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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

MICROZOOPLANKTON FEEDING IMPACT IN THREE DIFFERENT EUROPEAN COASTAL SYSTEMS

ISABEL CRISTINA GOMES TEIXEIRA

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Dissertação de Candidatura ao grau de Doutor em Ciências do Meio Aquático submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientador – Doutor Francisco Gómez Figueiras

Categoria – Professor de Investigação

Afiliação – Instituto de Investigacións Mariñas do Consejo Superior de Investigaciones Cientificas. Vigo, Espanha.

Co-orientador – Doutor Alexandre Lobo da Cunha

Categoria – Professor Associado

Afiliação –Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

No cumprimento do disposto no decreto-lei nº 216/92 de 13 de Outubro declara-se que a autora desta tese participou na concepção e execução do trabalho experimental que esteve na origem dos resultados apresentados, bem como na sua interpretação, e na redacção dos respectivos manuscritos.

Nesta tese, o trabalho apresentado nos capítulos 2-5 contribuiu para os seguintes artigos científicos:

- Capítulo 2:

Teixeira IG & Figueiras FG (aceite) Feeding behaviour and non-linear responses in dilution experiments in a coastal upwelling system. Aquatic Microbial Ecology.

- Capítulo 3:

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- Capítulo 4:

Teixeira IG, Crespo BG, Nielsen TG & Figueiras FG (para ser enviado) Stratification-mixing cycles and plankton dynamics in a shallow water system: implications for benthic-pelagic coupling.

- Capítulo 5:

Teixeira IG, Crespo BG, & Figueiras FG (para ser enviado) Role of microzooplankton during a *Phaeocystis* sp. bloom in the Oosterschelde (SW Netherlands).

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RESUMO

Neste trabalho, procedeu-se ao estudo do impacto alimentar do microzooplâncton sobre a comunidade planctónica em três sistemas costeiros na Europa: a Ría de Vigo em Espanha, o Limfjord na Dinamarca e o Oosterschelde na Holanda. Este estudo foi efectuado através da aplicação do método de diluição (Landry & Hassett 1982) associado à contagem e identificação por microscopia, o que permitiu determinar simultaneamente a taxa de crescimento dos diversos componentes da comunidade planctónica, autotróficos e heterotrófcos, e o seu consumo pelo microzooplâncton. O método de diluição, apesar de ser a técnica experimental mais usada para determinar o impacto alimentar do microzooplâncton nos sistemas marinhos, pode apresentar vários problemas relacionados com o correcto funcionamento dos pressupostos básicos nos quais se baseia. Na primeira parte deste trabalho (capítulo 2), a violação destes pressupostos básicos foi analisada relativamente à obtenção de respostas não-lineares, que teoricamente deveriam ser lineares. Estas respostas não-lineares, que são observadas frequentemente mas raramente analisadas, foram relacionadas com o comportamento alimentar não-linear do microzoplâncton, o qual atinge uma taxa de ingestão máxima e aumenta a sua selectividade por determinadas presas face a grandes concentrações de alimento. Esta conclusão, que não invalida o uso desta técnica experimental, mostra a necessidade de introdução de várias mudanças no protocolo experimental de modo a optimizar a sua aplicação.

Nos três capítulos seguintes (3, 4 e 5), a avaliação do impacto alimentar do microzooplâncton nos três sistemas costeiros referidos atrás, ofereceu uma visão desse impacto em diferentes comunidades biológicas e face a diferentes condições ambientais. O estudo sazonal efectuado no sistema de afloramento costeiro da Ría de Vigo (capítulo 3) mostrou que o impacto do microzooplâncton sobre os vários grupos planctónicos foi variável ao longo do ano. O consumo de organismos heterotróficos foi constante e relativamente mais importante durante condições de *downwelling*, contrastando com as condições de afloramento, quando a principal fonte alimentar para o microzooplâncton foi o fitoplâncton, nomeadamente diatomáceas. No Limfjord (capítulo 4), a hidrodinâmica do sistema mostrou ser muito importante para o acoplamento entre os sistemas bentónico e pelágico. Os episódios de mistura favoreceram este acoplamento, com uma cadeia trófica curta onde o impacto do microzooplâncton foi mais pequeno que durante os períodos de estratificação, durante os quais a rede trófica microbiana foi favorecida. No Oosterschelde (capítulo 5), o microzooplâncton teve grande importância durante um bloom da haptófita colonial *Phaeocystis* sp., tanto através do consumo directo desta alga, como pelo

consumo de organismos heterotróficos que foram favorecidos pela matéria orgânica dissolvida libertada durante o colapso do bloom.

As principais conclusões deste trabalho estão relacionadas com a validação do uso do método de diluição, mesmo quando são encontradas respostas não-lineares, e com a determinação do importante papel que o microzooplâncton tem nos sistemas costeiros como consumidor dos diversos grupos planctónicos autotróficos e heterotróficos. O conhecimento da importância do microzooplâncton nos sistemas marinhos ajuda a uma melhor compreensão do funcionamento destes sistemas, permitindo desenvolver modelos e previsões mais precisas para as diversas condições ambientais.

ABSTRACT

In this work, the microzooplankton feeding impact on the plankton community has been assessed in three systems along the European coast: an embayment in Spain (Ría de Vigo), a shallow fjord in Denmark (Limfjord) and a shallow basin in the Netherlands (Oosterschelde). This was achieved by performing the dilution method (Landry & Hassett 1982) associated with microscopic enumeration and identification, which allowed determining the production of the several components of the plankton community, both autotrophic and heterotrophic, and their consumption by microzooplankton. The dilution method, albeit being the most widely used experimental technique to estimate the microzooplankton feeding impact in marine systems, can present several problems regarding the correct accomplishment of the basic assumptions on which it relies. In the first part of this work (section 2), the violation of these basic assumptions was analysed in relation to the observed deviations from linearity in a relationship that theoretically should be linear. Non-linear responses, which are also frequently reported in the literature but rarely analysed, were related to the non-linear feeding behaviour of the microzooplankton, which at high food availability reaches the maximum ingestion rate and enhances their selectivity for certain preys. These findings, which do not invalidate the use of this technique, provide further evidence for the introduction of some changes in the protocol in order to optimize its use.

In the following three sections (3, 4 and 5), the estimation of the microzooplankton feeding impact in the three coastal systems referred above, provided an insight into the impact of microzooplankton on different communities and under contrasting environmental conditions. In the seasonal study performed in the coastal upwelling system of the Ría de Vigo (section 3), the microzooplankton feeding impact on the several plankton groups varied over the year. Predation on heterotrophs was very constant and relatively more important during downwelling conditions, when consumption of phytoplankton was low. During upwelling, the main food source for microzooplankton was phytoplankton, mainly diatoms. In the Limfjord (section 4), the hydrodynamics of the system, characterized by stratification-mixing cycles during 9 successive sampling days, was very important for the coupling between the benthic and pelagic systems. Mixing events enhanced this coupling, favouring a short food chain where the impact of microzooplankton was lower. In contrast, stratification periods favoured the microbial food web and the decoupling between benthic and pelagic communities. In the Oosterschelde (section 5), microzooplankton was observed to have an important role during a bloom of the colonial haptophyte *Phaeocystis* sp., either from direct consumption of this algae, or from consumption of heterotrophs

which were favoured by the dissolved organic matter released during the collapse of the bloom.

Major findings from this work are related to the validity of the use of the dilution technique, even when non-linear responses are obtained, and to the determination of the important role that microzooplankton plays in coastal systems as consumers of the several autotrophic and heterotrophic plankton groups. The understanding of the importance of microzooplankton in marine systems aids to a better knowledge of their functioning, enabling to develop more accurate models and predictions under the several environmental conditions.

RÉSUMÉ

Dans cette étude, l'impact alimentaire du microzooplancton sur la communauté planctonique a été évaluée dans trois systèmes des côtes Européennes : La ria de Vigo en Espagne, Limfjord au Danemark et Oosterschelde en Hollande. Ce travail a été effectué à l'aide de la méthode de dilution (Landry et Hassett, 1982), associée à une énumération et identification microscopique qui a permis de déterminer la production de plusieurs composants de la communauté planctonique autotrophe ainsi qu' heterotrophe et leur consommation par le microzooplancton. La méthode de dilution, bien qu'étant la méthode la plus couramment employée pour évaluer l'impact de la prédation du microzooplancton sur les systèmes marins, peut présenter certains inconvénients selon si les hypothèses qu'elle implique sont vérifiées ou non. Dans la première partie de cette étude (chapitre 2), la violation de ces hypothèses de base est analysée en fonction de la déviation par rapport à la linéarité dans une relation qui devrait être linéaire en théorie. La réponse non-linéaire, fréquemment rapportée dans la littérature, mais rarement analysée, est reliée au comportement non-linéaire de la prédation du microzooplancton, qui, lorsque la nourriture est abondante, atteignent le taux maximum d'ingestion et augmentent leur sélectivité pour certaines proies. Ces résultats, qui n'invalident pas l'utilisation de cette technique, apportent des arguments supplémentaires pour l'introduction de certains changements dans le protocole afin d'améliorer son usage.

Dans les chapitres suivants (3, 4 et 5), l'estimation de l'impact alimentaire du microzooplancton sur les trois systèmes côtiers précédemment cités apportent de nouveaux éléments quant à l'impact du microzooplancton sur les différentes communautés et sous des conditions environnementales différentes. Dans l'étude saisonnière effectuée dans le système d'upwelling de la ria de Vigo (chapitre 3), L'impact du microzooplancton sur divers groupes de plancton s'avère varier tout au long de l'année. La prédation sur les hétérotrophes est trés constante et légèrement plus élevée durant des conditions de downwelling, lorsque la consommation de phytoplancton est faible. En période d'upwelling, la principale source d'alimentation du microzooplancton est le phytoplancton, principalement les diatomées. Dans le Limfjord (chapitre 4), l'hydrodynamique du système, caractérisée par des cycles de mélange et stratification durant 9 jours successifs s'est révélée très importante pour le couplage entre les systèmes benthiques et pélagiques. Les épisodes de mélange augmentent le couplage, favorisant une chaine alimentaire courte avec un impact plus faible du microzooplancton. Au contraire, les épisodes de stratification favorisent la chaine alimentaire microbienne et un découplage entre les communautés benthiques et pélagiques. A Oosterschelde

(chapitre 5), le microzooplancton s'est avéré avoir un impact important durant la floraison de haptophyte *Phaeocystis* sp., soit par consommation directe de cette algue, soit par consommation d'heterotrophes qui est favorisée par la dissolution de matière organique durant l'effondrement de la floraison.

Les principaux résultats de cette étude sont reliés à la validité de l'utilisation de la méthode de dilution, même lorsque la réponse non-linéaire a été obtenue et a la détermination du rôle important que joue le microzooplancton dans les systèmes côtiers en tant que consommateur de plusieurs groupes autotrophes et hétérotrophes. Une meilleure compréhension de l'importance du microzooplancton dans les systèmes marins participe à une meilleure connaissance de leur fonctionnement, permettant le développement de modèles plus précis et des prédictions selon les différentes conditions environnementales.

1.
GENERAL INTRODUCTION

1.1. A FEW WORDS ABOUT MICROZOOPLANKTON

In early views of marine pelagic food webs, large diatoms are directly consumed by copepods, which then are eaten by fish. Nowadays, this classical food chain is known to be insufficient to fully explain the fluxes of organic matter and energy in pelagic systems, which led to recognise the importance of the microbial food web (Pomeroy 1974, Azam et al. 1983, Sherr & Sherr 1988).

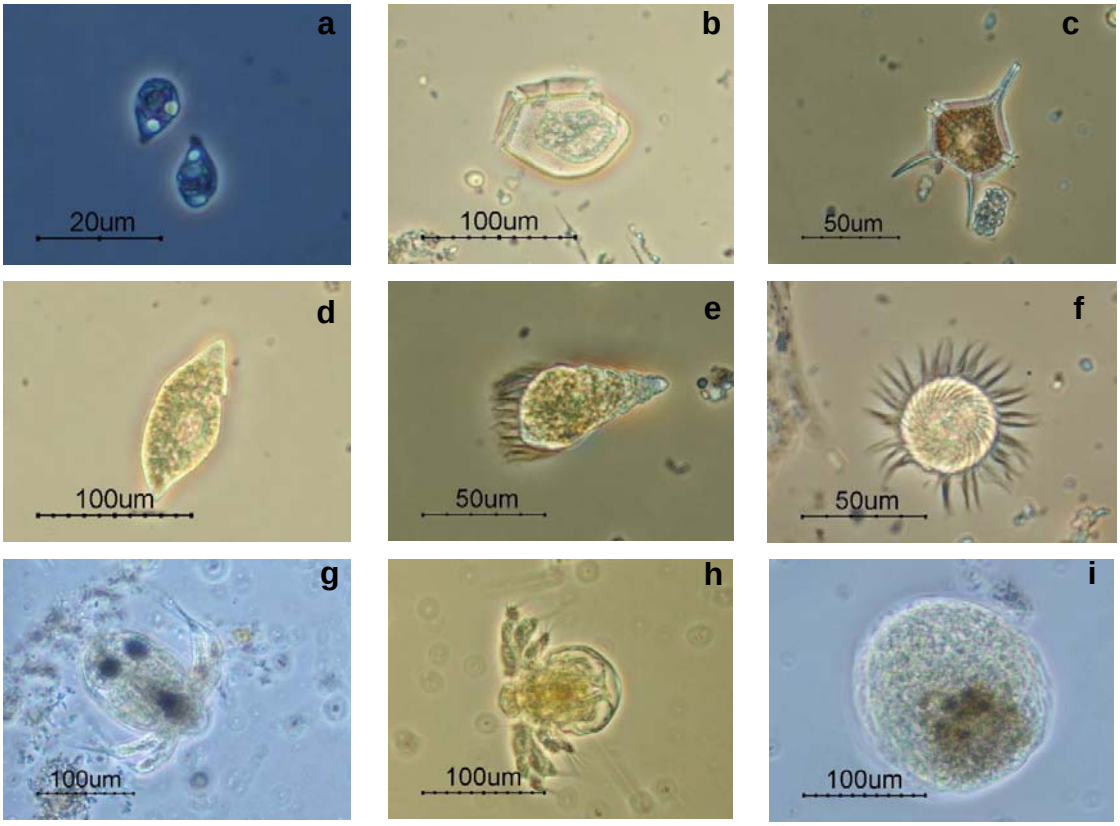

Fig. 1.1. Several microzooplankton organisms. (a) the flagellate *Leucocryptos* sp., (b) the thecate dinoflagellate *Dinophysis acuta*, (c) the thecate dinoflagellate *Protoperidinium diabolus*, (d) the naked dinoflagellate *Gyrodinium* cf. *fusiforme*, (e) the ciliate *Strombidium strobilum* formerly *Laboea strobila*, (f) the ciliate *Lohmaniella spiralis*, (g) and (h) copepod nauplii, (i) Bivalve larvae.

Microzooplankton, defined as the phagotrophic organisms of the plankton community that pass through a 200µm mesh (Beers and Stewart 1969, Capriulo 1990), is a key component of microbial food webs. This functional group comprises both protists and metazoan organisms. Among protists, microzooplankton is represented by dinoflagellates (Fig. 1.1b-d), other flagellates (Fig. 1.1a), ciliates (Fig. 1.1e & f) and sarcodines. The metazoan component of microzooplankton is mainly composed of larval stages, including both holo- and meroplanktonic forms. Naupliar larvae of copepods (Fig. 1.1g & h) are common within holoplankton, while bivalve larvae are frequent in the meroplankton (Fig. 1.1i).

Microzooplankton is ubiquitous and abundant, being found from polar to tropical regions, and from highly productive coastal regions to the oligotrophic open ocean (e.g. Paranjape 1987, Strom & Strom 1996, Froneman & McQuaid 1997, Caron et al. 2000, Putland 2000, Strom et al. 2001, Olson & Strom 2002, Verity et al. 2002, Kim et el. 2007).

1.2. THE ECOLOGICAL FUNCTION OF MICROZOOPLANKTON

As a component of the microbial food web, microzooplankton plays at least three fundamental roles in the pelagic system: i) it consumes other plankton organisms (Fig. 1.2 - A), ii) it mediates in the release of several chemical compounds to the surrounding medium (Fig. 1.2 - B) and iii) it is consumed by other organisms (Fig. 1.2 - C).

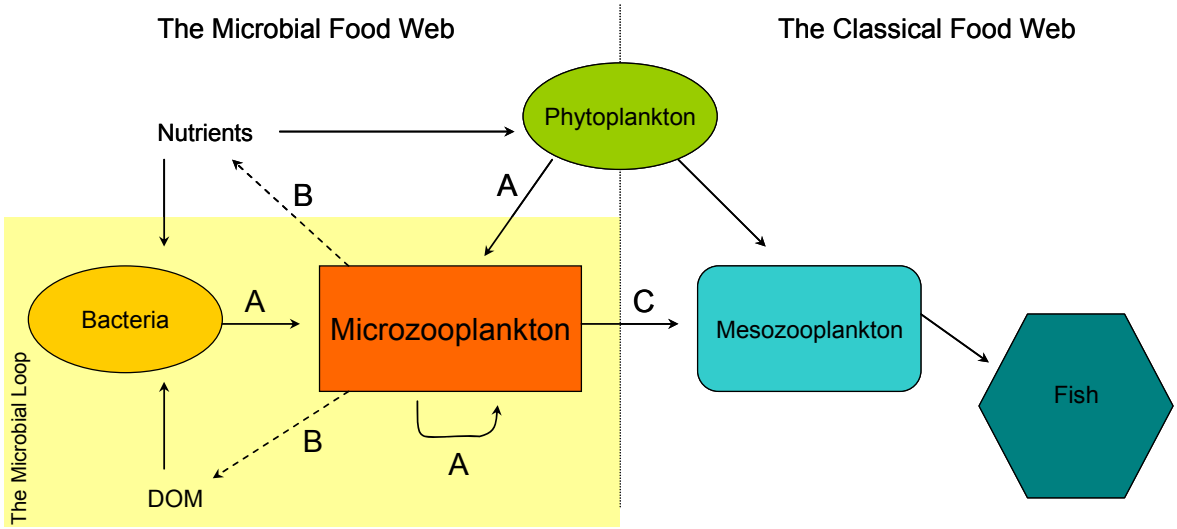

Fig. 1.2. Schematic representation of a pelagic food web showing the microbial food web (left panel) and the classical food chain (right panel). Yellow coloured area represents the microbial loop. Letters represent the several roles of microzooplankton in marine systems: A – as consumers, B – as responsible for the release of several products and C – as food for other organisms. Continuous arrows represent uptakes of matter and dashed arrows represent release. Release of dissolved compounds by other organisms than microzooplankton are not represented.

1.2.1. Microzooplankton as consumer

Within the plankton community, microzooplankton is an important consumer of bacteria and phytoplankton, being able to follow their changes in biomass very closely and thus exerts a tight control on their abundances (Sherr et al. 1986, Sherr & Sherr 1994, 2002). Microzooplankton bacterivory is the main mechanism by which the dissolved organic matter released by biological processes and then taken up by bacteria is transferred through the food web (Azam et al. 1983). The primary bacterivores in aquatic

systems are small flagellates (Fenchel 1982b, Andersen & Fenchel 1985, Fenchel 1986), including the pico-sized forms (Rassoulzadegan & Sheldon 1986, Vaqué et al. 1992, Calbet et al. 2001). However, ciliates, heterotrophic dinoflagellates and metazoan microzooplankton have also been observed consuming bacteria (Lessard & Swift 1985, Rassoulzadegan & Sheldon 1986, Turner & Tester 1992). Microzooplankton organisms are also the dominant herbivores in many marine systems, exerting an impact on phytoplankton communities habitually higher than the impact of mesozooplankton, which classically were considered the main herbivores (Landry et al. 1997, Calbet 2001, Vargas & González 2004, Putland & Iverson 2007). According to the average values provided by Calbet & Landry (2004), more than a half of primary production in marine systems is channelled through microzooplankton. Phytoplankton preys include all size classes, from small cyanobacteria and picoeukaryotes to large chain forming diatoms (Paranjape 1990, Caron et al. 1991, Quevedo & Anadón 2001, Sherr & Sherr 2007).

In addition, several microzooplankton organisms feed on other microzooplankton, which release some preys from their predators and therefore creates trophic cascades (Calbet & Landry 1999, Calbet et al. 2001). Thus, heterotrophic flagellates feed on other smaller heterotrophic flagellates (Calbet & Landry 1999, Calbet et al. 2001) and both are consumed by ciliates and dinoflagellates (Verity 1991, Weisse & Scheffel-Möser 1991, Jeong et al. 2007). Ciliates were observed feeding on dinoflagellates and vice-versa (Hansen 1991a, Bockstahler & Coats 1993, Jacobson & Anderson 1996), while some dinoflagellate species feed on other dinoflagellates (Jeong et al. 1997), including individuals of the same species (Jacobson & Anderson 1986, Latz & Jeong 1996). Dinoflagellates can also feed on early naupliar stages of copepods (Jeong 1994), whereas these have been observed to feed on protozoan microzooplankton (Paffenhöfer 1998). Ciliates also prey other ciliates (Capriulo et al. 1991).

Although scarcely reported, other food sources for microzooplankton can include dissolved organic matter (Sherr 1988, Tranvik et al. 1993), viruses (González & Suttle 1993), copepod eggs (Jeong 1994) and bivalve gametes (Galvão et al. 1989).

1.2.2. Microzooplankton as responsible for the release of several compounds

As a consequence of its feeding activity, microzooplankton releases undigested components of their ingested preys in the form of dissolved organic matter (Nagata & Kirchman 1991, 1992, Strom et al. 1997, Nagata 2000, Ward & Bronk 2001). Dissolved inorganic nutrients, mostly ammonium and phosphate (Goldman & Caron 1985, Caron & Goldman 1990, Neuer & Franks 1993, Dolan 1997, Gaul et al. 1999) are other final products of the feeding activity of microzooplankton. Therefore, microzooplankton feeding

activities provide substrates for further growth of their preys, particularly heterotrophic bacteria (Jumars et al. 1989) and phytoplankton (Dolan 1997).

