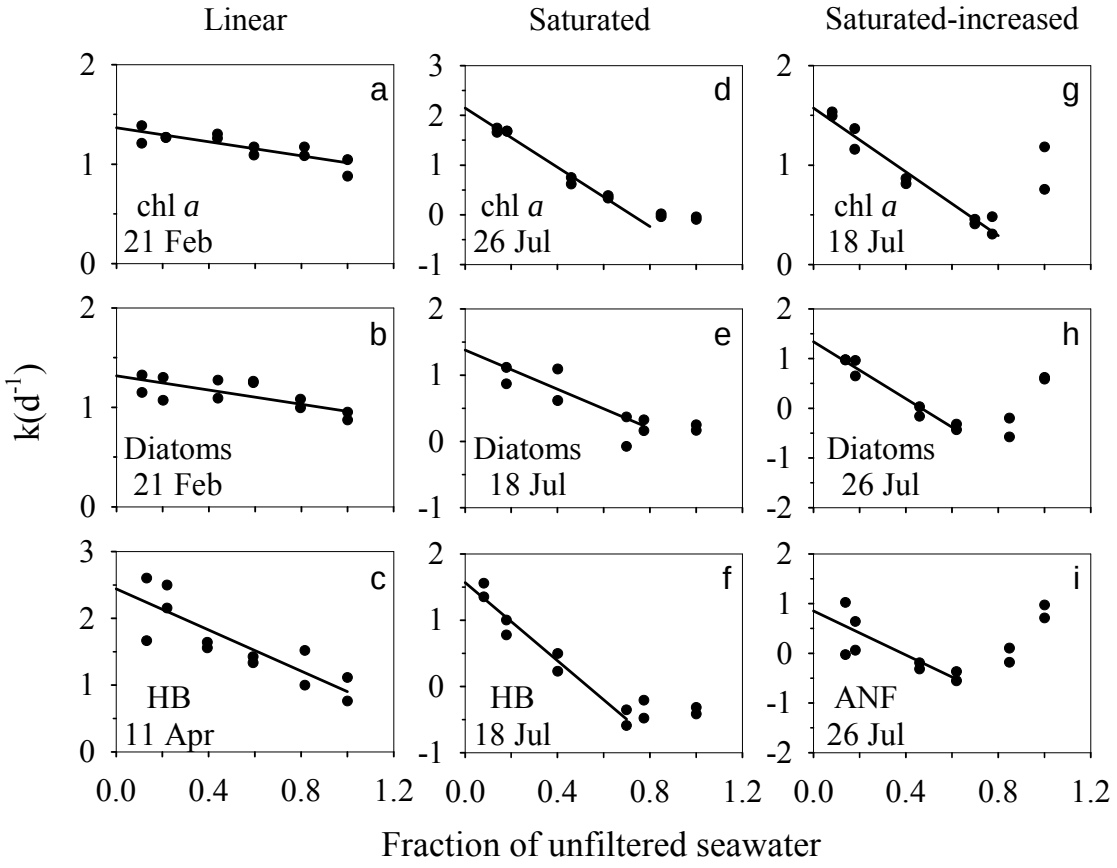

Fig. 2
Teixeira & Figueiras

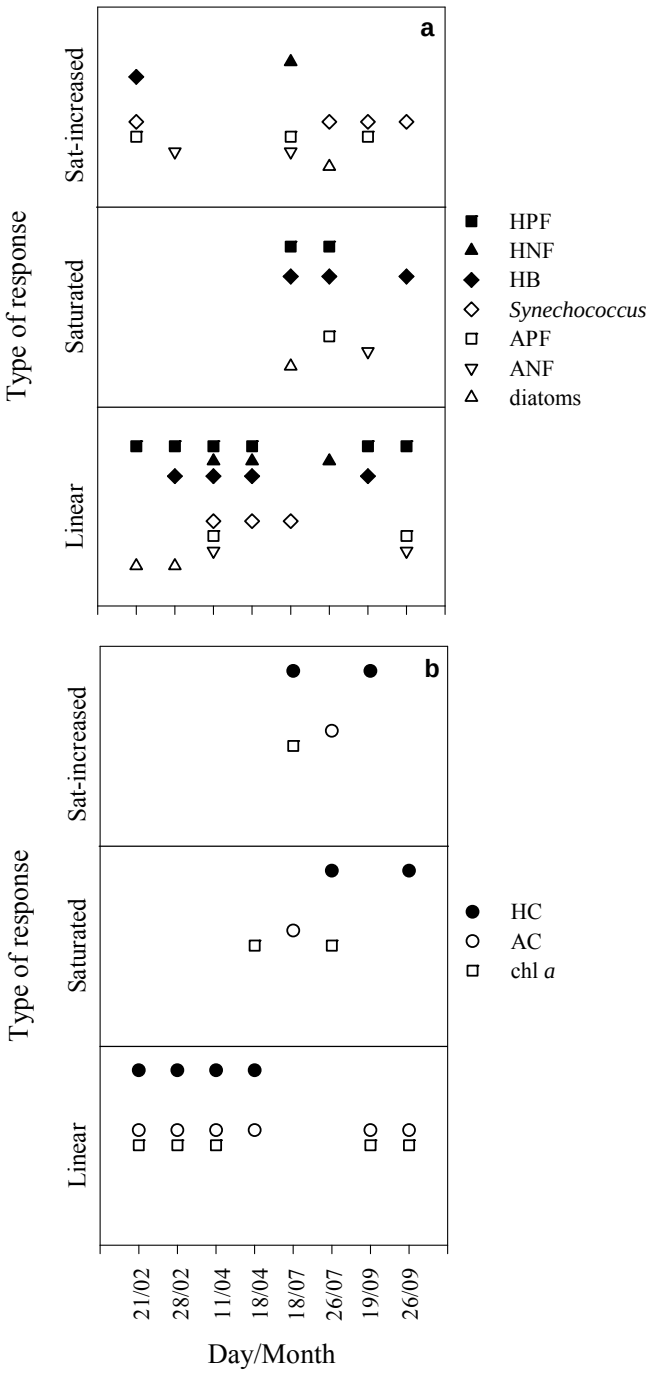

Fig. 3