1.2.3. Microzooplankton as food for higher trophic levels

On other hand, microzooplankton organisms serve as food for higher trophic levels (Sherr et al. 1986, Stoecker & Capuzzo 1990, Gifford 1991, Atkinson 1996, Calbet & Saiz 2005). Due to their cell size (Berggreen et al. 1988), nutritional composition (Stoecker & Capuzzo 1990, Klein Breteler et al. 1999) and swimming behaviour (Jonsson & Tiselus 1990, Kiørboe & Visser 1999), microzooplankton is often the preferred food of copepods. Moreover, it has been shown that the fecundity and growth of copepods is enhanced when their food based on phytoplankton is supplemented with microzooplankton (Kleppel 1993, Klein Breteler et al. 1999). Like copepods, microzooplankton is consumed by cladocerans (Turner et al. 1988), larval and post-larval ctenophores (Stoecker et al. 1987), fish larvae (Fukami et al. 1999), oysters (Dupuy et al. 1999) and mussels (Wong et al. 2003, Nielsen & Maar 2007). This trophic link emphasises the importance of microzooplankton within the pelagic system, since it establishes the connection between the microbial food web and the classical food chain, bringing the biomass of small organisms consumed by microzooplankton to larger consumers, unable to consume them directly.

At a global scale and through these roles, microzooplankton mediates in the biogeochemical cycling of several elements directly connected with earth's climate regulation. In particular, the role of microzooplankton as intermediary in the carbon and sulphur cycles has been specifically highlighted.

The biological pump, which is the downward flux of carbon resulting from the gravitational settling of organic carbon and carbonate fixed by autotrophs in the surface layer, is considerably reduced when trophic transfers occur through the microbial food web (Longhurst 1991, Wassmann 1998). This is because microzooplankton excretions are slow-sinking and labile materials that are rapidly remineralized within the upper layers of the water column (Stoecker 1984, Michaels & Silver 1988, Longhurst & Harrison 1989). Consequently, microzooplankton activity reduces the carbon export to the deep ocean and therefore acts decreasing the sequestration of CO_2 from the atmosphere (Legendre & Le Fèvre 1995).

The role of microzooplankton in the sulphur cycle is related to their feeding impact on dimethylsulfoniopropionate (DMSP)-producing species (Malin 1997). DMSP, which is the cellular precursor of dimethylsulfide (DMS), is a compound mainly produced by haptophytes and dinophytes, though other phytoplankton species can produce it as well.

Oceanic DMS is the main natural source of sulphur to the atmosphere, where its oxidation products form aerosol particles that will be involved in scattering the solar radiation. The conversion of DMSP into DMS appears to be favoured by the presence of grazers and their feeding activities (Belviso et al. 1990, Wolfe et al. 2000, Simó et al. 2002).

To quantify the role of the microzooplankton in the cycling of these elements is a difficult task, but it is important to consider their contribution to improve our forecasting capability and so anticipate the responses of the pelagic system to the global change.

1.3. MICROZOOPLANKTON FEEDING STRATEGIES

The ability of microzooplankton to feed on different types and sizes of preys is related to the taxonomic diversity of this functional group, which includes forms with different feeding strategies and behaviour (Tillmann 2004).

1.3.1. Food capture and ingestion mechanisms

Food capture can occur through three main mechanisms: (i) filter feeding, consisting in the passage of the water through a sieving mechanism; (ii) predatory hunting, which is the active search of preys; and (iii) passive feeding, where predators wait for preys to trap them (Fenchel 1982a, Fenchel 1986, Capriulo 1990, Capriulo et al. 1991, Kiørboe & Titelman 1998). Several types of food capture mechanisms can be found within each microzooplankton group. Thus some ciliates and flagellate species feed by filter feeding, while other are active predators. Dinoflagellates, which typically search for their prey, include some species which are passive feeders.

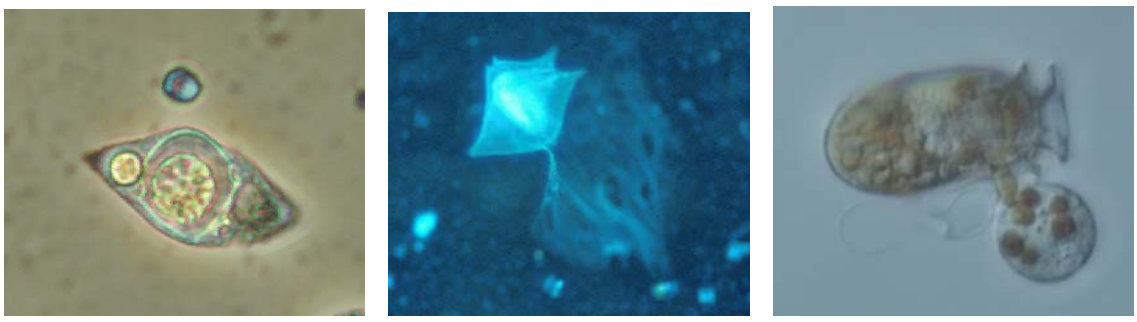

Fig. 1.3. Feeding mechanisms of dinoflagellates. (a) Direct engulfment of *Scrippsiella trochoidea* by *Gyrodinium* cf. *fusiforme* (photo from Teixeira, I.G.). (b) *Protoperidinium* sp. with a feeding veil (photo from Figueiras, F.G.). (c) *Dinophysis acuminata* feeding on *Mesodinium rubrum* through a feeding tube (photo from Park et al. 2006)

Following the contact between preys and predator, ingestion can also occur in several ways. Dinoflagellates are the microzooplankton organisms that present the highest variety of feeding mechanisms (Hansen & Calado 1999, Jacobson 1999). Direct engulfment of the whole prey (Fig. 1.3a) is widespread and occurs in many naked dinoflagellate genera (Gaines & Elbrächter 1987, Hansen 1991b), but it was also described for a few thecate species (Jeong et al. 1997, Jeong et al. 1999). Within thecate dinoflagellates, pallium feeding, in which the prey is surrounded by a pseudopodium (the pallium) originating from the flagella pore (Fig. 1.3b) and digestion takes place outside the main cell body, is common (Gaines & Taylor 1984, Jacobson & Anderson 1986). Another group of dinoflagellates uses a feeding tube (Fig. 1.3c) to suck the contents of their prey (Hansen 1991a, Hansen & Calado 1999). In the other microzooplankton groups, feeding occurs usually through direct engulfment of the whole prey (Capriulo et al. 1991).

1.3.2. Food selection

Microzooplankton can be extremely selective with their preys, but this selectivity is based on several criterions. The size of the prey plays the major role in the selection of food by each microzooplankton organism (Hansen et al. 1994). The suitable size range of preys depends directly on the feeding apparatus of the predator, with the maximum size being set up by the size of the oral aperture (e.g. the cytostome of ciliates), and the minimum size being determined by the efficiency in the capture (e.g. the cilliary distance of filter feeders). In this context, dinoflagellates with their capacity of extracellular feeding are less limited by the size of prey than other microzooplankton organisms. Potential preys for dinoflagellates are almost all type of particles present in the ocean, including bacteria, nanoflagellates, microalgae and microzooplankton, eggs of copepods, marine snow and injured metazoans (reviewed in Jeong 1999). Exceptions to this rule also occur, since there are a number of observations reporting that naked ciliates may also ingest extremely large food particles (Smetacek 1981, Paranjape 1990, Aberle et al. 2007). In addition to prey size, feeding can also be determined by the nutritional value of the prey (Stoecker et al. 1986, Verity 1988, Verity 1991, Jürgens & DeMott 1995, John & Davidson 2001), the physiological state of the predator (Christaki et al. 1998) and the presence of metabolites or toxicity in preys (Wolfe et al. 1997, Lewitus et al. 2006). Ultimately, the degree of food selection will depend on the concentration of preys, and so it has been stated that food selection increases with the increase in food abundance (Heinbokel 1978, Stoecker et al. 1986, Jürgens & DeMott 1995, John & Davidson 2001).

Thus, microzooplankton organisms, through their feeding activities, not only control the abundance of their preys, they also shape taxonomically the plankton community (e.g. Burkill et al. 1987, Paranjape 1990, Olson & Strom 2002, Stelfox-Widdicombe et al. 2004).

1.4. MICROZOOPLANKTON FEEDING IMPACT IN MARINE SYSTEMS

In a global analysis performed with values reported in the literature about microzooplankton grazing on phytoplankton, Calbet & Landry (2004) observed that for the different marine systems studied, which were classified according to distance from shore, the average values for microzooplankton impact on primary production varied within a narrow range (Table 1.1). However, behind these average values, which definitely demonstrate the importance of microzooplankton in marine food webs, the impact of these consumers shows high variability associated with seasonal variations in the systems (Landry & Calbet 2004). Variability in hydrographic conditions, nutrient inputs and levels of primary production, supporting different plankton communities, must thus force contrasting patterns in the impact of microzooplankton.

Table 1.1. Regional comparisons of average chlorophyll *a* (chl *a*) concentrations and percentage of primary production (%PP) grazed daily by microzooplankton, according to Calbet & Landry (2004).

Region	chl *a* (mg m^{-3})	%PP grazed
Oceanic	0.58 ± 0.03	69.6 ± 1.5
Coastal	3.06 ± 0.53	59.9 ± 3.3
Estuarine	13.0 ± 1.8	59.7 ± 2.7

1.4.1. Oceanic and oligotrophic regions

As main consumer of small cells, the importance of microzooplankton has been traditionally linked to the stratified and oligotrophic areas of the ocean (Ryther 1969, Cushing 1989), where pico- and nanoplankton dominates (e.g. Li et al. 1983, Verity et al. 1996, Lessard & Murrell 1998). Thus, several studies have shown that microzooplankton organisms are the primary grazers of phytoplankton in these zones (Landry et al. 1993, 1997, 1998, Verity et al. 1993, Lessard & Murrell 1998, Quevedo & Anadón 2001, Liu et al. 2002), where according to Calbet & Landry (2004), microzooplankton consumes 70% of primary production (Table 1.1).

In these regions of the ocean, small prokaryotes and eukaryotes are transferred through a food web characterized by a high number of trophic steps (e.g. Weisse & Scheffel-Möser 1991, Calbet & Landry 1999, Calbet et a. 2001), with small flagellates being the main microzooplankton group (Calbet 2008). Consequently, most of the organic matter photosynthesised in these regions is remineralised in the upper layers of the water column and the microbial food web is low efficient transferring matter and energy to higher trophic levels.

1.4.2. Coastal and productive regions

Short food chains have been traditionally linked to coastal and productive regions, including upwelling areas. In these productive regions, chain-forming diatoms dominate and they could be directly consumed by mesozooplankton (Ryther 1969). Thus, these areas should be characterized by efficient trophic transfers, owing to the low number of trophic steps involved, that allow sustaining high fish production. Although the role of microzooplankton in these zones was considered irrelevant until recent times, it is now well known that pico and nanophytoplankton are also found in these highly productive regions of the world ocean, sometimes dominating over larger phytoplankton species (e.g. Sherr et al. 1986, Froneman & McQuaid 1997, Putland 2000, Kim et al. 2007). Moreover, heterotrophic dinoflagellates form a significant proportion of the microzooplankton community in these areas (Strom & Strom 1996, Kim et al. 2007, Jyothibabu et al. 2008) and it is now documented that they feed on large diatoms (Jeong 1999, Sherr & Sherr 2007). Thus, recent studies in coastal systems have demonstrated that microzooplankton can be a significant mortality source for phytoplankton (Calbet & Landry 2004), and that its impact is high not only when small phytoplankton forms dominate (Paranjape 1987, Paranjape 1990, Putland 2000, Strom et al. 2007), but also during periods with dominance of large phytoplankton (Neuer & Cowles 1994, Strom & Strom 1996, Strom et al. 2001, Olson & Strom 2002, Stelfox-Widdicombe et al. 2004, Leising et al. 2005, Kim et al. 2007). According to Calbet & Landry (2004) microzooplankton consumes ~60% of primary production in coastal systems (Table 1.1). Besides its impact on primary production, its role as bacterivores or omnivorous is also important in these systems (Fonda Umani & Beran 2003, Vargas & González 2004, Vargas et al. 2007). The assessment of the role of microzooplankton in coastal and productive systems is thus essential in order to definitely consider these organisms as important components of these systems channelling a significant fraction of matter and energy.

1.5. ESTIMATING THE MICROZOOPLANKTON FEEDING IMPACT: THE DILUTION METHOD

The methods used to estimate microzooplankton feeding impact can be divided in three main categories: (1) inferences from natural communities, (2) tracer techniques and (3) community manipulations (Gifford 1988, Landry 1994). Inferential approaches use quantifiable characteristics of field collected samples (e.g. pigment degradation products, stage of cell division, vacuole contents, and digestive enzymes) as a base to estimate feeding rates. Tracer methods use either radioisotopes or fluorescent stains to label the target prey population. Community manipulations include size fractionation or dilution to

separate preys from consumers as well as the use of selective metabolic inhibitors to suppress growth or grazing of plankton components.

Among these experimental techniques, the seawater dilution method, introduced in 1982 (Landry & Hassett 1982), is the method most currently used to estimate the impact of microzooplankton on phytoplankton. Additionally, this method has been also applied to estimate bacterivory (e.g. Landry et al. 1984, Tremaine & Mills 1987). The fundament of the dilution technique relies on the reduction of the encounter rates between predators and preys through the dilution of the original water sample with filtered water from the same location. It is an attractive technique, conceptually simple and of easy execution that requires little manipulation of the community, hence minimizing the adverse effects of handling on individual organisms. Furthermore, it provides growth and mortality rates of the prey simultaneously. Assuming that the growth of the prey is exponential, the change in its abundance over time can be defined by the following equation:

$$C_t = C_0 e^{kt} \tag{1.1}$$

Where C_t and C_0 are the final and initial abundances of the prey in the time interval t, and k is the net growth rate of the prey. The net growth rate is thus the result of processes of growth and mortality:

$$k = \ln\left(\frac{C_t}{C_0}\right)\frac{1}{t} = \mu - m \tag{1.2}$$

Where μ is the specific growth rate of the prey and m is the mortality rate. As the dilution of the natural water sample reduces the encounter rates between predators and their preys, the mortality rates will be reduced by the dilution factor X, but the specific growth rates will be kept constant:

$$k = \mu - mX \tag{1.3}$$

Thus, through the dilution of the natural sample at several levels, and whenever these assumptions are met, i.e., the prey grows exponentially and is equal at all dilution treatments, and microzooplankton consumption increases linearly with prey abundance, a negative linear relationship between the net growth rate of preys (k) and the dilution factor (X) should be obtained (Fig. 1.4).

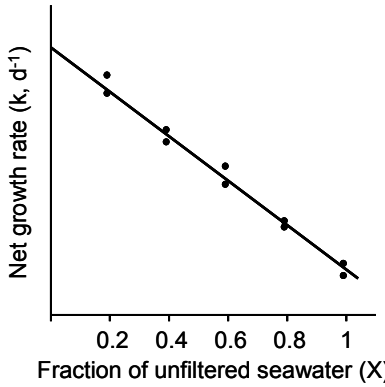

Fig. 1.4. Linear feeding response resulting from plotting net growth rates of the prey against the correspondent dilution factor (or fraction of unfiltered seawater).

From this linear relationship, the specific growth rate (the growth rate in the absence of predators) is given by the y-axis intercept (μ) and the slope (m) is the mortality rate due to microzooplankton (equation 1.3).

For the calculation of net growth rates (equation 1.2), it is necessary to control the abundance of preys at the beginning and the end of the incubation time (usually 1 day). This can be achieved by monitoring changes in bulk properties, such as total chlorophyll concentration (as originally proposed by Landry & Hassett 1982), size-fractionated chlorophyll concentration (e.g. Froneman & Balarin 1998, Strom et al. 2001, Verity et al. 2002), changes in pigment composition determined by High Performance Liquid Cromatography (HPLC) analysis (e.g. Burkill et al. 1987, McManus & Ederington-Cantrell 1992, Gaul & Antia 2001), changes in cell numbers measured by flow cytometry (e.g. Landry et al. 1995, Liu et al. 2002, Worden & Binder 2003) or changes in cell abundances determined by conventional microscopy (e.g. Landry et al. 1984, Paranjape 1990, Fonda Umani & Beran 2003). The choice of the technique associated with the dilution method to determine changes in prey populations must be adapted to the aim of the study. The most widely used is the determination of changes in chlorophyll *a*, either bulk or size-fractionated, because of its greater simplicity and promptness to obtain the results. However, it only provides microzooplankton impact on phytoplankton without further details on the taxonomic composition of the preys consumed. Therefore, the technique has been sometimes improved by determining changes in several pigments using HPLC. For determining changes in other components of plankton communities due to microzooplankton feeding impact, cell counts by flow cytometry or microscopy should be used.

Regardless of its extended use, the dilution method has been the target of several critics concerning the correct accomplishment of its basic assumptions (e.g. Gallegos 1989, Evans & Paranjape 1992, Ayukai 1996, Dolan et al. 2000, Dolan & McKeon 2004).

Namely, the occurrence of non-linear responses in the relationship between the net growth rates and the dilution factor, implying the violation of the assumption that microzooplankton impact increases linearly to prey abundance, revealed to be very common. Two types of non-linear responses were described: a “threshold feeding response”, where deviations from linearity occur at highest dilution levels (Lessard & Murrel 1998, Gaul & Antia 2001) (Fig. 1.5a) and a “saturated feeding response”, where deviations occur at lowest dilutions (e.g. Gallegos 1989, Landry et al. 1993, Strom et al. 2001) (Fig. 1.5b).

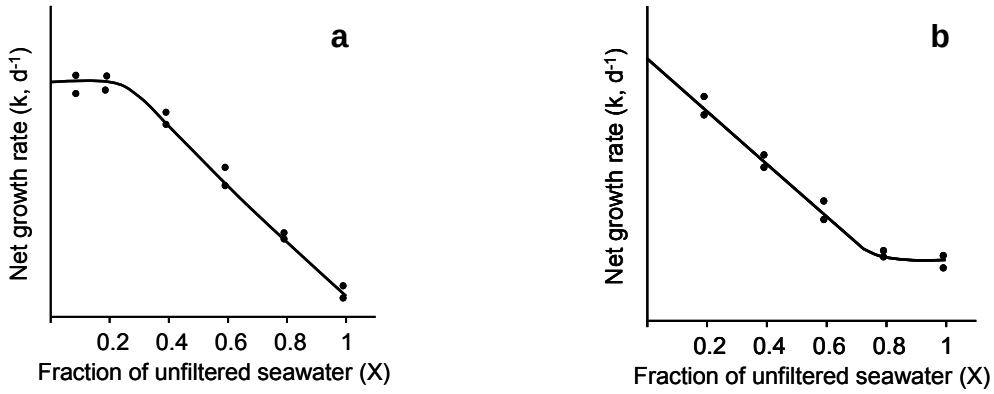

Fig. 1.5. Non-linear responses between the net growth rate and the dilution factor habitually observed during dilution experiments. (a) Threshold feeding response. (b) Saturated feeding response.

The first type of response (Fig. 1.5a), which was attributed to very low food levels that would cease microzooplankton feeding activities, was reported in very few works. However, the second type of responses (Fig. 1.5b), attributed to an excess of food that would saturate ingestion (Gallegos 1989, Moigis 2006), is extremely common in literature (e.g. McManus & Ederington-Cantrell 1992, Landry et al. 1993, Strom et al. 2001, Leising et al. 2005). Despite several studies provided solutions for the correct calculation of rates, once linear regression is not adequate (Gallegos 1989, Evans & Paranjape 1992, Redden et al. 2002), the explanation for the appearance of these types of responses in dilution experiments was never much explored. Ultimately, violation of the other basic assumptions related to nutrients or microzooplankton dynamics within the dilution series could also be in the origin of the appearance of non-linear responses (Elser & Frees 1995, Dolan et al. 2000, Agis et al. 2007). As the dilution technique is the methodology most widely used to estimate the microzooplankton feeding impact on plankton communities, it is essential to know how it operates in order to understand the reasons behind the appearance of non-linear responses.

1.6. OBJECTIVES OF THIS WORK

After this introduction, in which the importance of microzooplankton in pelagic marine systems has been highlighted, and the problems inherent to the technique most commonly used to estimate its impact on plankton communities have been mentioned, the objectives of this work are:

1. Understand the appearance of non-linear responses in the dilution experiments, regarding the correct accomplishment of the basic assumptions of the technique (Section 2)
2. Estimate the microzooplankton feeding impact on the several components of the plankton community (autotrophic and heterotrophic) in three European coastal systems with high differences in nutrient status, hydrodynamic conditions and plankton communities: the Ría de Vigo in Spain (Section 3), the Limfjord in Denmark (Section 4) and the Oosterschelde in Holland (Section 5). The Ría de Vigo is a very dynamic embayment, affected by seasonal upwelling events which introduce nutrient-replete subsurface water from the shelf and fuel phytoplankton communities. The Limfjord, on the contrary, is a more confined shallow system with a high residence time, where stratification and mixing events induced by meteorological forcing characterize the main hydrodynamics of the system. The Oosterschelde basin is also a shallow water system, but with very strong tidal flows, which induce vertical mixing.

2.

FEEDING BEHAVIOUR AND NON-LINEAR RESPONSES IN DILUTION EXPERIMENTS IN A COASTAL UPWELLING SYSTEM*

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 lowest 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.

*The research work presented in this section is also a contribution to the paper:

Teixeira IG & Figueiras FG (in press) Feeding behaviour and non-linear responses in dilution experiments in a coastal upwelling system. Aquatic Microbial Ecology

2.1. 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}):

$$k = \mu - mX \tag{2.1}$$

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.

2.2. MATERIALS AND METHODS

2.2.1. Experimental setup

A total of 8 dilution experiments (Landry & Hasset 1982) were done in February, April, July and September 2002, two times each month, in the coastal upwelling system of the Ría de Vigo (NW Iberia). Salinity and temperature were recorded with a SBE 9/11 CTD probe attached to a rosette sampler. Samples for inorganic nutrients concentration determinations were collected in 50 ml polyethylene bottles and maintained refrigerated until their analysis in the laboratory within 2 h of collection using standard segmented flow analysis procedures. 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. 2.1) with a 30 l Niskin bottle that was dipped twice at the surface.

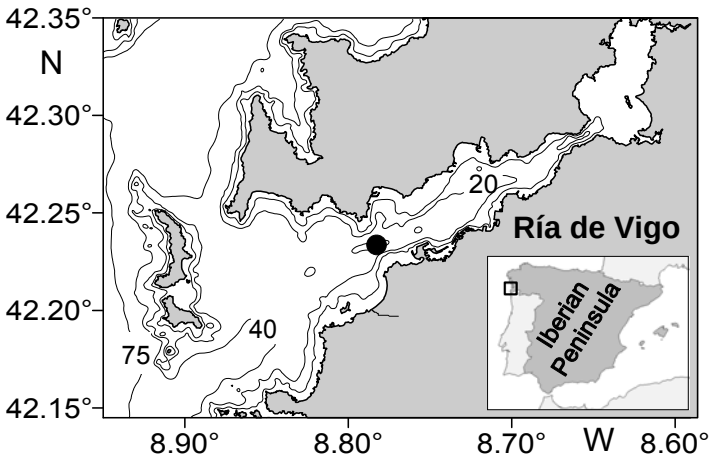

Fig. 2.1. Map of the Ría de Vigo with the location of the sampled station.

Water from the first dip was gravity filtered through a 0.2 µm 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.

2.2.2. Analyses

Chl *a* was determined by fluorometry after filtering subsamples of 250 ml through 25 mm 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 & Fuhrman (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 bibliography (e.g. Lessard & Swift 1986, Larsen & Sournia 1991) 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 other flagellates (>20µm) and Putt & Stoecker (1989) for ciliates.

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

$$k = \frac{1}{t} \cdot \ln\left(\frac{C_t}{C_0}\right) \tag{2.2}$$

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.

2.3. RESULTS AND DISCUSSION

2.3.1. Feeding responses

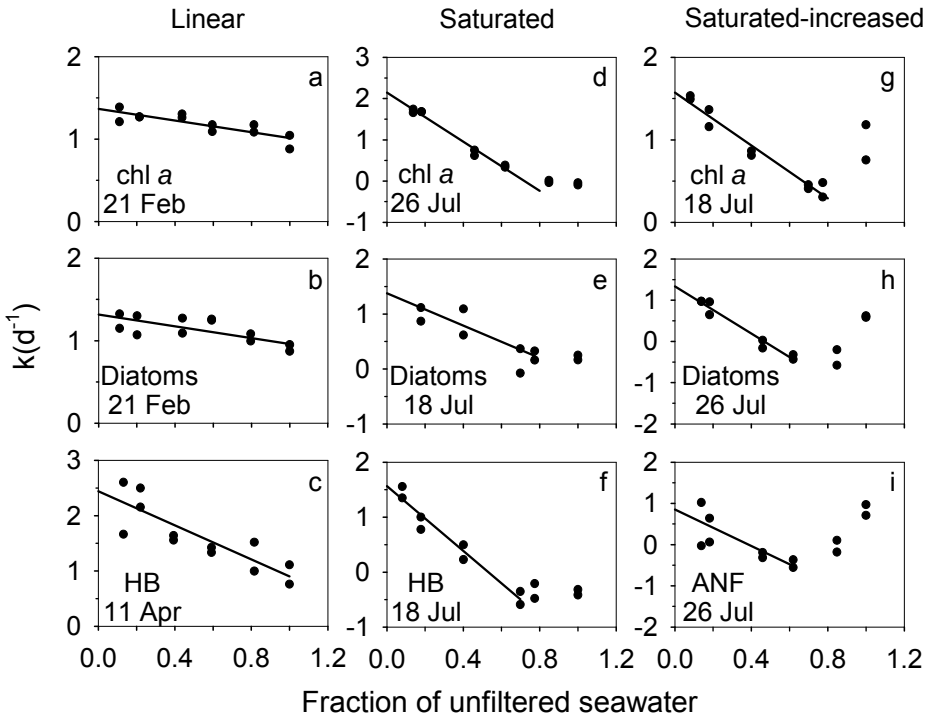

Fig. 2.2. Examples of the 3 types of responses obtained in the 8 dilution experiments made in the Ría de Vigo. (a to c) linear, (d to f) saturated, (g to i) saturated-increased responses (see text for more details). HB, heterotrophic bacteria; ANF, autotrophic nanoflagellates.

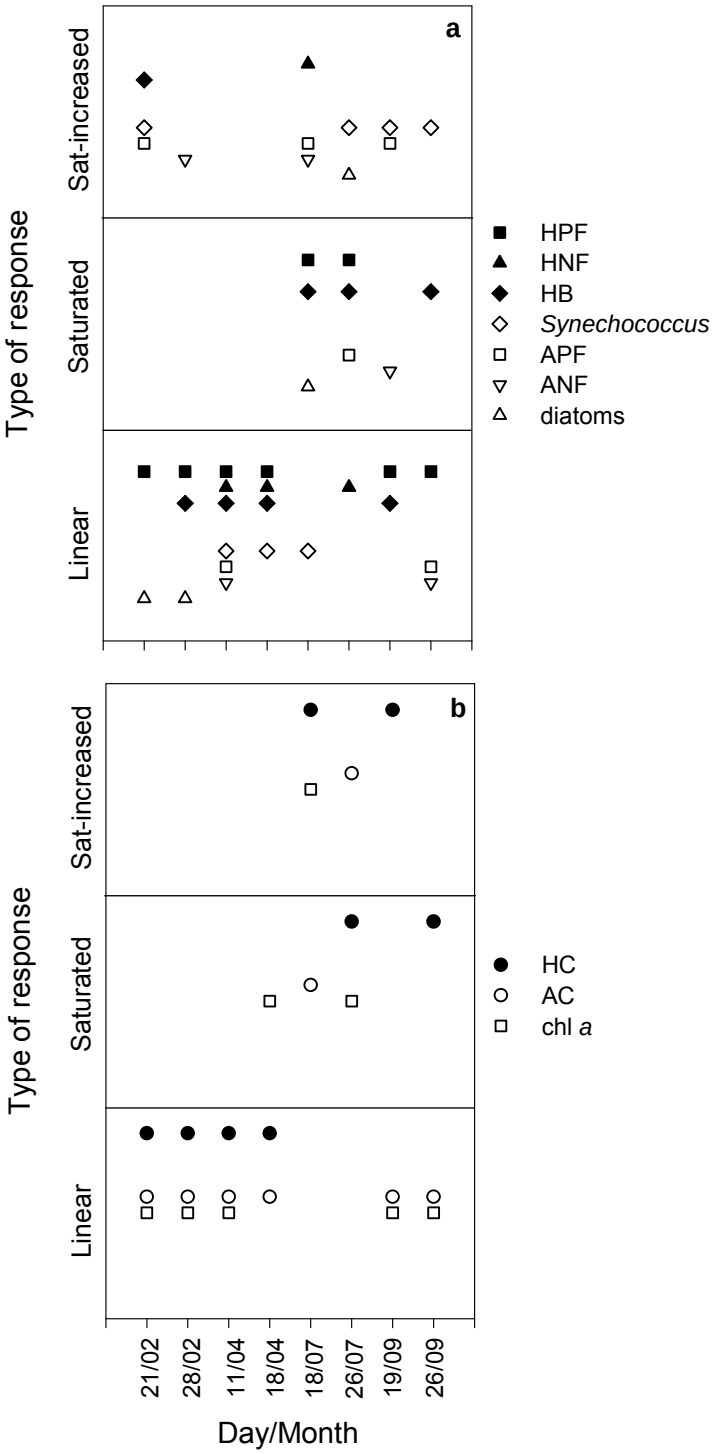

Fig. 2.3. Distribution of the 3 types of responses found in the 8 dilution experiments for (a) plankton groups and (b) bulk plankton properties. ANF, autotrophic nanoflagellates; APF, autotrophic picoflagellates; HNF, heterotrophic nanoflagellates; HPF, heterotrophic picoflagellates; HB, heterotrophic bacteria; AC, total autotrophic carbon; HC, total heterotrophic carbon. Y-axis is dimensionless.

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. 2.2 & 2.3, growth and mortality rates are given in section 3 - Tables 3.2 & 3.3).

In addition to linear (Figs. 2.2a-c) and saturated feeding responses (Figs. 2.2d-f), a third type of response (Figs. 2.2g-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. 2.3). 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 rate increases proportionally with the increase in food abundance (represented by the fraction of unfiltered seawater in equation 2.1). 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 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.

2.3.2. 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). 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.

Table 2.1. 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	Temperature	DIN	HPO_4^{2-}	Chl *a*	TC	Microzoo
	psu	ºC	µmol l^{-1}	µmol l^{-1}	mg m^{-3}	mg m^{-3}	%
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

Initial nutrient concentrations (Table 2.1) 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. 2.3). On September 19 the three responses occurred (Fig. 2.3). Besides, saturated and saturated-increased responses were also

obtained for heterotrophic plankton organisms in July (Fig. 2.3), 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 responses in our experiments, despite nutrient influence was not specifically assessed.

2.3.3. 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):

$$m_X = cZ_X \tag{2.3}$$

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.

(a) 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 2.2). 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. 2.3).

Table 2.2. 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

According to equation (2.3) 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. 2.4). Saturated-increased responses occurred when microzooplankton biomass did not change during the experiment (Fig. 2.4a, Table 2.2), as well as when there was an increase (Fig. 2.4b) or decrease (Fig. 2.4c) 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.

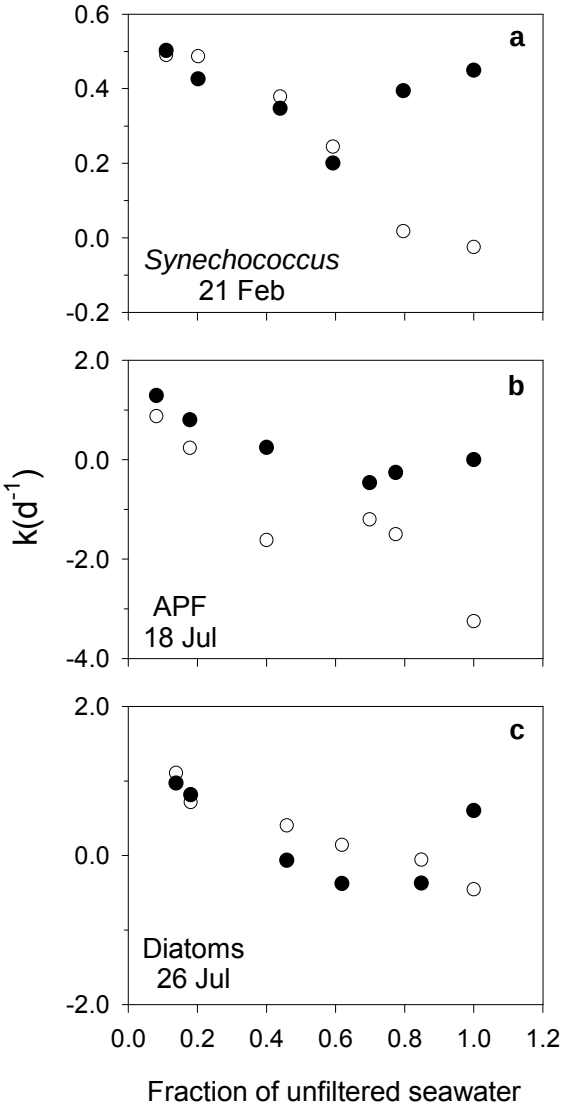

Fig. 2.4. Real (black circles) and simulated (open circles) responses of 3 dilution experiments. Simulated responses were made with the microzooplankton biomass at the end of the experiments and assuming a constant maximum clearance rate. These maximum clearance rates were estimated, using the equation (2.3) 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 minimum at the highest dilutions.*

(b) 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):

$$I = cP \qquad (2.4)$$

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.

$$I_{max} = cte = cP \qquad (2.5)$$

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 2.1) 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. 2.3), 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 2.1). 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 and diverse 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 2.1, see also section 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 (2.4) 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 ≥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 (2.4). Clearance rates were then used in equation (2.3) 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. 2.5) 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. 2.5a), the release of pressure on the more abundant prey B allows it to grow, whereas for the case of passive selection (Fig. 2.5b) 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.

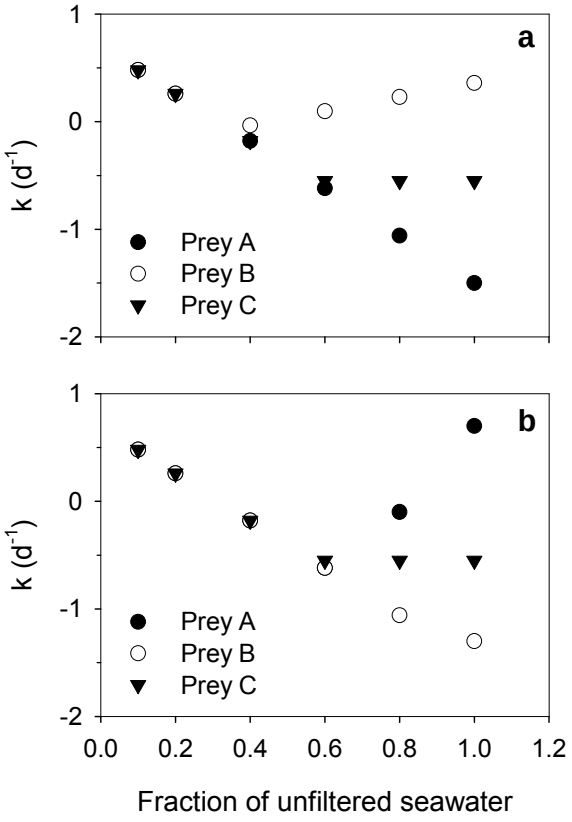

Fig. 2.5. Output of the simulations made with dilution experiments considering (a) active and (b) passive selection of preys. See text for details.

2.3.4. Some considerations on viral influence

It must be noted that in the present analysis the viral 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 viral 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.

2.3.5. 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. 2.3a) 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. 2.3a), because grazing on diatoms can release of predation other species. In situations with relatively low abundance of food, such as that in April, selection of food should be less feasible (Fig. 2.3a). In general, HB and HPF appear to be suitable food for microzooplankton, because responses (with the exception of HB on February 21) were linear or saturated (Fig. 2.3a). In contrast, *Synechococcus* frequently showed saturated-increased responses, mainly in summer and autumn (Fig. 2.3a). 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. 2.3b), which might be attributed to the different AC:chl *a* ratios of the several phytoplankton species and then result in different feeding responses for these two bulk properties.

2.3.6. 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.

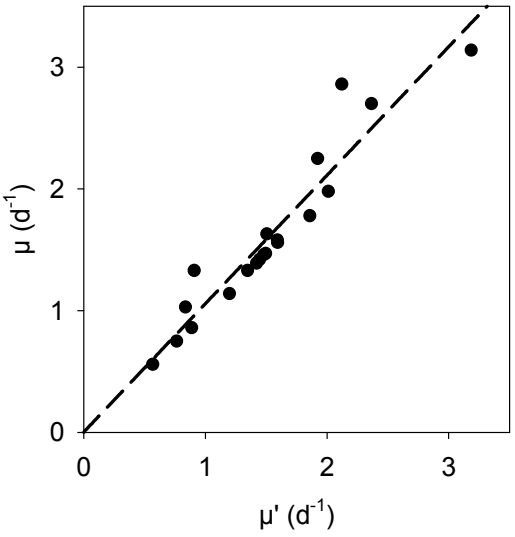

Fig. 2.6. Relationship between the specific growth rates estimated from the regressions of the linear part in non-linear responses of dilution experiments (μ) and the specific growth rates estimated considering the final concentration of microzooplankton (μ') (see also Fig. 2.4 and Table 2.2).

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 2.4, that is, using final microzooplankton biomasses and assuming maximum clearance rates (Fig. 2.6). 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).

3.

MICROZOOPLANKTON FEEDING IMPACT IN THE COASTAL UPWELLING SYSTEM OF THE RÍA DE VIGO (NW IBERIA)*

ABSTRACT

The dilution technique, combined with identification and enumeration of pico-, nano- and microplankton by microscopy, was used to estimate the impact of microzooplankton on the several groups of the plankton community in the Ría de Vigo, a coastal embayment on the NW Iberian upwelling system. Microzooplankton revealed as a significant consumer of autotrophic and heterotrophic plankton in this system, where it can feed up to 93% of standing stocks and more than 100% of productions. Heterotrophic bacteria and heterotrophic picoflagellates experienced the highest and constant impact, with on average 75-84% of their standing stocks and 85-102% of their productions being channelled through the microbial food web. Pico- and nanophytoplankton were also consumed, although maximum grazing occurred on diatoms during upwelling events, coinciding with highest primary production. Predation on pico-nanoheterotrophs was especially relevant under downwelling conditions, when the total carbon consumed was considerably lower than during upwelling. The results suggest that the existence of a multivorous food web, extending from the microbial loop to the herbivorous food web, could be a major feature in coastal upwelling systems. The microbial loop, which occurs as a permanent background in the system, would contribute to maintain the microbial food web during downwelling, while the herbivorous food web could coexist with a microbial food web based on large diatoms during upwelling. The multivorous food web would hence divert diatoms from sinking and favour the retention of organic matter in the water column, which could enhance the energy transfer to higher pelagic trophic levels in coastal upwelling systems.

*The research work presented in this section is also a contribution to the paper:

Teixeira IG, Figueiras FG, Crespo BG & Piedracoba S (submitted to Journal of Plankton Research) Microzooplankton feeding impact in the coastal upwelling system of the Ría de Vigo (NW Iberia).

3.1. INTRODUCTION

While the role of heterotrophic nano- and microplankton (hereafter microzooplankton) is widely recognised in oligotrophic regions of the ocean, where it plays a fundamental function transferring matter and energy from the dominant small-sized organisms to large consumers (Ryther 1969), its importance in coastal upwelling systems has been classically undervalued. Traditionally, it has been accepted that short food chains, in which large phytoplankton can pass directly to zooplankton and then to larger animals, prevail in coastal upwelling systems. However, microzooplankton is abundant in upwelling regions, and information on its importance has considerably increased during the last decades (Painting et al. 1992; Neuer & Cowles 1994, García-Pámanes & Lara-Lara 2001, Vargas & González 2004). Thus, it is now well known that microzooplankton not only feeds on small phytoplankton, because it also impacts on communities dominated by large phytoplankton (Calbet 2008). Particularly, heterotrophic dinoflagellates are now considered as major herbivores of large and chain-forming diatoms (Sherr & Sherr 2007). Moreover, microzooplankton can consume heterotrophic plankton, bacteria or other phagotrophic organisms (Azam et al. 1983, Rassoulzadegan & Sheldon 1986, Jeong 1999).

Microzooplankton, through these complex trophic interactions within the microbial food web can modulate biogeochemical fluxes in coastal upwelling systems. Short food chains, owing to the few steps involved, are more efficient than microbial food webs transferring energy to higher trophic levels, but they are also responsible of removal a large amount of material from the photic layer via rapid sinking of large diatoms and faecal material from large metazoans (Turner 2002). Therefore, the co-occurrence of the two trophic ways or the existence of a “multivorous food web” (Legendre & Rassoulzadegan 1995), in which microzooplankton is a key player, could contribute to reduce carbon losses from the photic layer, while still retaining enough efficiency in the energy transfer to high trophic levels. To know the role that the microzooplankton plays in coastal upwelling systems is hence fundamental to advance in our understanding of carbon fluxes in these highly productive oceanic areas.

Reports on the importance of microzooplankton in the Iberian upwelling are scarce. Despite some studies, through indirect approaches, suggest that microzooplankton activity in this upwelling area must be important (Figueiras & Ríos 1993, Bode & Varela 1994, Bode et al. 2004), estimates of microzooplankton grazing activity were only determined by Fileman & Burkill (2001). The Ría de Vigo (Fig. 1) is a bay on the Galician coast (NW Iberia) where coastal upwelling, induced by northerly winds, introduces subsurface nutrient-rich water through the bottom from spring to autumn. During the rest of the year, the dominant southerly winds cause downwelling (Fraga 1981). Relaxation

and even opposite events can however occur within each season, as response to short-time variations in the wind regime driven by small fluctuations in the large-scale climatology of the North Atlantic. Plankton composition in this system is that typical of temperate coastal regions, but it is also influenced by the hydrographic variability imposed by upwelling-downwelling events (Figueiras et al. 2002). Thus, large diatoms are abundant in spring, whereas in summer the plankton community is composed of heterotrophic and autotrophic organisms, with autotrophy (diatoms) dominating during upwelling events and heterotrophy (dinoflagellates and ciliates) attaining greater importance during relaxations. Large pigmented dinoflagellates, sometimes forming toxic blooms, are common in autumn, while small flagellates dominate in winter. Pico- and nanophytoplankton are present in the system all through the year, though their contribution to the plankton community is higher in winter, because peaks of biomass during upwelling are due to the increase in the abundance of diatoms (Figueiras et al. 2002, Arbones et al. 2008). Therefore, it could be expected that this high variability in plankton composition and size structure affects carbon fluxes in this coastal upwelling system.

The aim of this work was to quantify for the first time in the coastal upwelling system of the Ría de Vigo (NW Iberia), the feeding impact of microzooplankton on the several autotrophic and heterotrophic plankton groups (≤200 µm) under different hydrographic conditions. It was achieved by performing dilution experiments (Landry & Hassett 1982) associated with identification and enumeration of plankton components by microscopy.

3.2. MATERIALS AND METHODS:

The experimental procedure and analyses are the same described in section 2.

3.2.1. Growth and mortality rates

Instantaneous growth (μ, d^{-1}) and mortality (m, d^{-1}) rates for each plankton group, chl *a*, total carbon biomass (TC), total autotrophic carbon biomass (AC) and total heterotrophic carbon biomass (HC) were estimated by linear regression of the correspondent net growth rates k (d^{-1}) against the dilution factor X (Landry & Hassett 1982):

$$k = \mu - mX \tag{3.1}$$

In cases of saturated and saturated-increased feeding responses, μ was obtained by regression of the linear part of the response and m was calculated by the difference between μ and the net growth rate in the undiluted sample (see section 2).