Teixeira & Figueiras

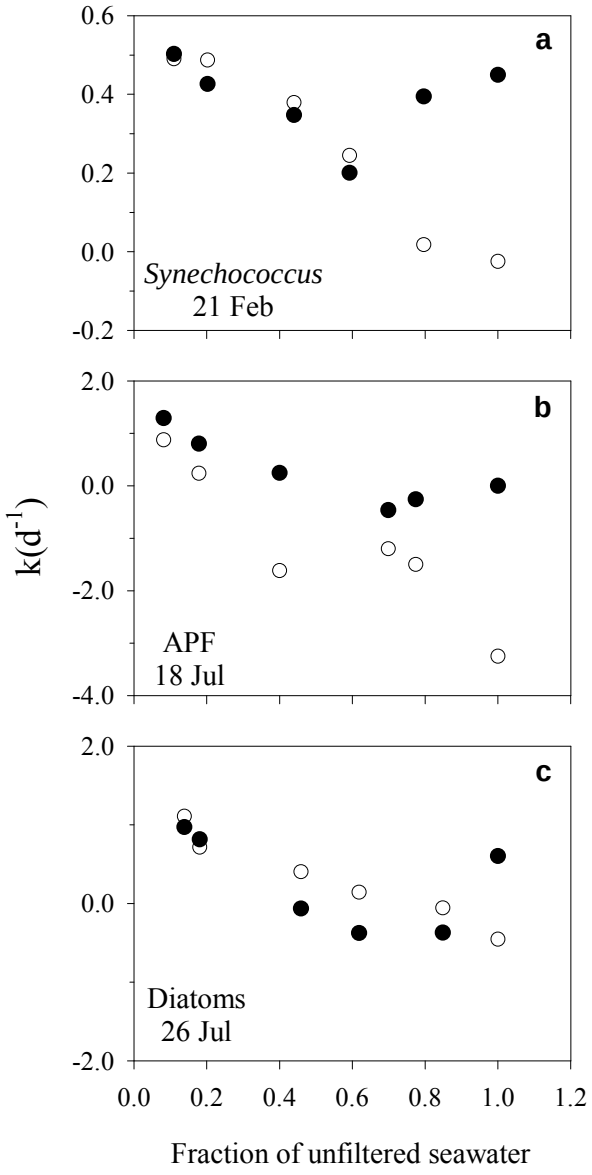

Fig. 4
Teixeira & Figueiras

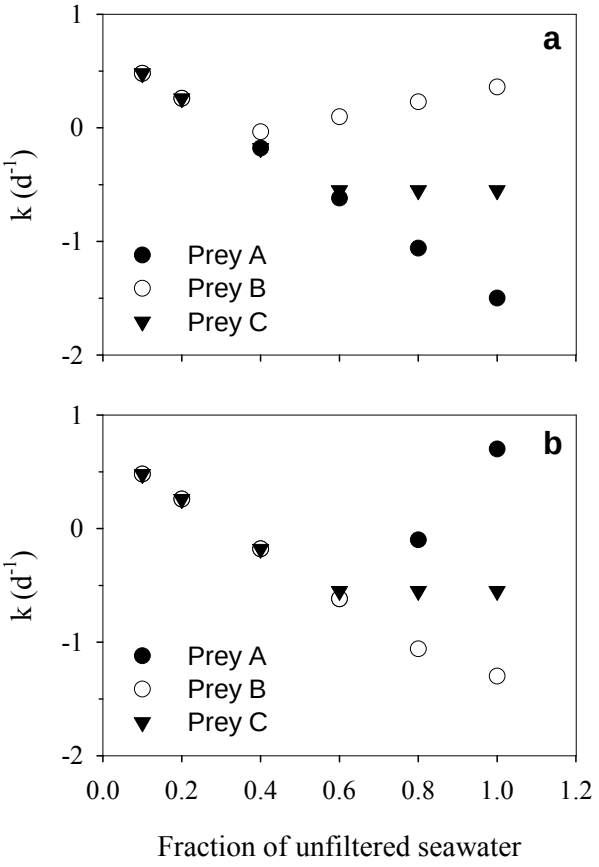