The quantity of carbon and chl *a* consumed (*G*, mg $m^{-3}d^{-1}$) and produced (*P*, mg $m^{-3}d^{-1}$) were calculated as:

$$G = m \times C_m \tag{3.2}$$

$$P = \mu \times C_m \tag{3.3}$$

where C_m (mg m^{-3}) is the average chl *a* or carbon biomass during incubation time and is calculated as:

$$C_m = C_0 \left[e^{(\mu - m)t} - 1 \right] / (\mu - m)t \tag{3.4}$$

Therefore the daily impact on production (*%P*, d^{-1}) can be estimated as:

$$\%P = \frac{G}{P} \times 100 = \frac{m}{\mu} \times 100 \tag{3.5}$$

The impact on the standing stock (*%SS*, d^{-1}) was obtained as:

$$\%SS = (1 - e^{-m}) \times 100 \tag{3.6}$$

3.3. RESULTS

3.3.1. Hydrography

The rapidly changing hydrographic conditions commonly observed in the region were also found during the four sampling periods. Thus, upwelling which characterised the first two days of sampling in February (Figs. 3.1a-c), quickly reverted to downwelling on the third day (February 25) to persist until the end of the sampling. The two dilution experiments of this month were done under these two contrasting conditions. The opposite situation occurred in April (Figs. 3.1d-f), when the water column at the beginning of sampling was still responding to a previous downwelling event. Then, after a weak upwelling event, which did not reach the surface, the water column became stratified. Again, the two dilution experiments were performed under these two different environmental conditions. July (Figs. 3.1g-i) showed a stratified water column in which a

short relaxation separated two upwelling events, during which the dilution experiments were done. Downwelling was persistent during the sampling of September (Figs. 3.1j-l). Although nitrate concentrations in the surface layer were <1 μmol l^{-1} in April and September, concentrations of total inorganic nitrogen <1 μmol l^{-1} were only recorded in April (section 2). Further details on the hydrographic conditions can be found in Piedracoba et al. (2005).

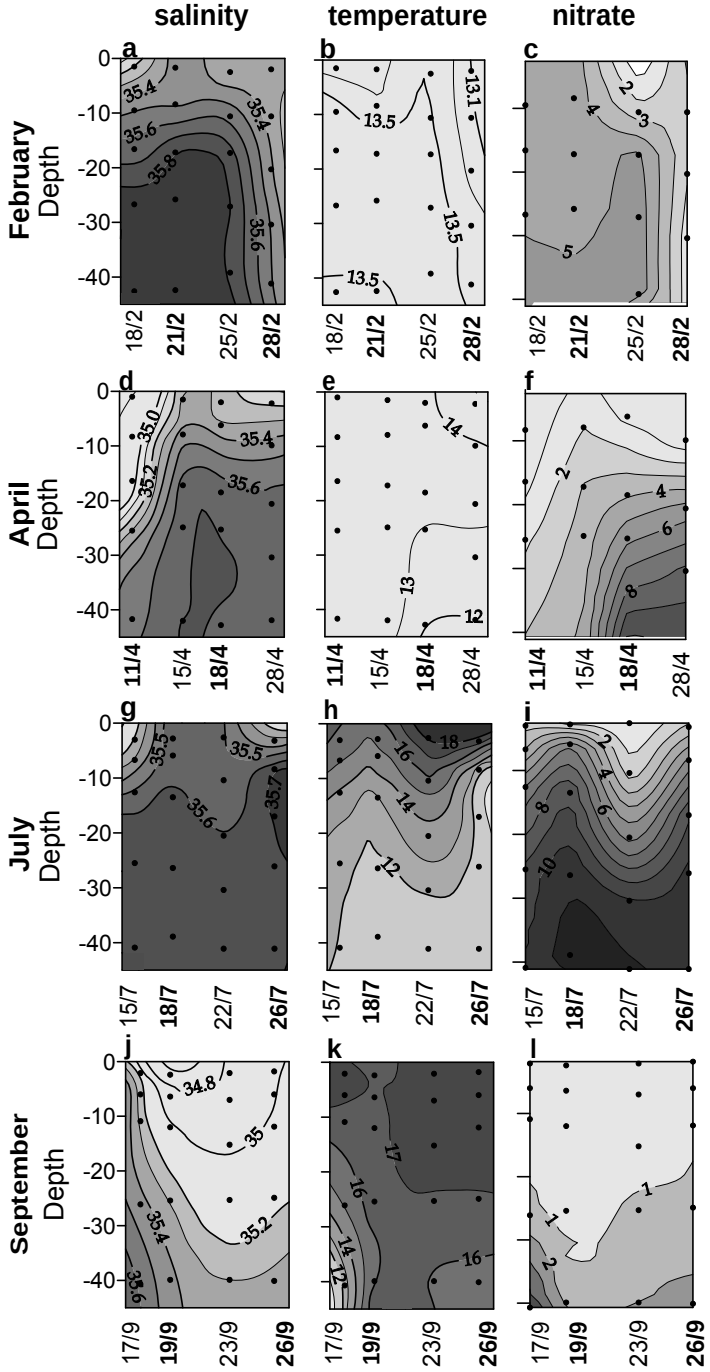

Fig. 3.1. Variations in salinity (psu), temperature (ºC) and nitrate concentration (μmol l^{-1}) in the water column during the four sampling periods: February, April, July and September 2002. The days when the dilution experiments were performed are in bold.

3.3.2. Plankton biomass and composition

Total plankton (≤200 µm) biomass, though variable, showed a clear seasonal trend (Fig 3.2a), with low values in winter (February) and high values in summer (July) and the beginning of autumn (September). The highest biomass (819 mg C m^{-3}) was recorded during the first upwelling event of July, while the lowest (84 mg C m^{-3}) coincided with the downwelling of the end of February. The plankton community was dominated by autotrophs (67 ± 13% of the total plankton biomass) during the four sampling periods, although autotrophy was more evident in summer and autumn (76 ± 12% in July and September) than in winter and spring (58 ± 5% in February and April). Changes in total plankton biomass were caused by variations in autotrophic biomass (slope = 1.07 ± 0.09, $r^2 = 0.96$, $p < 0.001$), because changes in heterotrophic biomass were not significant.

Diatoms plus autotrophic nanoflagellates (ANF) and autotrophic dinoflagellates (ADF) accounted for the largest fraction (84-99%) of the autotrophic biomass (Fig. 3.2b). Diatoms, which were always present, were especially abundant in the upwelling of July, representing >90% of the autotrophic biomass. ADF, present since April (2-3 mg C m^{-3}), attained the highest biomass during the downwelling of September, when accounted for 44% and 25% of the total autotrophic biomass on September 19 and 26, respectively. Biomass of ANF (30 ± 17 mg C m^{-3}) and their contribution to autotrophic biomass (27 ± 5%) was higher in February and April than in July and September (13 ± 5 mg C m^{-3} and 4 ± 3%, respectively). Synechococcus-type cyanobacteria and autotrophic picoflagellates (APF) only accounted for a very small fraction of the total autotrophic biomass, 2 ± 2% and 4 ± 5% respectively (data not shown). Although chl a followed a similar evolution to that of AC (Fig. 3.2b), both variables were not significantly correlated ($r = 0.65$; $p = 0.08$), reflecting the variable AC:Chl a ratios which fluctuated between a minimum value of 19 on April 11 and a maximum value of 106 on July 18.

The diatom community (Table 3.1) was dominated by *Skeletonema* cf. *costatum* on February 21, but changed to dominance of other larger chain-forming species (*Thalassiosira rotula* and *Chaetoceros* spp.) on February 28. *Chaetoceros* spp., *Pseudo-nitzschia* cf. *seriata*, *Detonula pumila* and *T. rotula* were the more abundant species in April. Small chain-forming diatoms (*Leptocylindrus danicus* and small *Chaetoceros* spp.) accounted for 74% of the total carbon biomass (606 mg C m^{-3}) on July 18. The same species remained abundant on July 26. *Proboscia alata* dominated on September 19, while *Skeletonema* cf. *costatum*, *Chaetoceros* spp., *Leptocylindrus danicus* and *Thalassiosira nana* were abundant on September 26. The large pigmented species *Ceratium fusus* and *C. furca* were especially abundant in September, when dominated the ADF community (Table 3.1).

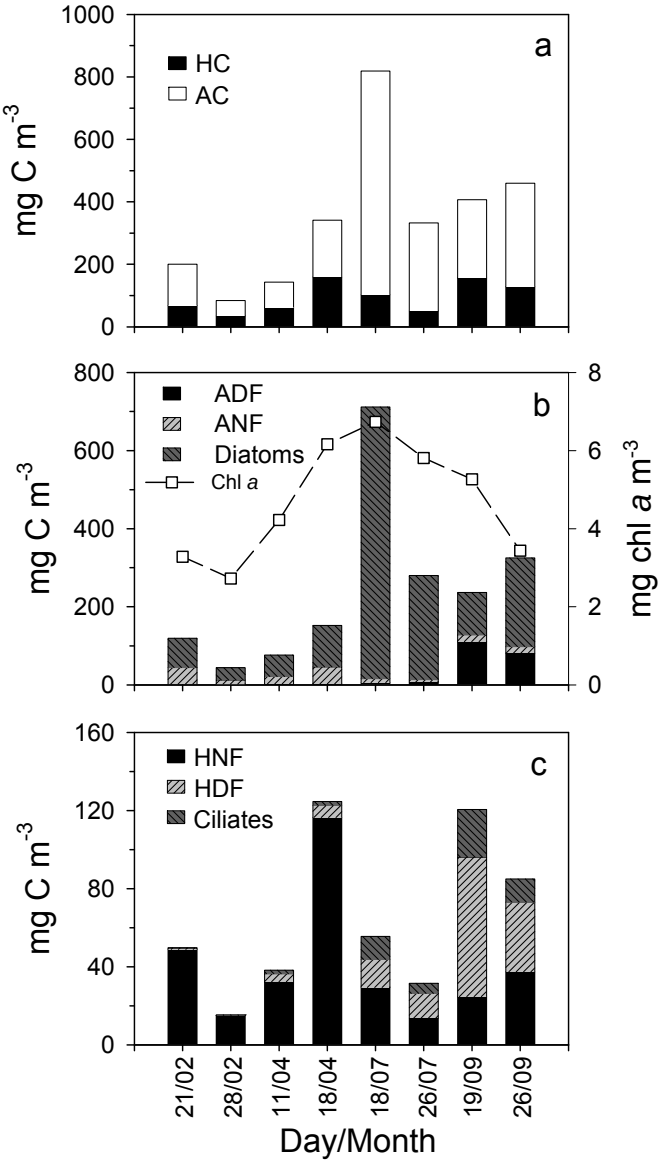

Fig. 3.2. Initial concentrations in the dilution experiments of (a) total heterotrophic and autotrophic carbon, (b) chl *a* and carbon of the main autotrophic plankton groups, and (c) carbon of the main heterotrophic plankton groups. HC, total heterotrophic carbon; AC, total autotrophic carbon; ADF, autotrophic dinoflagellates; ANF, autotrophic nanoflagellates; HNF, heterotrophic nanoflagellates; HDF, heterotrophic dinoflagellates.

Table 3.1. Initial biomass (mg C m^{-3}) of the plankton species or groups more abundant for each experiment. ADF, autotrophic dinoflagellates; HDF, heterotrophic dinoflagellates; Un., unidentified.

Plankton species or groups	21/02	28/02	11/04	18/04	18/07	26/07	19/09	26/09
Diatoms								
Chaetoceros spp.		4.19	15.11	54.71	283.11	219.39	2.84	53.09
Detonula pumila			10.92	8.01				
Guinardia delicatula			0.96	1.21	68.44	3.23		0.95
Leptocylindrus danicus			0.90	2.67	319.92	20.51	17.43	41.02
Proboscia alata					0.99	0.99	79.82	26.32
Pseudo-nitzschia cf. *seriata*			12.10	12.26				0.70
Skeletonema cf. *costatum*	71.81	0.64						56.16
Thalassiosira nana	2.02		0.77	1.96	1.78		1.49	41.91
Thalassiosira rotula		25.56	8.69	19.36		0.88		0.73
ADF								
Ceratium furca		0.04					68.08	31.24
Ceratium fusas			0.06		0.30	0.07	28.43	35.74
Amphidoma caudata		0.11						
Goniodoma sphaericum	0.07							
Gymnodinium agiliforme	0.08		0.33		0.84	5.85	0.05	0.05
Gymnodinium cf. *varians*				1.79	0.83	0.80	3.06	1.76
Scrippsiella trochoidea			0.29	0.05	1.52	0.19	0.34	0.10
Un. naked dinoflagellate <50µm	0.12	0.04	1.37	0.33	0.83			6.63
HDF								
Dinophysis acuminata					1.11		46.01	4.45
Gyrodinium spp.	0.03	0.01	0.36	0.13	1.41	0.81	0.09	2.12
Gymnodinium spp. 20-50µm		0.01	0.05	2.41				
Noctiluca scintillans					7.82	8.69		5.21
Protoperidinium spp.	0.06	0.02	0.31	0.10	1.04	0.83	3.37	8.02
Gymnodinium spp. <20µm	0.46	0.41	1.70	2.63	0.77		8.84	2.32
Un. naked dinoflagellate <20µm			0.97	0.27		0.93	1.62	
Ciliates								
Un. aloricate choreotrichs >50µm			0.41	0.37	3.48			1.02
Un. aloricate choreotrichs 20-50µm			0.10	0.94	2.30	0.59	5.82	1.94
Un. aloricate choreotrichs <20µm	0.10	0.10	0.09	0.39	1.75	3.24	1.69	2.07
Strombidium spp.	0.02	0.08	0.68	0.25	1.25	0.84	12.16	5.50
Tintinnida	0.03						0.83	

Heterotrophic plankton biomass, which varied between 35 mg C m^{-3} on February 28 and ~160 mg C m^{-3} on April 18 and September 19 (Fig. 3.2a), was basically composed of heterotrophic nanoflagellates (HNF), heterotrophic bacteria (HB), heterotrophic dinoflagellates (HDF) and heterotrophic picoflagelates (HPF), with the four groups representing 93 ± 6% of the total heterotrophic biomass. While the biomasses of HB (17 ± 9 mg C m^{-3}) and HPF (13 ± 5 mg C m^{-3}) and their contributions to heterotrophic biomass (20 ± 9% and 14 ± 4%, respectively) were relatively constant (data not shown), those of HNF and HDF varied (Fig. 3.2c). HNF were more important in winter and spring (60 ±

15%), when they reached the highest biomass (116 mg C m^{-3} on April 18), than in summer and autumn (25 ± 6%; 26 ± 10 mg C m^{-3}). In contrast, the contribution of HDF was high in summer and autumn (28 ± 13%), attaining the highest biomass (71 mg C m^{-3}) on September 19. The biomass of HDF was low (0.11-0.6 mg C m^{-2}) in winter, but began to increase in spring (4-6 mg C m^{-3}) (Fig. 3.2c). Small naked species (<50 µm) dominated in spring and large forms (91 ± 5% of HDF biomass) were more abundant in summer and autumn (Table 3.1). *Noctiluca scintillans*, *Gyrodinium* spp. and *Protoperidinium* spp. were common in July, while *Dinophysis acuminata* and *Protoperidinium* spp. prevailed in September (Table 3.1). The biomass of heterotrophic ciliates was positively correlated with the biomass of HDF ($r = 0.96$; $p < 0.001$) (Fig. 3.2c), varying between ~0.20 mg C m^{-3} in winter and 24 mg C m^{-3} on September 19. Aloricate choreotrichs >20 µm (Table 3.1) were the major components, accounting for 77 ± 1% of the total biomass of ciliates.

Metazoa ≤200 µm were only present in very few samples at low abundance and their contribution to microzooplankton biomass and the dynamics of the microbial food web was not considered.

3.3.3. Growth and mortality rates

Growth and mortality rates of autotrophic (Table 3.2) and heterotrophic components (Table 3.3) of the plankton community were highly variable. Diatoms were grazed during the two upwelling events of February and July. Small autotrophs (ANF, APF and Synecchoccocus) were also grazed, even though not significant responses were found in some experiments. HB and HPF showed significant responses in all experiments, indicating that they were continuously consumed at relatively high rates ($m \geq 0.94$ d^{-1} for HB; $m \geq 1.27$ d^{-1} for HPF).

Rates obtained for chl *a* and autotrophic carbon (AC), both representing changes in the autotrophic community, were not always comparable. In the two experiments of February growth and mortality rates of chl *a* and AC were similar to the rates obtained for diatoms. Growth rates of chl a were extremely low in April ($0.07 \leq \mu \leq 0.09$ d^{-1}), contrasting with those of AC ($0.31 \leq \mu \leq 0.42$ d^{-1}). During the upwelling of July, when diatoms accounted for >90% of the AC and >80% of the TC (Figs. 3.2a & b), growth and mortality rates of AC (μ = 1.33, 1.35 d^{-1}; m = 1.10, 0.74 d^{-1}) and diatoms (μ = 1.33 d^{-1}; m = 1.13, 0.73 d^{-1}) were similar (Table 3.2) and also comparable to the rates obtained for TC (μ = 1.32, 1.43 d^{-1}; m = 1.04, 0.85 d^{-1}; Table 3.3). In contrast, rates of chl *a* were different (μ = 1.57, 2.15 d^{-1}; m = 0.58, 2.22 d^{-1}; Table 3.2). Growth and grazing rates of AC and chl *a* were also different during the downwelling of September (Table 3.2). Growth rates of autotrophs were generally higher than their mortality rates (Fig. 3.3a), whereas growth and mortality rates of heterotrophs were more tightly coupled (Fig. 3.3b), particularly those of HB and HPF.

Table 3.2. Growth (μ, d^{-1}) and mortality (*m*, d^{-1}) rates obtained from dilution experiments for autotrophic plankton; $p < 0.05$. APF, autotrophic picoflagellates; ANF, autotrophic nanoflagellates; AC, total autotrophic carbon; chl *a*, chlorophyll *a*; ns, not significant; [a]saturated response; [b]saturated-increased response. See text and section 2 for more details.

Date	*Synechococcus*			APF			ANF			Diatoms			AC			chl *a*		
(2002)	*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 3.3. Growth (μ, d^{-1}) and mortality (*m*, d^{-1}) rates obtained from dilution experiments for heterotrophic and total plankton; $p < 0.05$. HB, heterotrophic bacteria; HPF, heterotrophic picoflagellates; HNF, heterotrophic nanoflagellates; HC, total heterotrophic carbon; TC, total carbon; ns, not significant; [a]saturated response; [b]saturated-increased response. See text and section 2 for more details.

Date	HB			HPF			HNF			HC			TC		
(2002)	*m*	μ	r^2	*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	0.23	0.94	0.90[b]
28 Feb	1.31	2.24	0.92	1.41	1.25	0.66	ns	ns	ns	1.05	1.57	0.87	0.62	1.13	0.83
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	0.59	0.79	0.95
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	0.42	0.14	0.98[a]
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]	1.04	1.32	0.92[a]
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]	0.85	1.43	0.98[b]
19 Sep	1.82	2.01	0.99	1.88	1.39	0.90	ns	ns	ns	0.63	0.62	0.93	0.26	0.25	0.97
26 Sep	0.97	1.14	0.92[a]	2.15	1.95	0.91	ns	ns	ns	0.46	0.80	0.81	0.13	0.32	0.53[b]

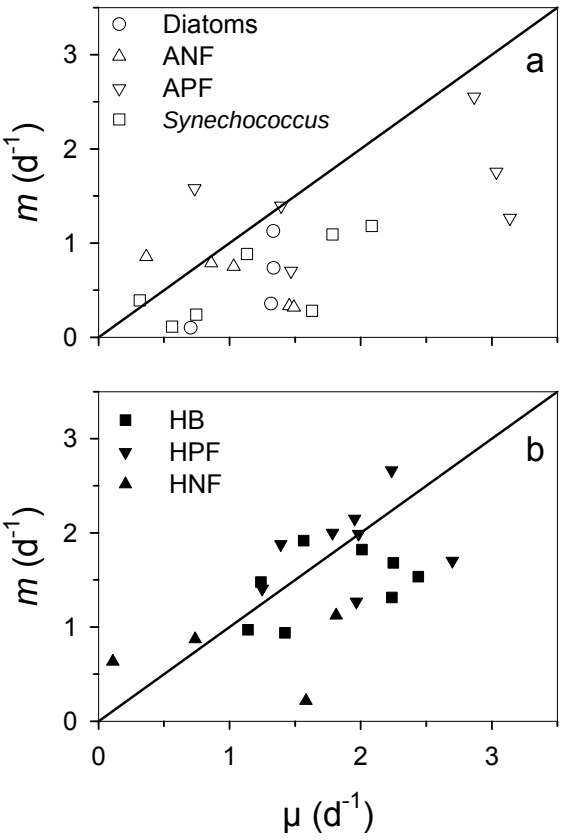

Fig. 3.3. Growth (μ) versus mortality (m) rates for (a) autotrophic and (b) heterotrophic organisms. ANF, autotrophic nanoflagellates; APF, autotrophic picoflagellates; HB, heterotrophic bacteria; HPF, heterotrophic picoflagellates; HNF, heterotrophic nanoflagellates. The lines represent the 1:1 relationship.

3.4.4. Impact of microzooplankton on plankton community

Carbon consumption and production (Figs. 3.4 & 3.5) derived from the rates obtained for TC, AC and HC (Tables 3.2 & 3.3) were not significantly different ($0.32 \leq p \leq 0.99$, t-test for two samples) from the corresponding estimates obtained by the addition of the carbon consumed and produced in the several plankton components with significant responses in the dilution experiments, suggesting that these plankton groups were those which were actually growing and were consumed.

The highest consumption of plankton biomass occurred during the upwelling of July, with 987 mg C m^{-3} d^{-1} being consumed in the first experiment and 383 mg C m^{-3} d^{-1} in the second (Fig. 3.4a). Most of this carbon was autotrophic (89% on July 18 and 73% on July 26), mainly diatoms (88% on July 18 and 70% on July 26) (Fig. 3.4b). TC consumed was considerably lower in the other experiments, varying between 125 mg C m^{-3} d^{-1} on April 18 and 66 mg C m^{-3} d^{-1} on February 21 and September 26 (Fig. 3.4a). Diatoms were also grazed during the upwelling of February 21 (45 mg C m^{-3} d^{-1}) (Fig. 3.4b), when they

accounted for 90% and 68% of the AC and TC consumed, respectively. In the other experiments, the AC consumed (1-25 mg C m^{-3} d^{-1}) corresponded to pico- and nanophytoplankton, which in general were consumed at very low rates (13 ± 8 mg C m^{-3} d^{-1}). Consumption of chl *a* did not follow that of AC (Fig. 3.4b), showing apparent deviations in April and July. These deviations were not only due to variations in AC:Chl *a* ratios, they were also caused by differences in the mortality rates (Table 3.2), which were not correlated.

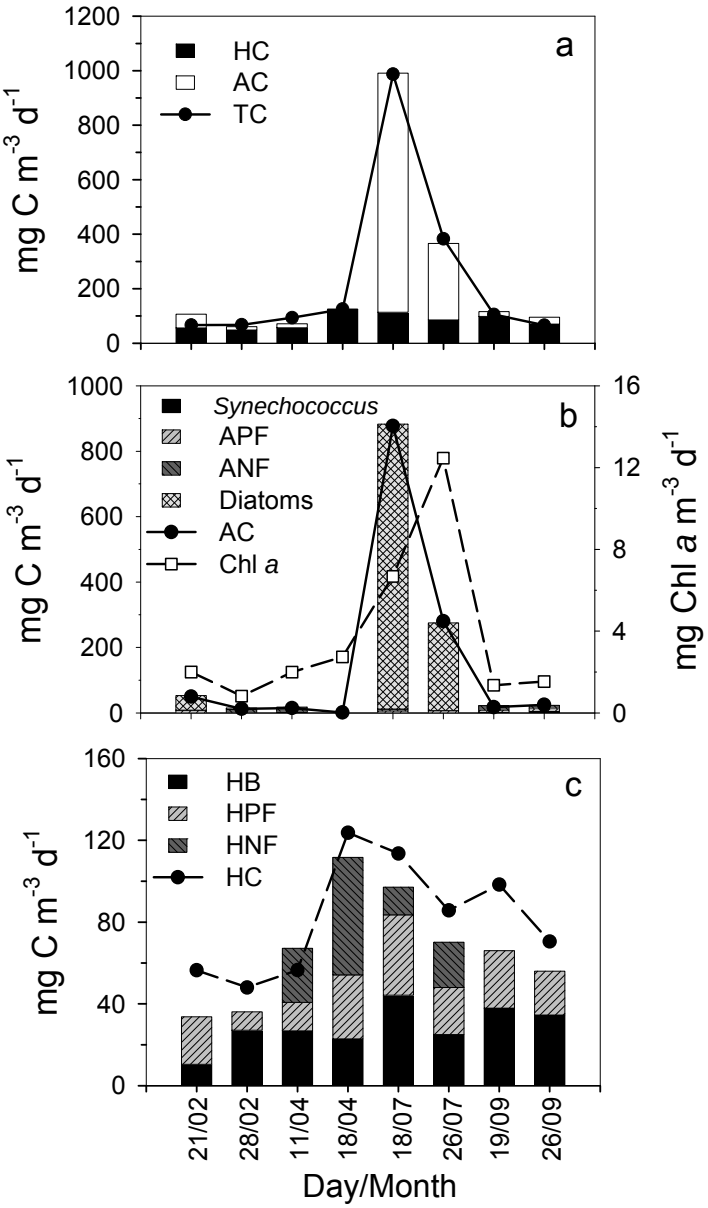

Fig. 3.4. Consumption of (a) total plankton carbon, (b) chl a and autotrophic plankton carbon and (c) heterotrophic plankton carbon. HC, total heterotrophic carbon; AC, total autotrophic plankton; TC, total carbon; APF, autotrophic picoflagellates; ANF, autotrophic nanoflagellates; HB, heterotrophic bacteria; HPF, heterotrophic picoflagellates; HNF, heterotrophic nanoflagellates.

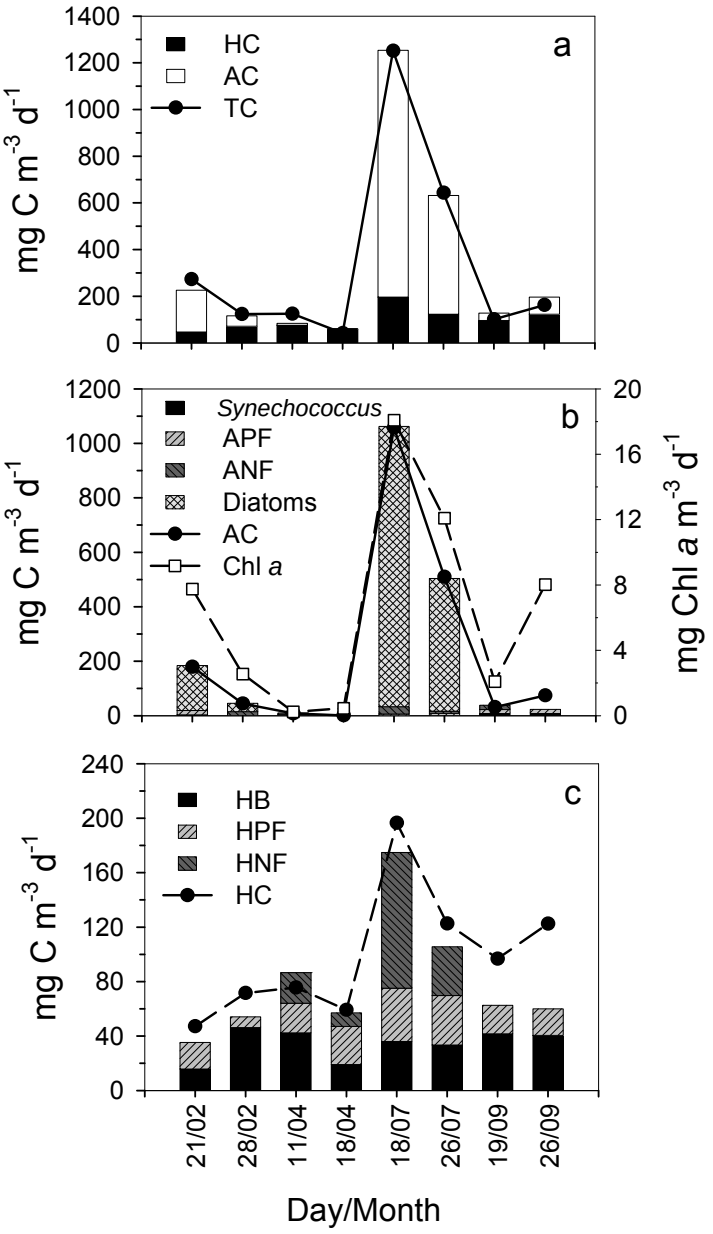

Fig. 3.5. Production of (a) total plankton carbon, (b) chl a and autotrophic plankton carbon and (c) heterotrophic plankton carbon. HC, total heterotrophic carbon; AC, total autotrophic plankton; TC, total carbon; APF, autotrophic picoflagellates; ANF, autotrophic nanoflagellates; HB, heterotrophic bacteria; HPF, heterotrophic picoflagellates; HNF, heterotrophic nanoflagellates.

Below the strong variations in carbon consumption caused by grazing on diatoms (Figs. 3.4a & b), there was a rather constant predation (67 ± 27 mg C m^{-3} d^{-1}) on heterotrophic carbon (Figs. 3.4a & c). It mainly occurred on HB (29 ± 10 mg C m^{-3} d^{-1}) and HPF (24 ± 10 mg C m^{-3} d^{-1}) with HNF being only consumed during April (26-57 mg C m^{-3} d^{-1}) and during the upwelling of July (14-22 mg C m^{-3} d^{-1}) (Fig. 3.4c). Predation on HC was especially relevant during non-upwelling conditions, when it represented >60% of the TC

consumed and >90% in some occasions (April 18, Fig. 3.4a). HB and HPF accounted for 81 ± 10% of the total picoplankton (autotrophic and heterotrophic) biomass consumed.

Consumption of TC (Fig. 3.4a) was strongly correlated (r^2 = 0.94, $p < 0.001$) with TC production (Fig. 3.5a), and it was basically due to the correlation between production and consumption of AC (r^2 = 0.97, $p < 0.001$), which in fact was due to the coupling between consumption and production of diatoms (r^2 = 0.97, $p < 0.001$). Thus, the upwelling of July showed the highest TC production, with 1251 and 643 mg C m^{-3} d^{-1} on July 18 and July 26 (Fig. 3.5a), when diatoms (Fig. 3.5b) accounted for 82% and 76% of the TC production, respectively. The contribution of diatoms (165 mg C m^{-3} d^{-1}) to AC production (179 mg C m^{-3} d^{-1}) and TC production (273 mg C m^{-3} d^{-1}) was also important during the upwelling of February 21 (Figs. 3.5a & b). Production of chl *a* and AC were correlated (r^2 = 0.86, $p < 0.001$) (Fig. 3.5b), and it was mainly due to the correlation between both growth rates (r^2 = 0.71, $p < 0.01$). Therefore, consumption and production of chl *a* were not correlated. Consumption and production of HC were also not correlated (Figs. 3.4c & 3.5c).

Table 3.4. Percentages of production daily removed by microzooplankton. ANF, autotrophic nanoflagellates; APF, autotrophic picoflagellates; chl *a*, chlorophyll a; AC, total autotrophic carbon; HNF, heterotrophic nanoflagellates; HPF, heterotrophic picoflagellates; HB, heterotrophic bacteria; HC, total heterotrophic carbon; na, not applicable.

Plankton group	21 Feb	28 Feb	11 Apr	18 Apr	18 Jul	26 Jul	19 Sep	26 Sep
Autotrophs								
Diatoms	27	14	na	na	85	55	na	na
ANF	na	73	234	na	23	0	92	21
APF	48	na	216	na	100	89	40	58
Synechococcus	20	na	57	124	78	17	32	61
chl *a*	26	32	871	617	37	103	65	19
AC	28	28	184	124	83	55	56	33
Heteterotrophs								
HNF	na	na	118	578	14	62	na	na
HPF	119	113	65	112	100	63	135	110
HB	66	59	63	119	123	75	91	85
HC	120	67	75	209	58	70	102	58
Total Carbon	24	55	75	299	79	60	104	41

Despite the strong correlations between primary production and grazing, the impact of microzooplankton on primary production was highly variable (Table 3.4). Grazing, although low (Fig. 3.4b), largely exceeded primary production during the downwelling of April, when primary production was also extremely low (1-8 mg C m^{-3} d^{-1}) (Fig. 3.5a), and grazing occurred on pico- and nanophytoplankton. Thus, when these two experiments are not considered, the impact of microzooplankton on AC production decreases from a mean value of 74 ± 55% (range 28-184%) to 47 ± 22% (range 28-83%) (Table 3.4). The impact

on the production of chl *a* also showed a marked decrease after removing these two experiments, from 221 ± 331% (range 19-871%) to 47 ± 32% (range 19-103%).

Between 58% and 209% (average 95 ± 51%) of the HC production was consumed by microzooplankton (Table 3.4). The highest percentage was recorded on April 18, when consumption on AC (1 mg C m^{-3} d^{-1}) was the lowest (Fig. 3.4b) and almost all consumption (125 mg C m^{-3} d^{-1}) occurred on HC (Fig. 3.4a). Between 63 and 135% (average 102 ± 26%) of the HPF production and between 59 and 123% (average 85 ± 25%) of the HB production were removed by microzooplankton (Table 3.4). Considering all plankton biomass, microzooplankton consumed between 24 and 299% (average 92 ± 87%) of TC production.

The impact on the standing stocks was less variable (Table 3.5), with ~40% of the stocks of AC, chl *a* and TC being daily removed by microzooplankton. The standing stock of HC was slightly more affected (57 ± 11%), and again HPF (84 ± 7%) and HB (75 ± 9%) experienced the highest impact.

Table 3.5. Percentages of the standing stocks daily removed by microzooplankton. ANF, autotrophic nanoflagellates; APF, autotrophic picoflagellates; chl *a*, chlorophyll a; AC, total autotrophic carbon; HNF, heterotrophic nanoflagellates; HPF, heterotrophic picoflagellates; HB, heterotrophic bacteria; HC, total heterotrophic carbon; na, not applicable.

Plankton group	21 Feb	28 Feb	11 Apr	18 Apr	18 Jul	26 Jul	19 Sep	26 Sep
Autotrophs								
Diatoms	30	9	na	na	68	52	na	na
ANF	na	53	57	na	28	0	55	27
APF	50	na	79	na	75	92	72	83
Synechococcus	11	na	69	32	59	24	21	66
Chl *a*	30	21	46	43	44	89	21	22
AC	30	19	54	32	67	52	39	44
Heteterotrophs								
HNF	na	na	58	47	19	68	na	na
HPF	93	76	72	86	86	82	85	88
HB	61	73	78	77	85	81	84	62
HC	58	65	55	63	56	72	47	37
Total Carbon	20	46	45	34	65	57	23	12

3.4. DISCUSSION

The hydrographic conditions and the associated plankton communities observed during the four sampling periods were representative of the typical seasonal cycle in the Ría de Vigo (Figueiras & Ríos 1993). Overall, major variations in plankton biomass occurred due to variations in autotrophic biomass (Fig. 3.2). Specifically, diatoms were the main responsible for the peaks of biomass and primary production recorded in response to

upwelling events, which agrees with prior reports for this area and the established picture of phytoplankton dynamics, according to which nutrient inputs cause major variations in phytoplankton through the addition of large size classes (Chisholm 1992, Cermeño et al. 2006, Arbones et al. 2008). Among heterotrophs, picoheterotrophic organisms showed a constant background of biomass, while microzooplankton presented a seasonal succession also typical of this system (Figueiras & Ríos 1993). Thus, large forms of heterotrophic dinoflagellates and ciliates appeared through summer and autumn, while small flagellates were relatively more important in winter and spring (Fig. 3.2, Table 3.1). Therefore, our experiments can be considered as representative of the several environmental conditions regularly found in the Ría de Vigo.

3.5.1. Microzooplankton impact on phytoplankton:

Although both chl *a* and AC are estimates of phytoplankton biomass, we obtained different growth and mortality rates calculated by changes in these two variables. Moreover, growth rates of chl *a* and AC followed the same pattern but mortality estimates were not correlated at all. Several reasons can be behind these differences, and it is difficult to undoubtedly explain their occurrence. First, they could result from the different AC:chl *a* ratios that were observed during the samplings (Fig. 3.2b). Second, the different phytoplankton species can contribute with different percentages to the AC and chl *a* pools, and the selective grazing on some phytoplankton groups or species could decouple the overall mortality estimates derived from both variables (section 2). Finally, the use of pigments as an index for changes in phytoplankton community has some inherent problems, mainly related to the incomplete degradation of chl a inside predators at the beginning or the end of the incubation (e.g. Barlow et al. 1988, Waterhouse & Welschmeyer 1995).

Despite the obvious differences between the rates estimated through changes in chl *a* and AC, the mean impact of microzooplankton for all experiments on the phytoplankton standing stock (~40%) and primary production (47%, excluding the April experiments) were very similar for both variables. These values also compare well with other estimates reported for coastal waters (e.g. Gallegos 1989, Calbet & Landry 2004) and coastal upwelling systems (Neuer & Cowles 1994, Vargas & González 2004, Vargas et al. 2007), including those estimates found during an upwelling/relaxation event along the NW Iberian shelf (Fileman & Burkill 2001).

Although pico- and nanoplankton were predated, the total carbon biomass consumed by microzooplankton was tightly coupled with primary production, which in the Ría de Vigo was basically due to diatoms (Fig. 3.5). Consequently, highest consumption occurred during upwelling conditions and on diatoms (Fig. 3.4), which agrees with prior evidences

found for other coastal upwelling systems (Neuer & Cowles 1994, Vargas et al. 2007) Grazing on diatoms, mainly on large and chain-forming species has been attributed to large heterotrophic dinoflagellates, which are capable to consume organisms larger than themselves (Sherr & Sherr 2007). The high grazing on diatoms during the upwelling of July (Fig. 3.4b) coincided with the presence of large heterotrophic dinoflagellates (*Noctiluca, Protoperidinium, Gyrodinium* spp. etc) in the microplankton community, an association which has been found in other occasions when grazing on chain-forming diatoms occurred (e.g. Neuer & Cowles 1994, Strom & Strom 1996, Kim et al. 2007). Some authors refer that ciliates would also feed on diatoms (e.g. Paranjape 1990, Aberle et al. 2007). Hence we cannot discard that part of the high consumption of diatoms observed in July was due to ciliates, when large aloricate choreotrichs were present in the microzooplankton community (Table 3.1). In contrast, grazing on diatoms in February could hardly be attributed to these large dinoflagellates and ciliates, because their abundances were very low in a microzooplankton community dominated by HNF (Fig. 3.2c). However, it has also been reported that HNF are able to feed on diatoms of their same size range (Goldman and Caron 1985), and though the dominant diatom on February 21 was the chain-forming *Skeletonema* cf. *costatum*, its cells are small (~5µm) and without protrusions, spines or other formations that could make difficult feeding. Therefore, we could tentatively conclude that *S.* cf. *costatum* on February 21 was consumed by HNF.

3.5.2. Microzooplankton impact on heterotrophic plankton:

The role of microzooplankton as consumers of heterotrophic plankton has long been recognized (Azam et al. 1983). Bacterivory (and predation of small bacterivores), transfers through the food web the dissolved organic compounds released into the medium by biological processes and assimilated by HB. Despite the importance of this process for the cycling of matter in marine systems, the microzooplankton impact on heterotrophs is not frequently quantified. Previous studies in the coastal upwelling system of Chile, using a modelling approach, suggested that microzooplankton can consume a significant fraction of heterotrophs (Vargas & González 2004, Vargas et al. 2007). Here, we directly measured this consumption through the dilution technique and effectively observed that microzooplankton consumed an important part of the heterotrophic biomass in the coastal upwelling system of the Ría de Vigo. Predation on HB and HPF was relatively important and constant (Fig. 3.4c), indicating that large fractions of their standing stocks and productions were channelled through the microbial food web (Tables 3.4 & 3.5). In fact, this tight coupling could explain the relative constant biomasses of HB and HPF found over the year in this upwelling system, suggesting an efficient top-down control on these

organisms by microzooplankton. Small flagellates are considered the main bacterivores, but ciliates and heterotrophic dinoflagellates can also consume HB (Fenchel 1982b, Lessard & Swift 1985, Rassoulzadegan & Sheldon 1986). As at least one of these bacterivores was present in the microzooplanktonic community of the Ría de Vigo (Fig. 3.2c), HB could be always consumed. HB can also be controlled by HPF (Rassoulzadegan & Sheldon 1986, Calbet et al. 2001), but in the Ría de Vigo both groups were heavily consumed, which impedes to infer definitive conclusions about to what extent HB were consumed by HPF. Alternatively, the small size of HPF would permit their control by the same groups controlling HB (Lessard & Swift 1985, Rassoulzadegan & Sheldon 1986, Calbet et al. 2001). In addition, HNF were consumed in April and July, coinciding with the increase in the biomass and size of ciliates and heterotrophic dinoflagellates (Fig. 3.2c, Table 3.1), two groups that ingest small flagellates (Verity 1991, Jeong 1999). In fact, predation on HNF was important during April (Fig. 3.4c), when consumption of phytoplankton was extremely low (Fig. 3.4b), and the microbial food web was largely sustained by heterotrophs (Fig. 3.4a).

3.5.3. Conclusion

These results demonstrate the importance of microzooplankton in the consumption of other plankton organisms in the Ría de Vigo. Microzooplankton not only feeds pico- and nanoplankton, it also consumes large diatoms, and in this way contributes to establish a multivorous food web in coastal upwelling systems (Legendre & Rassoulzadegan 1995). This multivorous food web probably extends from the microbial loop to the herbivorous or classical food web. A rather constant carbon flow through the microbial loop was present in the system as a permanent background. Therefore, the microbial loop was relatively more important during non-upwelling conditions, when predation on autotrophs was very low or nil, and the microbial web was basically maintained by pico- and nanoheterotrophs. A microbial food web based on large diatoms could coexist with the classical food web and the microbial loop during upwelling. Despite the fate of phytoplankton blooms in coastal upwelling systems is largely controlled by hydrodynamics, the multivorous food web should facilitate the retention of organic matter in the water column through limiting sinking, which in turn should enhance the energy transfer to higher pelagic trophic levels. Once the multivorous food web seems to be a common feature in coastal upwelling systems (Neuer and Cowles 1994, Vargas & González 2004, Vargas et al. 2007), the microzooplankton-associated pathway, which channels a significant part of plankton biomass, should be considered as an important component of the pelagic food webs in these systems.

4.

STRATIFICATION-MIXING CYCLES AND PLANKTON DYNAMICS IN A SHALLOW WATER SYSTEM: IMPLICATIONS FOR BENTHIC-PELACIG COUPLING*

ABSTRACT

The biomass and production of phytoplankton and bacteria and the feeding impact of microzooplankton on the plankton community in a shallow water system in Denmark (Limfjord) were analysed during a 9-day period. During the campaign, the water column changed between stratified and mixed conditions, which influenced the dominant processes in the pelagic system. During strong stratification, phytoplankton was mainly controlled by microzooplankton grazing. A mixing event, which homogenised the water column, provided food to a benthic community dominated by mussels and also affected microzooplankton, which decreased its feeding impact. The nutrient input occurring during mixing and the subsequent stabilization of the water column provided the ideal conditions for the recovery of phytoplankton. This recovery was accompanied by a change in phytoplankton species composition and an increase in microzooplankton grazing. Microzooplankton, which was also a significant consumer of bacteria during all sampling period, however was not the only consumer controlling phytoplankton. Although the microbial food web was an important route for total plankton carbon during all sampling period, with ~60% of the standing stock and ~100% of production being daily consumed by microzooplankton, the classical food web was favoured under mixing conditions, when the benthic-pelagic coupling was stronger. Stratification-mixing cycles, occurring during short-time periods, appear to be a key mechanism to maintaining the equilibrium of the biological communities in this system.