Fig. 5
Teixeira & Figueiras

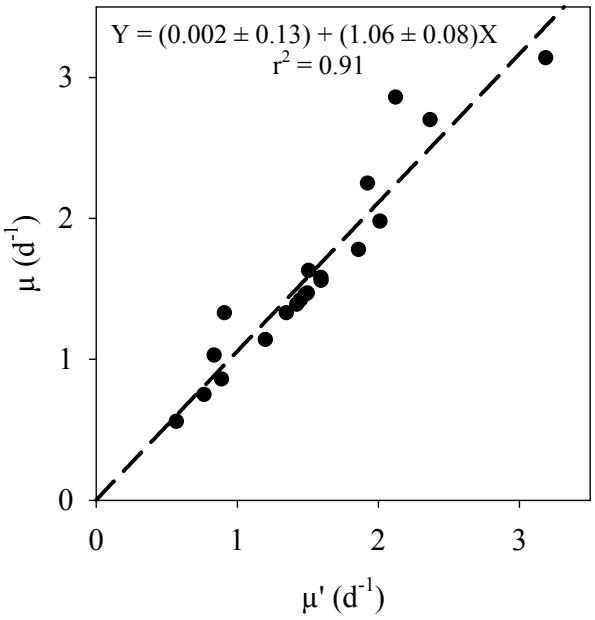

Fig. 6
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