*The research work presented in this section is also a contribution to the paper:
Teixeira IG, Crespo BG, Nielsen TG & Figueiras FG (to be submitted) Stratification-mixing cycles and plankton dynamics in a shallow water system: implications for benthic-pelagic coupling.

4.1. INTRODUCTION

Phytoplankton is controlled by physical (e.g. light and water motion), chemical (e.g. nutrients) and biological (e.g. predation) factors that act simultaneously regulating the species composition and their succession in the water column (e.g. Smayda 1980). Among all these factors, turbulence and nutrient availability have been assigned as the most important (Margalef 1978). Thus, it is well established that under mixed and nutrient-replete conditions there is a dominance of large and non-motile species, namely diatoms, while in stratified and nutrient-depleted waters small motile forms, as small flagellates, are favoured. Phytoplankton community composition is, in turn, a determinant factor influencing the trophic pathways in the pelagic system (Legendre & Rassoulzadegan 1996). In stratified water columns, with dominance of small phytoplankton, remineralization processes within the microbial food web predominate and so export is reduced. In contrast, well mixed water columns, in which large phytoplankton prevails, have a higher export potential, fuelling upper trophic levels through a short food chain. Mixing is especially relevant in shallow water systems with dense populations of benthic filter feeders, because it supplies the food needed for their growth that under stratified conditions is limited (Frechette & Bourget 1985, Wiles et al. 2006). Prolonged periods of stratification habitually lead to a depletion boundary layer near the bed of benthic suspension feeders (Muschenheim & Newell 1992, Ackerman et al. 2001, Nielsen & Maar 2007), which can only be disrupted by mixing events (Wiles et al. 2006).

The Limfjord is a shallow water body (mean depth ~4.5 m) in peninsular Denmark (Fig. 4.1) with a low tidal range (≤0.2 m) and relatively long residence time (~225 days), which sustains a high biomass of the blue mussel *Mytilus edulis* important for the local economy (Dolmer & Frandsen 2002). This is an eutrophic system, because of the high nutrient input from the watershed, in which primary production frequently exceeds 1 g C m^{-2} d^{-1} in summer. In response to meteorological forcing, the water column in the Limfjord periodically changes between stratified and mixed conditions and it has been suggested that these stratification-mixing cycles are essential to the supply of food to the mussel bed (Dolmer 2000a, Wiles et al. 2006). While vertical mixing would provide food to mussels, short-lived stratification periods should allow to phytoplankton recovers from the grazing pressure of mussels. Therefore, knowing plankton dynamics in this shallow water system is fundamental to attain a better understanding of the benthic-pelagic coupling and so contribute to improve the management of the shellfish industry.

As part of a multidisciplinary study, a fieldwork of 9 days was performed in this shallow water system. Here, we report the dynamics of phytoplankton and bacteria as well as the impact of microzooplankton on plankton populations ≤200 μm which occurred in response to the hydrographic variability observed during this short time period. The aim was to

understand the hydrodynamical and biological processes that regulate plankton dynamics and their coupling with benthic communities.

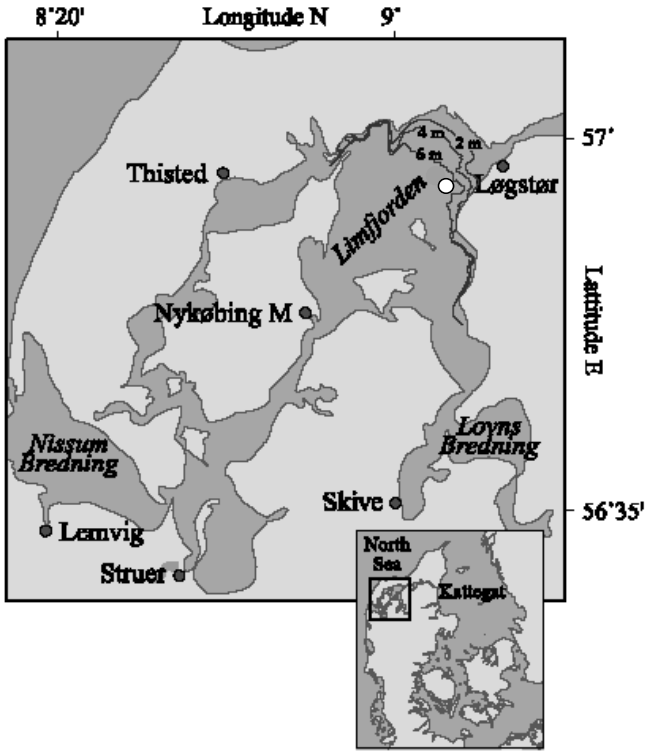

Fig. 4.1. Map of the Limfjord. The white circle near Løgstør on the NE indicates the study site.

4.2. MATERIALS AND METHODS

4.2.1. Sampling and hydrography

Sampling took place onboard of the R/V *Genetica II* from 27 May to 4 June 2003 at one station close to a mussel bed where the water depth was approximately 6 m (Fig. 4.1). Seawater samples were taken at 3 depths (0.5 m, 3.5 m and 5.5 m) using 30 litre Niskin bottles.

Vertical profiles of temperature and salinity were recorded using a CTD (GMI AROP2000). Temperature and salinity were used to calculate the water column stability in the form of the potential energy anomaly parameter φ (J m^{-3}) according to Simpson & Bowers (1981):

$$\varphi = \frac{1}{h} \cdot \int_0^h (\hat{\rho} - \rho) g z dz; \hat{\rho} = \frac{1}{h} \cdot \int_0^h \rho dz \qquad (4.1)$$

where $\rho(z)$ is the density profile (kg m^{-3}) and h is the water depth (m). A large positive value of φ indicates strong stratification while a negative value informs on an unstable water column.

4.2.2. Nutrients and chlorophyll *a*

Samples to determine nutrient concentrations (PO_4^{3-}, NO_3^-, NH_4, SiO_4^{3-}) were frozen immediately after collection. Measurements were done later using an automatic nutrient analyser according to Grasshoff (1976). All nutrients were analysed in duplicated with a precision of 0.06, 0.1, 0.3 and 0.2 µmol l^{-1} for phosphorous, nitrate, ammonia and silicate, respectively.

For chlorophyll a (chl *a*) determinations, three replicates of 50 ml were filtered onto 25 mm Whatman GF/F filters. Pigments were extracted overnight in 5 ml 96% ethanol in the dark and chl *a* concentration was determined against a chl *a* standard after reading fluorescence before and after acidification on a Turner Designs Model 700 Fluorometer.

4.2.3. Phytoplankton biomass and primary production

Samples for microplankton counts were preserved in Lugol's iodine solution and sedimented in composite sedimentation chambers. Diatoms, dinoflagellates, other flagellates >20 µm and ciliates were identified and counted to the species level, when possible, using an inverted microscope. Phototrophic dinoflagellates, flagellates and ciliates were differentiated following literature (e.g. Lessard & Swift 1986, Larsen & Sournia 1991) and using epifluorescence microscopy. Cell biovolumes were calculated from the dimensions and shapes according to Hillebrand et al. (1999). Plasmatic volumes of diatoms calculated according to Smayda (1965) and total cell volume of dinoflagellates, other flagellates >20µm and ciliates were converted to cell carbon following Strathmann (1967) for diatoms and dinoflagellates, Verity et al. (1992) for flagellates and Putt & Stoecker (1989) for ciliates.

Phototrophic pico- (≤2 µm) and nanoflagellates (2-20 µm) were enumerated from subsamples of 10 and 40 ml respectively, which were fixed with buffered 0.2 µm filtered formalin (2% final concentration) and then filtered on 0.2 and 0.8 µm black Millipore polycarbonate filters, respectively. An epifluorescence microscope was used to identify and count *Synechococcus*-type cyanobacteria and phototrophic pico- and nanoflagellates. Cell biovolumes of *Synechococcus* were converted to carbon biomass following Bratbak & Dundas (1984). Carbon biomass of pico- and nanoflagellates were estimated according to Verity et al. (1992).

Primary production was measured as carbon assimilation after 24h *in situ* incubations. Samples were collected in tissue culture flasks (3 light and 2 dark flasks) and immediately inoculated with ca. 10 μCi of $NaH^{14}CO_3$. Then, the flasks were placed at the corresponding depth using a rope with specially constructed holders. The array was kept vertical in the water column using a weight at the bottom and a large buoy at the surface. After 24h of incubation samples were recovered and filtered through Whatman GF/F filters under low vacuum pressure. Filters were placed in vials and left in HCl fumes atmosphere for 12h to remove unassimilated ^{14}C. Radioisotope incorporation was determined with a scintillation counter. Quenching was corrected using the external standard and the channel ratio methods. Integrated primary production (mg C m^{-2} d^{-1}) was calculated using the trapezoid rule.

4.2.4. Bacterial abundance and production

For bacteria enumeration, 10 ml samples from each depth were preserved with 1 ml formalin in 20 ml glass vials and stored cold until processing. The bacteria were enumerated on a FACS Calibur flow cytometer (Becton & Dickinson) after staining the fixed cells with the nucleic acid stain SYBR Green 1 (Molecular Probes). One ml sample was added to 10 μl of a 100 times dilution of stock SYBR Green 1, and 10 μl of a suspension of 2 μm fluorescent beads (Polyscience). The number of cells was converted to bacterial biomass applying 20 fg C $cell^{-1}$ (Lee & Fuhrman 1987).

Bacterial production was measured using two methods: incorporation of ^3H-thymidine (Furhmann & Azam 1982) and ^3H-leucine (Kirchman et al. 1985). Four replicates of 10 ml seawater from every sampling depth were dispensed in 20 ml plastic vials and incubated with both isotopes for 2 hours. Incubations included 10 ml control samples prekilled with 500 μl TCA 100%. The samples were filtered on 0.2 μm celluloses-nitrate filters and washed 10 times with 5 % ice cold TCA. Samples were processed on a scintillation counter after addition of 10 ml Filtercount. The incorporated thymidine was converted to cell production (mg C m^{-1} h^{-1}) using a conversion factor of 1.1 x 10^8 cells mol^{-1} ^3H-thymidine incorporated (Riemann et al. 1987). The incorporated ^3H-leucine was converted to carbon according to Simon & Azam (1989). Since differences between the two methods were not significant ($0.35 \leq p \leq 0.63$; t-test for paired samples), bacterial production was considered as the mean value of the two methods.

4.2.5. Microzooplankton feeding impact

Microzooplankton feeding impact on the several plankton components ≤200 μm was estimated using the dilution technique (Landry & Hassett 1982) on 29, 31 May and 2 and 4 June 2003. Water was collected at dawn from the surface with a 30 l Niskin bottle. All

experimental containers, bottles and tubing were soaked in 10% HCl and rinsed with distilled water before each experiment. The seawater from a first dip of the Niskin bottle was gravity filtered through a 0.8/0.2 µm Suporcap filter into a 25 l container. Water from a second dip was gently transferred to another 25 l container. After inverse filtration of this water through 200 µm nylon nets to remove large zooplankton, duplicate dilutions were prepared in 2.3 l polycarbonate bottles at the laboratory. Six dilutions levels of 100, 80, 60, 40, 20 and 10% of 200 µm filtered seawater were performed. The dilution bottles were incubated for 24 h with running seawater at *in situ* temperature and surface light conditions. Samples to estimate plankton biomass of organisms ≤200 µm were taken from the initial 200 µm filtered seawater and from all experimental bottles at the end of the incubation.

To determine microplankton biomass, samples were processed as described before. Metazoa biomass was estimated following Berggreen et al. (1988) for copepod nauplii and Fotel et al. (1999) for bivalve larvae. Pico- and nanoplankton biomass was determined following the process previously described, but as we were also interested in heterotrophs, the samples were stained with DAPI at 0.1 µg ml^{-1} final concentration (Porter & Feig 1980). Heterotrophic bacteria and heterotrophic pico- and nanoflagellates were counted under UV light excitation in the epifluorescence microscope.

Changes in biomass between the beginning (C_0) and the end (C_t) of the incubation time (t = 1 day) were used to calculate the net growth rates (k, d^{-1}) of total plankton (≤200 µm) biomass as well as net growth rates of the several autotrophic and heterotrophic components of the plankton community:

$$k = \frac{1}{t} \cdot \ln\left(\frac{C_t}{C_0}\right) \tag{4.2}$$

Mortality rates (m, d^{-1}) and specific growth rates (μ, d^{-1}) are the slope and the y-axis intercept respectively of the linear regressions between the net growth rates (k) and the dilution factor (X) (Landry & Hassett 1982):

$$k = \mu - m \cdot X \tag{4.3}$$

For the cases of non-linear responses (saturated and saturated-increased), the specific growth rate (μ) was estimated as the y-axis intercept derived from the regression of the linear part of the response. The mortality rate (m) was then obtained solving the equation 4.3 for the undiluted (100%) sample (see section 2).

The daily impact of microzooplankton on the standing stock (%SS, d^{-1}) and production (%P, d^{-1}) were calculated as:

$$\%SS = 1 - e^{-m} \times 100 \quad (4.4)$$

$$\%P = \frac{m}{\mu} \times 100 \quad (4.5)$$

The quantity of carbon consumed (G, mg C m^{-3} d^{-1}) and produced (P, mg C m^{-3} d^{-1}) were calculated as:

$$G = m \times C_m \quad (4.6)$$

$$P = \mu \times C_m \quad (4.7)$$

where C_m (mg C m^{-3}) is:

$$C_m = C_0 \left[e^{(\mu - m)t} - 1 \right] / (\mu - m)t \quad (4.8)$$

4.3. RESULTS

4.3.1. Hydrography and nutrients

Detailed information on the physical properties of the water column has been provided by Wiles et al. (2006). Briefly, the water column was characterised by a progressive warming (Fig. 4.2a) and salinity increase (Fig. 4.2b) over the 9 sampling days. Although the water column was homogeneous with regard to temperature, salinity revealed two periods of stratification at the surface (28-30 May and 1-2 June) that affected the density field (Fig. 4.2c). Thus, the stability of the water column (Fig. 4.2d) was high during these two periods of haline stratification but showed a minimum value (0.27 J m^{-3}), which indicates strong mixing, on 31 May when the vertical distributions of both temperature and salinity were homogeneous (Fig. 4.2a & b). Another low φ value (0.53 J m^{-3}) was recorded on 3 June. During periods of high stability, the water column was also stratified near the bottom (Fig. 4.2c).

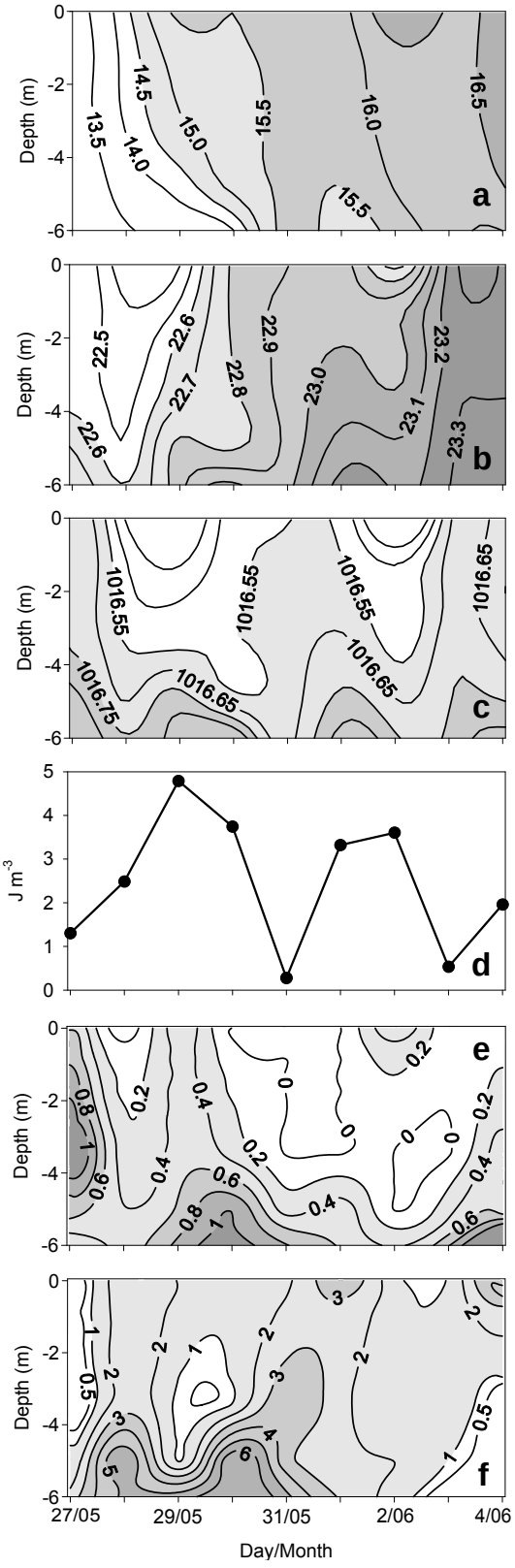

Fig. 4.2. Evolution of (a) temperature (ºC), (b) salinity (psu), (c) density (kg m^{-3}), (d) potential energy anomaly (J m^{-3}), (e) ammonium concentrations (µmol l^{-1}) and (f) silicate concentrations (µmol l^{-1}) in the water column during the sampling time.

Nitrate and phosphate concentrations were well below the detection levels during the whole sampling period (data not shown). Ammonium concentrations, which in general were higher near the bottom, were detected in the water column during the first half of the sampling (Fig. 4.2e). Silicate concentration was also higher in the bottom layer than in the rest of the water column (Fig. 4.2f), especially during the first half of the sampling period. The mixing event on 31 May caused the redistribution of silica within the whole water column. Coinciding with this redistribution of silica, ammonium was depleted.

4.3.2. Phytoplankton and primary production

Despite these stratification-mixing cycles observed in the hydrographic field, chl *a* concentration (Fig. 4.3a) and phytoplankton carbon biomass (Fig. 4.3b) were in general homogenously distributed in the water column. Nevertheless, both variables depicted higher values close to the bottom previously to the strong mixing event on 31 May. These high values coincided with stratification near the bottom (Fig. 4.2c). Chl *a* concentration gradually decreased from a mean value in the water column of ~4.5 mg chl *a* m^{-3} at the beginning of sampling to ~2.3 mg chl *a* m^{-3} on 31 May. Then, chl *a* concentration progressively increased to reach ~5 mg chl *a* m^{-3} on 3 June. Phytoplankton carbon biomass roughly followed the same pattern, but showing the lowest concentration (63 mg C m^{-3}) one day later, on 1 June, and a mean final concentration (103 mg C m^{-3}) on 3 June appreciably lower than the initial concentration on 27-28 May (167 mg C m^{-3}).

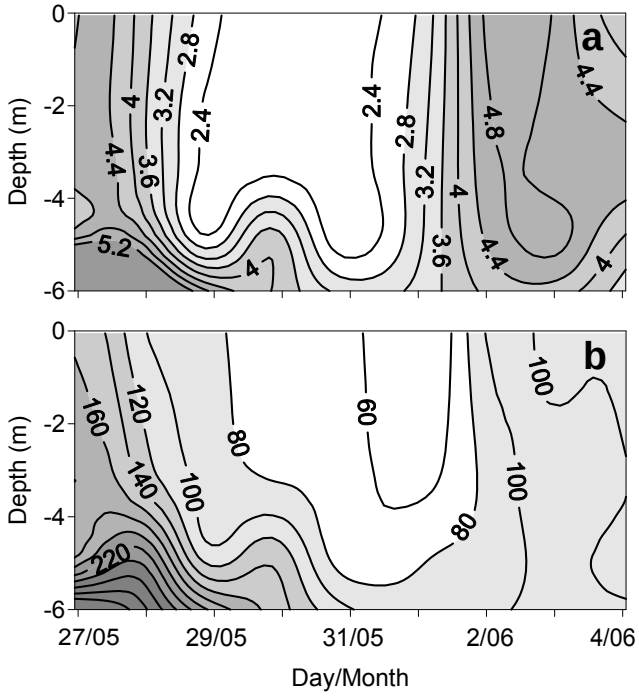

Fig.4.3. Evolution of (a) chl *a* concentration (mg chl *a* m^{-3}) and phytoplankton carbon biomass (mg C m^{-3}) in the water column during the sampling time.

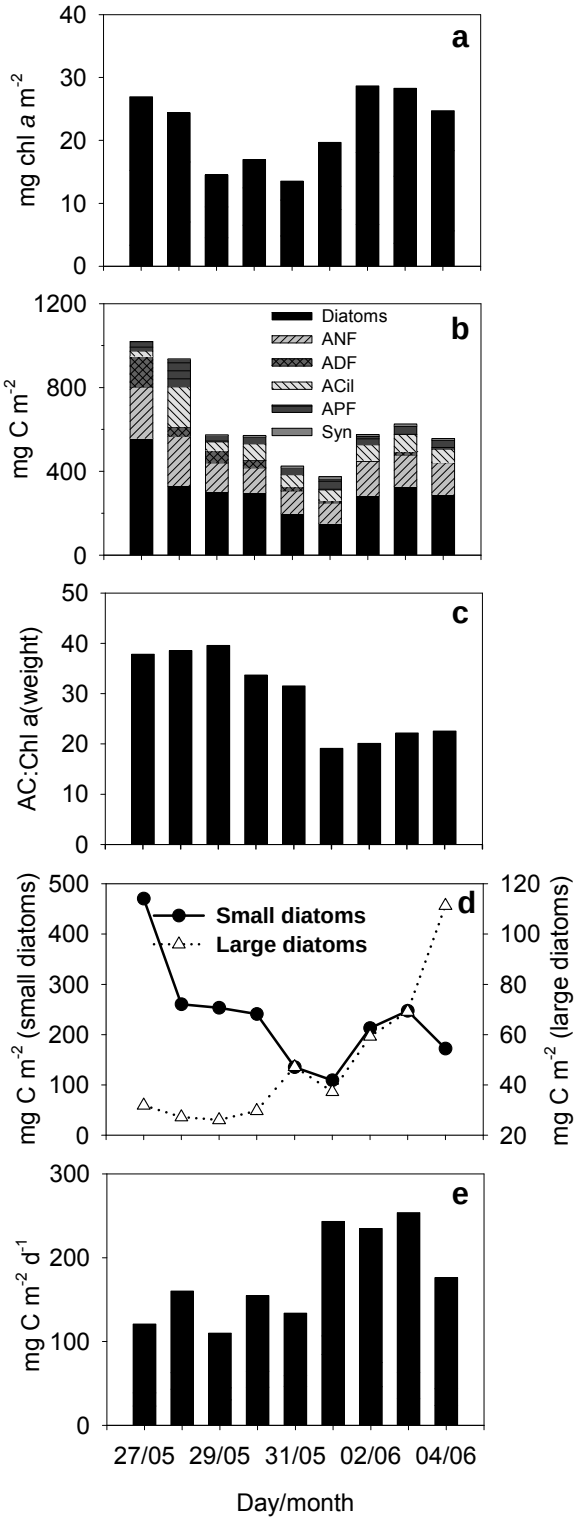

Fig. 4.4. Evolution of depth integrated (a) chl *a* concentration, (b) phytoplankton carbon biomass, (c) autotrophic carbon to chl *a* ratio, (d) biomass of small (*Chaetoceros socialis*, *Skeletonema* cf. *costatum*, *Thalasionema nitzschioides*) and large diatoms (mainly *Chaetoceros curvisetus* and *C. laciniousus*) and (e) primary production. ANF, autotrophic nanoflagellates; ADF, autotrophic dinoflagellates; ACil, autotrophic ciliates; APF, autotrophic picoflagellates; Syn, *Synechococcus*-like cyanobacteria.

Therefore, depth integrated chl *a* concentration (Fig. 4.4a) decreased from ~26 mg chl *a* m^{-2} on the first 2 days of sampling to ~15 mg chl *a* m^{-2} during the following 3 days. Then, integrated chl *a* concentration increased to values slightly higher (28 mg chl *a* m^{-2}) than the concentrations found during the first two days. Depth integrated phytoplankton carbon (Fig. 4.4b) decreased from 1020 to 376 mg C m^{-2} between the beginning of sampling and 1 June. After that day there was a slight increase up to ~600 mg C m^{-2}.

Diatoms (304 ± 112 mg C m^{-2}) and autotrophic nanoflagellates (157 ± 53 mg C m^{-2}) dominated within the autotrophic plankton community during all sampling period (Fig. 3b), accounting for 48 ± 6% and 25 ± 2% of the total phytoplankton biomass, respectively. Among diatoms, *Skeletonema* cf. *costatum*, *Thalassionema nitzschioides* and *Chaetoceros* spp. were the more abundant species. The ciliate *Mesodinium rubrum* (73 ± 48 mg C m^{-2}), dinoflagellates, mainly *Gymnodinium* cf. *varians* (38 ± 45 mg C m^{-2}), picoflagellates (50 ± 31 mg C m^{-2}) and *Synechococcus* (6 ± 4 mg C m^{-2}) accounted for the remaining 30% of the phytoplankton carbon (Fig. 4.4b).

Although both depth integrated chl *a* and phytoplankton carbon concentrations showed a quite similar time evolution, characterized by an initial decrease and a later increase (Fig. 4.4a & b), this later increase was considerably lower in phytoplankton carbon than in chl *a* concentration. Thus, it resulted in a substantial reduction in the autotrophic carbon to chl *a* (AC:Chl *a*) ratio (Fig 4.4c), which varied from a mean value of 36 ± 3 at the beginning of sampling to a mean value of 21 ± 2 on the last days. This variation in the AC:Chl *a* ratio coincided with a conspicuous change in the diatom community (Fig. 4.4d). Large *Chaetoceros* spp., which at the beginning of the observations constituted a small fraction (9%) of the total carbon of diatoms, increased following the mixing event on 31 May. At the end of the observations, on 4 June, large *Chaetoceros* spp. represented 39% of the total diatom carbon.

Primary production (Fig. 4.4e) basically followed the changes occurring in AC:Chl *a* ratios and the diatom community. Thus, integrated primary production was higher (227 ± 35 mg C m^{-2} d^{-1}) after the strong mixing on 31 May than previously (136 ± 22 mg C $m^{-2}d^{-1}$).

4.3.3. Bacterial biomass and production

Depth integrated bacterial biomass (Fig. 4.5a) increased from 668 mg C m^{-2} on the first day of sampling to 1016 mg C m^{-2} on the second day, but later showed a constant decrease at a rate of 63 ± 11 mg C m^{-2} d^{-1} (r^2 = 0.88), to a final value of 391 mg C m^{-2}. There were not significant differences in bacterial biomass between the three sampled depths ($0.61 \leq p \leq 0.86$; t-test for two samples).

In contrast, bacterial production (Fig. 4.5b) abruptly decreased from a mean value of 270 ± 22 mg C m^{-2} d^{-1} during the first two days of sampling to a mean value of 110 ± 33 mg C m^{-2} d^{-1} for the rest of the sampling days. Bacterial production was higher than primary production (BP:PP = 1.68 ± 0.48) only during the first three days. For the rest of the sampling, bacterial production was always lower than primary production (BP:PP = 0.58 ± 0.26).

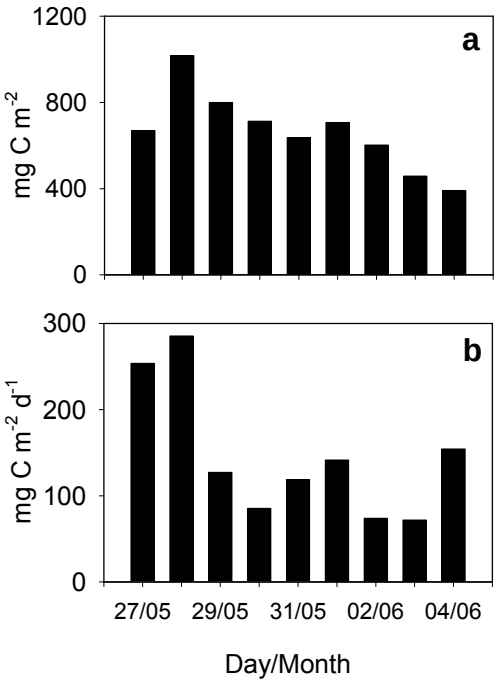

Fig. 4.5. Evolution of depth integrated (a) bacterial biomass and (b) bacterial production.

4.3.4. Microzooplankton feeding impact

The biomass of nano- and microheterotrophs (hereafter microzooplankton) at the beginning of the dilution experiments (Fig. 4.6) averaged 60 ± 13 mg C m^{-3}, representing 23 ± 5% of the total plankton (≤200 µm) biomass. The highest microzooplankton biomass, which also corresponded to the highest contribution of this group to the plankton community, was found on 31 May (77 mg C m^{-3} and 29%, respectively). Metazoan organisms, namely copepod nauplii and bivalve larvae (22 ± 12 mg C m^{-3}), heterotrophic nanoflagellates (HNF, 19 ± 7 mg C m^{-3}), ciliates, mainly aloricate choreotrichs <50 µm (11 ± 8 mg C m^{-3}) and dinoflagellates, both naked and armoured <50 µm (7 ± 6 mg C m^{-3}) were the groups that composed the microzooplankton community during the sampling period (Fig. 4.6). Metazoa dominated on 31 May and 4 June, when they accounted for 47% and 61% of the total microzooplankton biomass, respectively. HNF, with a contribution to total microzooplankton biomass always >20%, dominated on 2 June (46%), whereas ciliates were important during the two first experiments on 29 and 31 May (27%

and 25% of the total microzooplankton biomass. respectively). Afterwards, the biomass of heterotrophic ciliates declined to account only for 6% of the microzooplankton biomass on 4 June. The contribution of HDF to total microzooplankton biomass varied between 2% on 31 May and 25% on 2 June.

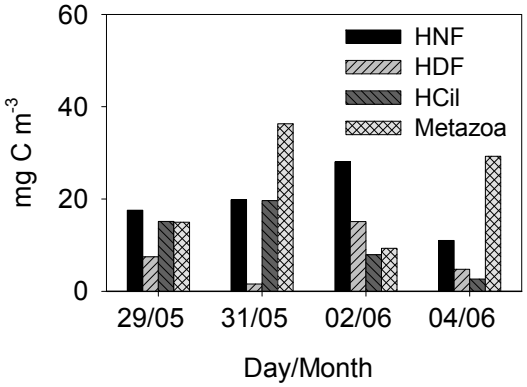

Fig. 4.6. Carbon biomass of the microzooplankton groups present in surface waters at the beginning of each dilution experiment. HNF, heterotrophic nanoflagellates; HDF, heterotrophic dinoflagellates; HCil, heterotrophic ciliates.

The dilution experiments showed that microzooplankton fed on both autotrophic and heterotrophic organisms (Table 4.1). Among phytoplankton, diatoms (specifically *Skeletonema* cf. *costatum*, small *Chaetoceros* spp. and *Chaetoceros socialis*), autotrophic nanoflagellates (ANF) and *Synechococcus* were grazed through the sampling period. Autotrophic picoflagellates (APF) were also grazed in three of the four experiments. The lowest growth and mortality rates were recorded for *Synechococcus* ($0.35 \leq \mu \leq 0.74$ d^{-1}; $0.39 \leq m \leq 0.67$ d^{-1}) and the highest for diatoms ($1.43 \leq \mu \leq 2.42$ d^{-1}; $1.28 \leq m \leq 2.05 d^{-1}$). Regarding heterotrophs, nanoflagellates (HNF), picoflagellates (HPF) and bacteria (HB) were the three groups that were consumed. Growth ($0.78 \leq \mu \leq 4.25$ d^{-1}) and mortality rates of these organisms were high ($0.80 \leq m \leq 1.76$ d^{-1}), except for HNF on 29 May when mortality rate was the lowest ($m = 0.29$). Despite the high variability recorded in the rates of the several plankton components, those obtained for total autotrophic biomass ($0.99 \leq \mu \leq 1.29$ d^{-1}; $0.86 \leq m \leq 1.20$ d^{-1}), total heterotrophic biomass ($0.74 \leq \mu \leq 0.89$ d^{-1}; $0.85 \leq m \leq 1.15$ d^{-1}) and total plankton biomass ($0.80 \leq \mu \leq 0.98$ d^{-1}; $0.67 \leq m \leq 1.11$ d^{-1}) were more similar, varying also within a narrower range.

Consequently, the daily impact of microzooplankton on the standing stocks of autotrophic ($64 \pm 5\%$), heterotrophic ($61 \pm 5\%$) and total plankton biomass ($60 \pm 8\%$) were very similar and showed low variability (Fig. 4.7a). Diatoms ($80 \pm 6\%$) among phytoplankton (Fig. 4.7b), and HPF ($73 \pm 12\%$) and HB ($67 \pm 8\%$) among heterotrophs (Fig. 4.7c), were the groups that suffered from the highest impact of microzooplankton on their standing stocks.

Table 4.1. Mortality (m, d^{-1}) and growth (μ, d^{-1}) rates obtained from the dilution experiments for autotrophic, heterotrophic and total plankton (≤200 µm); $p < 0.05$. ANF: autotrophic nanoflagellates, APF: autotrophic picoflagellates, AC: total autotrophic carbon, HNF: heterotrophic nanoflagellates, HPF: heterotrophic picoflagellates, HB: heterotrophic bacteria, HC: total heterotrophic carbon, TC: total carbon. ns: not significant, [a]saturated response, [b]saturated-increased response.

	29-May			31-May			2-Jun			4-Jun		
	m	***μ***	r^2	***m***	***μ***	r^2	***m***	***μ***	r^2	***m***	***μ***	r^2
Diatoms	1.47	1.43	0.94	1.28	1.74	0.97	1.70	2.12	0.99	2.05	2.42	0.94
Small Chaetoceros spp.	1.01	0.49	0.70	1.99	1.35	0.99[b]		ns			ns	
Chaetoceros socialis		ns			ns		2.37	2.97	0.99	3.39	3.56	0.88
Skeletonema cf. *costatum*	1.31	0.99	0.90	1.02	1.84	0.71	1.13	1.63	0.94		ns	
ANF	1.25	1.02	0.87	0.74	1.24	0.78	0.79	1.06	0.97[b]	0.82	0.86	0.93
APF	1.09	1.34	0.74		ns		0.95	0.52	0.84	0.98	0.38	0.71
Synechococcus	0.53	0.74	0.72	0.39	0.68	0.93[a]	0.64	0.47	0.92	0.67	0.35	0.82
AC	1.20	1.02	0.96	0.86	0.99	0.93	1.02	1.29	0.95[a]	1.06	1.12	0.97
HNF	0.29	1.20	0.93[a]	0.87	1.05	0.95	1.50	1.14	0.97[a]	1.12	1.44	0.94
HPF	0.83	1.97	0.89[a]	1.52	4.25	0.81	1.41	1.56	0.96	1.76	1.90	0.97
HB	1.17	0.78	0.91	0.80	0.90	0.83[a]	1.20	1.08	0.94[a]	1.28	0.83	0.93
HC	0.85	0.74	0.92	0.85	0.89	0.99[a]	0.94	0.86	0.94[a]	1.15	0.81	0.99
TC	0.98	0.85	0.95	0.67	0.80	0.93[a]	0.91	0.98	0.94[a]	1.11	0.93	0.99

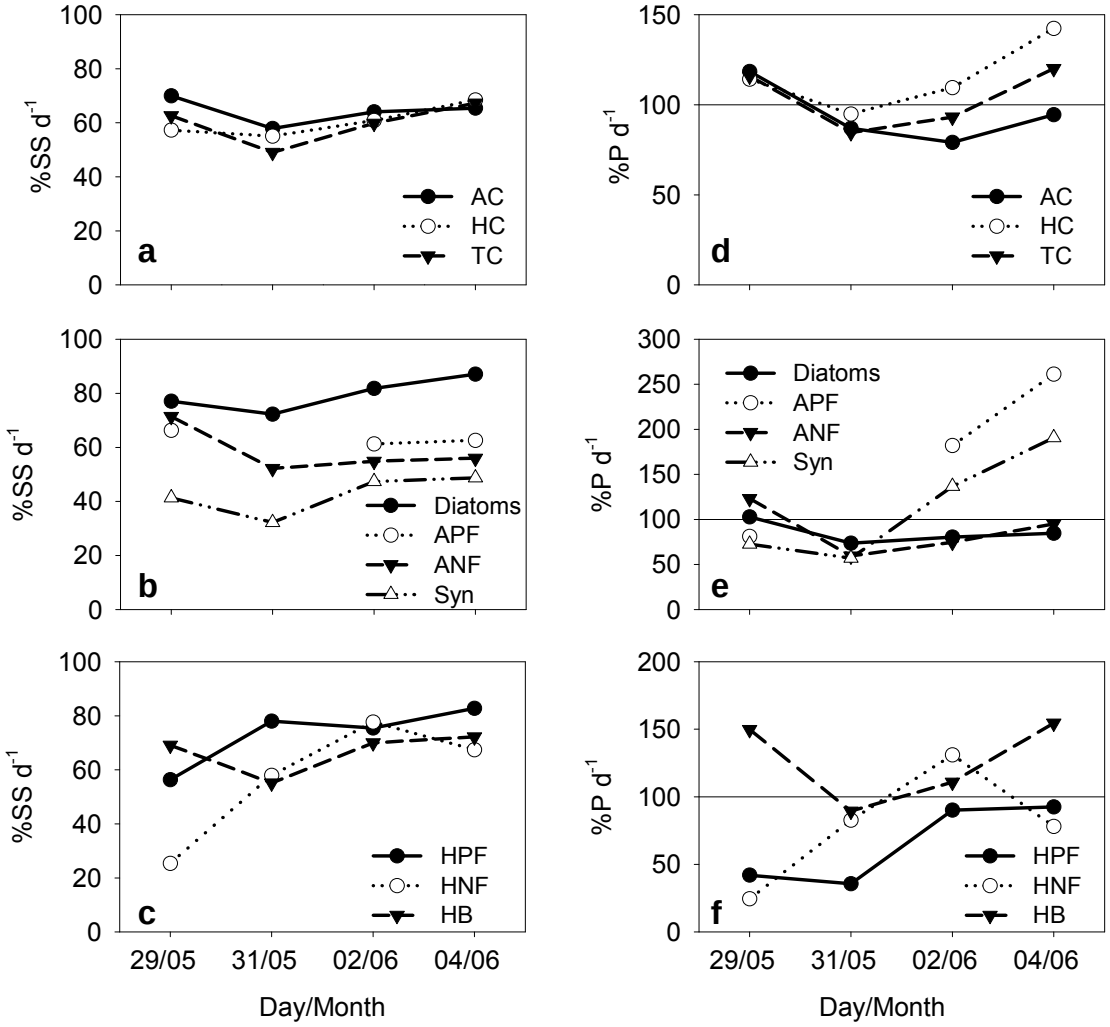

Fig. 4.7. Microzooplankton daily impact on the standing stock (%SS, left panels) and production (%P, right panels) of (a & d) total and autotrophic and heterotrophic plankton ≤200 µm, (b & e) autotrophic plankton groups and (c & f) heterotrophic plankton groups with significant regressions in the dilution experiments. AC, total autotrophic plankton; HC, total heterotrophic carbon; TC, total plankton carbon; APF, autotrophic picoflagellates; ANF, autotrophic nanoflagellates; Syn, *Synechococcus*-type cyanobacteria; HPF, heterotrophic picoflagellates; HNF, heterotrophic nanoflagellates; HB, heterotrophic bacteria. Note that the response of APF was not significant on 31 May (see Table 4.1).

Microzooplankton was able to virtually remove all production of the plankton community (≤200 µm) during the sampling period (103 ± 7%) (Fig. 4.7d), being the heterotrophic production (115 ± 20%) more affected than the autotrophic production (95 ± 17%). APF (131 ± 114%), *Synechococcus* (114 ± 62%) and HB (126 ± 31%) were the groups that experienced the highest impact on production (Fig. 4.7b & c). Remarkably, the impact on production decreased on 31 May (Figs. 4.7d), when less than 100% of

autotrophic and heterotrophic production was processed by microzooplankton (Fig. 4.7d). Later, the impact on autotrophic production (Fig. 4.7d) and in particular the impact on diatom and ANF production (Fig. 4.7e) remained below 100%, while the heterotrophic production (Fig. 4.7d), specifically HB production (Fig. 4.7f), returned to be fully consumed.

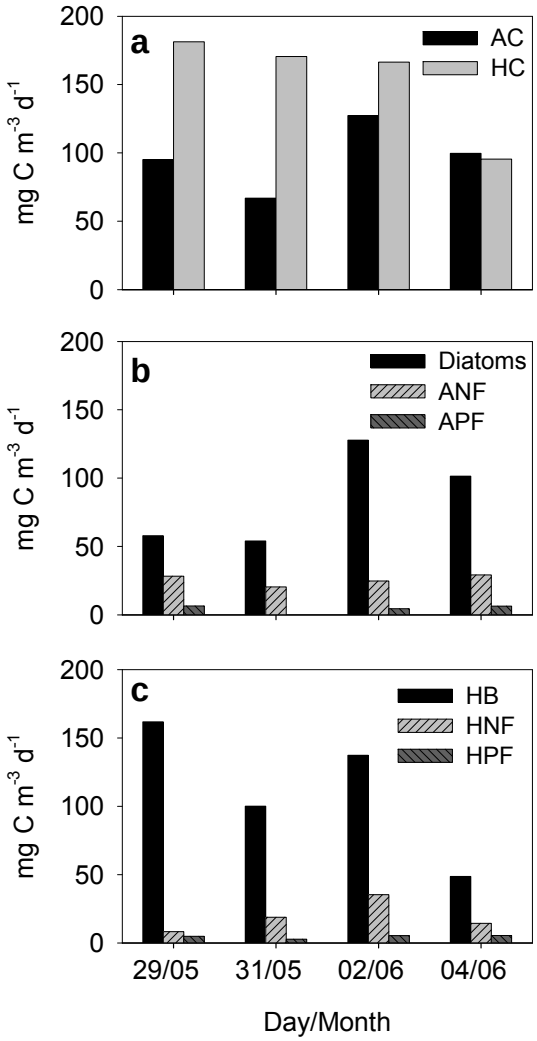

Fig. 4.8. Consumption (mg C m^{-3} d^{-1}) of (a) total autotrophic and heterotrophic plankton carbon, (b) carbon of the main autotrophic plankton groups and (c) carbon of the main heterotrophic plankton groups. AC, total autotrophic carbon; HC, total heterotrophic carbon; ANF, autotrophic nanoflagellates; APF, autotrophic picoflagellates; Syn, *Synechococcus*-type cyanobacteria; HB, heterotrophic bacteria; HNF, heterotrophic nanoflagellates; HPF, heterotrophic picoflagellates.

In terms of biomass, microzooplankton consumed more heterotrophic carbon (153 ± 39 mg C m^{-3} d^{-1}) than autotrophic carbon (97 ± 25 mg C m^{-3} d^{-1}). However, the two consumptions showed contrasting tendencies (Fig. 4.8a). While consumption of heterotrophic carbon continuously decreased from 181 to 95 mg C m-3 d^{-1} (r^2 = 0.74),

consumption of autotrophic carbon was higher after the mixing event on 31 May (113 ± 20 mg C m^{-3} d^{-1}) than before (81 ± 20 mg C m^{-3} d^{-1}). Consumption of heterotrophic carbon was largely based on HB (Fig. 4.8c), which accounted for 71% (r^2 = 0.80) of the total heterotrophic carbon consumed. Variations in the autotrophic carbon consumed were due to the consumption of diatoms (85 ± 36 mg C m^{-3} d^{-1}), and hence both consumptions were correlated (r^2 = 0.78). In contrast, consumption of ANF was relatively constant: 26 ± 4 mg C m^{-3} d^{-1} (Fig. 4.8b). Despite the high impact on the production of picoautotrophs (Fig. 4.7e), the biomass consumed of these organisms was very low (4 ± 3 mg C m^{-3} d^{-1} for APF and 0.74 ± 0.3 mg C m^{-3} d^{-1} for *Synechococcus*) owing to their lower standing stocks (Figs. 4.4b).

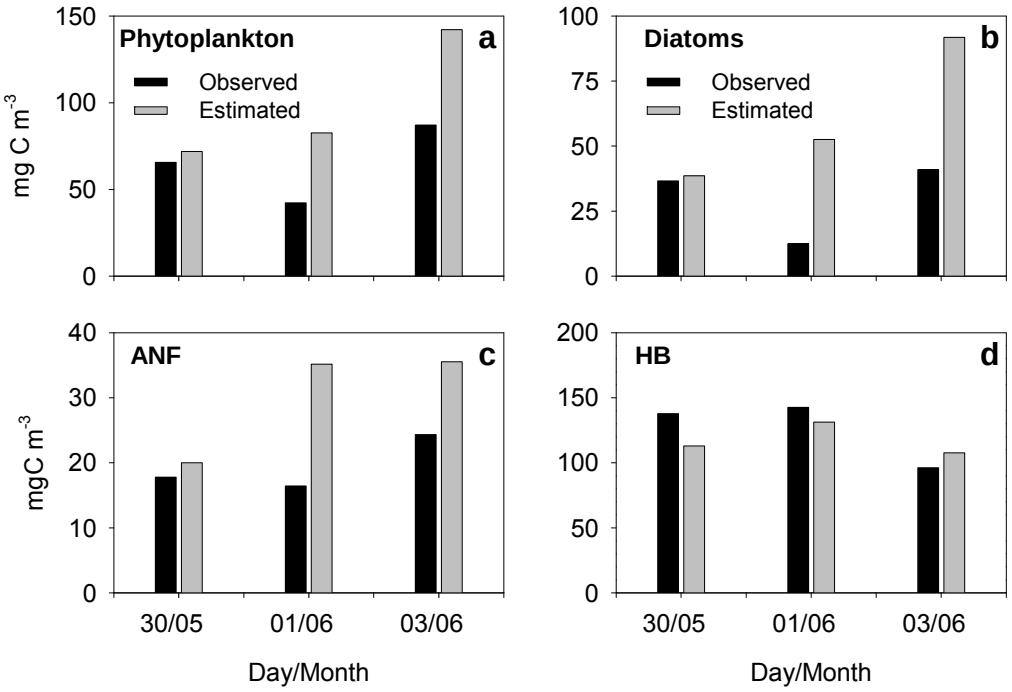

Fig. 4.9. Comparison between *in situ* observed and estimated carbon biomass in surface waters of (a) phytoplankton, (b) diatoms, (c) autotrophic nanoflagellates (ANF) and (d) heterotrophic bacteria (HB). Estimated carbon biomasses were calculated using the growth and mortality rates obtained from the dilution experiments started the previous day.

When growth and mortality rates derived from dilution experiments (Table 4.1) were used to forecast the expected plankton biomass in surface waters on the next day, the biomasses observed and predicted were in reasonable agreement for total phytoplankton, diatoms and ANF on 30 May (Figs. 4.9a-c), before the mixing event on 31 May. The differences between the biomasses predicted and observed were of ~2 mg C m^{-3} for diatoms and ANF and 6 mg C m^{-3} for total phytoplankton, which represent between 5%

and 12% of the biomass recorded *in situ*. However, differences were considerable on 1 and 3 June, after the mixing event, when the biomasses observed *in situ* were appreciably lower than the biomasses predicted (Figs. 4.9a-c). The overestimates varied between 40 and 55 mg C m^{-3} for total phytoplankton, 40 and 51 mg C m^{-3} for diatoms and 11 and 19 mg C m^{-3} for ANF, representing 63-95% of the *in situ* total phytoplankton biomass, 124-322% of the diatom biomass actually observed and 46-114% of the biomass recorded for ANF.

In contrast, the biomasses observed and predicted for HB were in reasonably good agreement on the three days (Fig. 4.9d). The HB biomass predicted was 82% of the biomass observed on 30 May and 92% of the biomass recorded on 1 June. On 3 June the HB biomass was overestimated in 11 mg C m^{-3}, which represents 12% of the *in situ* biomass.

4.4. DISCUSSION

Based on the evolution of the hydrographic field (Fig. 4.2), stratification-mixing cycles characterised the Limfjord during the sampling period. As determined by Wiles et al. (2006), solar heating promotes stratification and wind indirectly induces mixing through wave action. Advection is of minor importance for the hydrodynamics of the system, where flow velocities are usually <0.10 m s^{-1} and sometimes <0.04 m s^{-1} (Dolmer 2000b, Wiles et al. 2006). Thus, three main periods could be distinguished during our sampling: (1) a stratification period from the beginning of the sampling to 30 May, which was followed by (2) a strong mixing event occurring on 31 May and then (3) a less stratified period between 1 June and the end of the sampling. The biological processes and variables measured indicate that the strong mixing on 31 May induced significant changes in the plankton community with important consequences for the benthic-pelagic coupling in this shallow system.

4.4.1. Mixing and benthic-pelagic coupling

During the first stratification period (27-30 May), benthic and pelagic systems were apparently decoupled. In this period, microzooplankton grazing can be assigned as responsible for the dynamics of the phytoplankton community in the surface layers, as suggested by the matching between phytoplankton biomass recorded at the surface and that predicted considering only growth and mortality rates derived from dilution experiments (Figs. 4.9a-c). Microzooplankton consumed more carbon than was produced by phytoplankton (Fig. 4.7d) and, therefore, caused the reduction observed in phytoplankton carbon (Fig. 4.4b) and chl *a* concentration (Fig. 4.4a). The high impact of

microzooplankton led to a regenerated pelagic system with detectable ammonium concentrations in the surface layers (Fig. 4.2e), which probably fuelled phytoplankton growth. High bacterivory (Fig. 4.8c) and the elevated BP:PP ratios (1.68 ± 0.48) suggest that the microbial loop during this period was important, presumably channelling the dissolved organic matter that feeding activities released to the medium (Strom et al. 1997, Nagata 2000). The accumulation of chl *a* and phytoplankton biomass near the bottom (Fig. 4.3), probably resulting from sinking and later isolation from the upper layers by stratification, also indicates that mussels were not able to reach this food. Although not discernible with our sampling device, under stratified conditions mussels create a near boundary layer depleted of food that also impedes further supply of food from above (Muschenheim & Newell 1992, Ackerman et al. 2001, Nielsen & Maar 2007).

The mixing event on 31 May drastically changed this scenario, homogenising the water column and allowing a closer contact between pelagic and benthic communities. Namely, vertical mixing destroyed the stratification encountered in the bottom layers on the previous days, and probably eroded the near boundary layer providing food to mussels. Vertical mixing has been suggested as the main mechanism supplying food to mussels in this (Dolmer 2000a, b, Wiles et al. 2006) and other similar shallow systems with high biomass of benthic filter feeders (Møhlenberg 1995, Riisgård et al. 2004). Here, mussel feeding activity can be inferred from the differences found between the observed phytoplankton biomass at the surface and the biomass estimated considering microzooplankton as the only loss term (Fig. 4.9). This mixing event also induced a decrease in the microzooplankton impact (Figs. 4.7 & 4.8), though total microzooplankton biomass was higher on that day (Fig. 4.6). However, this increase in microzooplankton biomass was basically due to metazoa, namely bivalve larvae, which presumably were resuspended by mixing from the bottom. In contrast, the biomass of HDF decreased. Therefore, the lower impact of microzooplankton during this mixing event can be attributed to a change in microzooplankton composition (Lawrence et al. 2004). Furthermore, turbulence could have been strong enough to induce negative physiological effects on HDF (Berdalet 1992, Havskum 2003) and ciliates (Garstecki et al. 2002, Lawrence et al. 2004) leading to a decrease in microzooplankton feeding activity.

The mixing event, which also injected nutrients into the water column formerly accumulated in the bottom layers (Fig. 4.2) due to regeneration by benthic filter feeders (Nielsen & Maar 2007), stimulated the phytoplankton recovery during the following stratified period. This recovery was clearly viewed in primary production and chl *a* concentration and was accompanied by the continuous increase in the biomass of large diatoms (Fig. 4.4). Similar responses of phytoplankton during stratified periods following mixing events were also observed in neighbour shallow systems (Møhlenberg 1995,

Riisgård et al. 2004) However, the response recorded in Limfjord during this new stratified period differed from the previous one in several aspects. The homogeneous vertical distributions of chl *a* and phytoplankton biomass (Fig. 4.3) suggest that stratification was not strong enough to cause benthic-pelagic decoupling, and so the phytoplankton biomass recorded at the surface could not be estimated from growth and mortality rates due to microzooplankton grazing (Fig. 4.9). In addition, the low BP:PP ratio (0.58 ± .26) points to a minor importance of the microbial loop, a circumstance that is also supported by the undetectable levels of ammonium in the surface layer (Fig. 4.2e), suggesting that this nutrient was rapidly taken by phytoplankton.

4.4.2. Microbial versus classical food chain

Although variable in respect to the hydrographic conditions, the microbial food web was an important route in the transfer of phytoplankton and heterotrophic biomass through the pelagic food chain during all sampling period. Among all organisms, heterotrophic bacteria were found to be an important food source for microzooplankton in the Limfjord (Fig. 4.8c), agreeing with prior studies (Fenchel 1982b). In fact, the decrease in bacterial biomass and production (Fig. 4.5) could be explained by the tight control that microzooplankton exerted on this group, consuming higher quantities than those produced (Fig. 4.7f). Thus, bacterial biomass at the surface could be reasonably well predicted by the growth and mortality rates estimated for this group in dilution experiments (Fig. 4.9d). Diatoms were the most consumed group among phytoplankton (Fig. 4.8b), which agrees with other studies in coastal systems, where microzooplankton has been found to be an important consumer of these organisms (Neuer & Cowles 1994, Sherr & Sherr 2007, section 3).

Nevertheless, the importance of the microbial food web decreased during the mixing events when the classical food chain gained importance and phytoplankton was also apparently consumed by mussels. Assuming that phytoplankton growth and mortality due to microzooplankton did not change significantly in a well mixed vertical water column, the fraction of phytoplankton biomass removed by other organisms than microzooplankton can be estimated from the difference between the estimated and observed biomasses given in Fig. 4.9a. This simple calculation suggests that under strong stratified conditions, like those of the first period, less than 10% of the phytoplankton standing stock was directly channelled to the classical food chain, while under mixing or weak stratification ~60% of the phytoplankton stock followed this route. To what extent mussels were responsible for this pathway was not assessed in this work, however they should be significant consumers of phytoplankton (Frechette & Bourget 1985, Dolmer 2000a). Furthermore, mussels are also capable to feed on microzooplankton (Nielsen & Maar

2007), and mixing events would favour the direct consumption of microzooplankton by mussels, which could link the microbial and classical food webs releasing phytoplankton of grazing pressure from predators, microzooplankton and mussels.

4.4.3. Concluding remarks

These results demonstrated that stratification-mixing sequences in the Limfjord influenced the processes dominating in the pelagic system as well as benthic-pelagic coupling. Stratification periods, which favour the microbial food web and pelagic consumption, are disrupted by mixing events that provide food to mussels and distribute nutrients in the water column favouring phytoplankton growth when new stratification occurs. Whenever periodic, these sequences appear to contribute to the stability of the system, and hence to maintain pelagic and benthic communities in this coastal ecosystem.

5.

ROLE OF MICROZOOPLANKTON DURING A *PHAEOCYSTIS* SP. BLOOM IN THE OOSTERSCHELDE (SW NETHERLANDS)*

ABSTRACT

The feeding impact of microzooplankton on the plankton community in the Oosterschelde basin (SW Netherlands) was assessed between 4 and 13 May 2004, during a bloom of the haptophyte *Phaeocystis* sp. The evolution of this bloom was well represented in chlorophyll *a* concentration and *Phaeocystis* sp. abundance, which reached maxima values in the middle of sampling (~27 mg chl *a* m^{-3} and 3.4 x 10^4 cells ml^{-1}, respectively). The bloom collapse coincided with the last sampling day, when accentuated decreases in chlorophyll *a* concentrations (~11 mg chl *a* m^{-3}) and *Phaeocystis* sp. cells numbers (~1.3 x 10^4 cells ml^{-1}) were recorded. Microzooplankton organisms were significant consumers of both phytoplankton and heterotrophic plankton groups. *Phaeocystis* sp. was the most consumed organism in terms of quantities of biomass (336 ± 71 mg C m^{-3} d^{-1}), but the microzooplankton impact on its standing stock was lower comparatively to the impact on other less abundant organisms. Also, microzooplankton impact on the standing stock and production of *Phaeocystis* sp. was lower during the bloom period, when the colonial forms presumably predominated, than during the bloom collapse, when free-living cells were supposedly more abundant. The microzooplankton impact on heterotrophic organisms was comparatively higher than on phytoplankton, showing also an increasing tendency towards the end of sampling. Among heterotrophs, picoplankton experienced the highest impact on the standing stock (~75% d^{-1}) and production (~90% d^{-1}). These results demonstrate that during a *Phaeocystis* sp. bloom, the microbial food web was responsible for channelling a significant fraction of plankton biomass, either from direct consumption of *Phaeocystis* sp. cells or through consumption of heterotrophs, which would have been favoured by the high quantities of released organic matter during the bloom collapse.

*The research work presented in this section is also a contribution to the paper:

Teixeira IG, Crespo BG, & Figueiras FG (to be submitted) Role of microzooplankton during a *Phaeocystis* sp. bloom in the Oosterschelde (SW Netherlands)

5.1. INTRODUCTION

Phaeocystis (Prymnesiophyceae, Haptophyta) is a worldwide distributed phytoplankton genus with a significant role in biogeochemical processes, primarily due to the production of high amounts of organic carbon and the production of sulphur compounds involved in climate regulation (e.g. Schoemann et al. 2005). The major particularity of this organism is related to its polymorphic life cycle, in which the solitary stage characterized by flagellate or non-motile free-living small cells (3–9 µm) can alternate with the colonial stage composed of non-motile cells embedded in a mucilaginous matrix (e.g. Rousseau et al. 1994, 2007). When in the form of these mucilaginous colonies, which can reach several millimetres in diameter, *Phaeocystis* spp. frequently forms nearly mono-specific blooms of huge biomass in polar and temperate regions during the spring (e.g. Schoemann et al. 2005). The magnitude of these blooms is such that locally they can account for up to 70% of the annual primary production (Joiris et al. 1982).

Despite numerous investigations, several details on the life cycle of *Phaeocystis* spp. are still poorly understood, which must be due to its morphological and physiological variability and the contrasting environmental conditions of the different systems where this algae can temporally dominate. High nutrient concentrations and high irradiance have been assigned as responsible for promoting the transformation of solitary cells into colonial forms (Veldhuis et al. 1986, Verity et al. 1988a, Peperzak 1993). Conversely, the bloom demise, habitually occurring within a short time period, has been attributed to nutrient limitation and/or changes in temperature, both causing the colony disruption and the release of the colonial cells into the water column (Veldhuis et al. 1986, Verity et al. 1988b). Pelagic consumption, cell lysis and sedimentation are other processes that can be responsible for the sudden disappearance of biomass from the water column. (Wassmann 1994, Weisse et al. 1994, Brussaard et al. 1996).

The magnitude of the pelagic consumption of *Phaeocystis* spp. cells and derived material, which determines the fraction of biomass that is transferred through the food web, has major implications for the fluxes of matter and energy in the systems where this organism dominates. Colonial forms, protected by a tough skin (Hamm et al. 1999) that aids them to avoid viral infection and grazing on individual cells, apparently are suitable for consumption by copepods and larger animals. Thus, colonial forms could contribute to sustain the classical short food chain. In fact, it has been recently reported that colony formation and enlargement in *Phaeocystis* spp. is enhanced by the presence of micrograzers, which could indicate that this strategy corresponds to a mechanical defence against predation by microzooplankton (Jakobsen & Tang 2002, Tang 2003). However, the trophic significance of colonial biomass for metazoans is uncertain. Divergent reports

regarding ingestion rates, nutritive value of the prey and adverse effects on consumers are widespread through literature (Weisse et al. 1994). These contrasting results seem to be related to the variability in the size ratio between predators and colonies, the physiological state of preys and whether experiments were performed in laboratory with *Phaeocystis* spp. as single preys or *in situ* (Nejstgaard et al. 2007). In general, crustacean grazers depict a much lower grazing impact in the field than in the laboratory, which could be due to the presence of alternative preys *in situ*, and changes in the chemical properties of *Phaeocystis* spp. in the laboratory conditions probably favouring their consumption. Rather than consuming *Phaeocystis* spp. biomass during bloom periods, copepods have been observed to feed on alternative preys, namely microzooplankton (Hansen et al. 1993, Gasparini et al. 2000). In contrast, microzooplankton apparently shows preference by solitary cells of only a few micrometers (Admiraal & Venekamp 1986, Weisse & Scheffel-Moser 1990, Tang et al. 2001, Brussaard et al. 2005a), although large naked dinoflagellates, such as *Noctiluca scintillans* and *Gyrodinium spirale*, have been observed to feed on colonies (Jakobsen & Tang 2002, Stelfox-Widdicombe et al. 2004). In addition, both colony matrix disintegration and cell lysis at the end of the bloom period, which increase the quantities of dissolved organic matter in the water column (Alderkamp et al. 2007), should enter the microbial food web through enhanced bacterial production and microzooplankton bacterivory (van Boeckel et al. 1992, Fernández et al. 1992, Brussaard et al. 1995, Brussaard et al. 1996, Rousseau et al. 2000). Nevertheless, studies assessing the importance of microzooplankton during *Phaecoystis* spp. blooms are scarce (Nejstgaard et al. 2007).

As the fate of the high biomass produced during *Phaeocystis* spp. blooms has a major implication for biogeochemical cycles in marine systems, further assessment of the role of microzooplankton during these blooms is fundamental to reach a better understanding of the functioning of the marine systems dominated by these organisms. In this context, an intensive and multidisciplinary research was conducted in the Oosterschelde (SW Netherlands) (Fig. 5.1) between 4 and 13 May 2004, coinciding with the development and decay of a *Phaeocystis* sp. bloom when phytoplankton biomass, primary production and the microzooplankton feeding impact were studied.

5.2. MATERIAL AND METHODS

5.2.1. Experimental procedure

Sampling took place on board of R/V *Luctor* between 4 and 13 May 2004. Seawater samples to determine chlorophyll *a* (chl *a*) concentration, phytoplankton biomass and

primary production were taken at three depths (surface, middle and bottom) during high tide in a location close to a mussel bed (Fig. 5.1) using Niskin bottles. The sampling depths depended on the tidal range, which varied according to the sampling days. On 9 and 12 May samples were not taken. Temperature, salinity and light penetration in the water column were measured with a CTD to which a spherical PAR quantum sensor was attached.

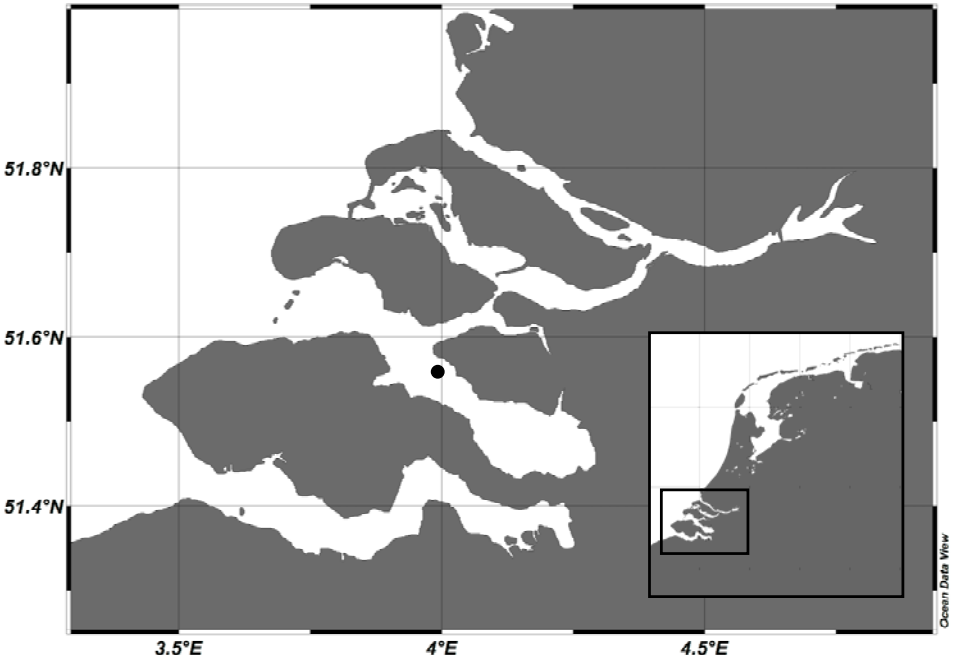

Fig. 5.1. Map of the Oosterschelde and adjacent basins with the location of the sampling site (black dot) and its position in the Dutch coast (inset).

5.2.2. Analyses

For chl *a*, three replicates of 50 ml were filtered onto GF/F filters. Pigments were extracted overnight in 5 ml 96% ethanol in the dark and chl *a* concentration was determined against a chl *a* standard after reading fluorescence before and after acidification on a Turner Designs Model 700 Fluorometer.

Samples for microplankton counts were preserved in Lugol's iodine solution and sedimented in composite sedimentation chambers. Diatoms, dinoflagellates, other flagellates >20 µm and ciliates were identified and counted at species level, when possible, using an inverted microscope. Differentiation of phototrophic species of dinoflagellates, other flagellates (hereafter, flagellates) and ciliates was basically done following Lessard and Swift (1986) and Larsen & Sournia (1991); however, epifluorescence microscopy was also used to assign phototrophy within these plankton groups. The biovolumes of each taxon were calculated from the dimensions and shapes according to Hillebrand et al. (1999). The plasmatic volumes of diatoms and total cell

volume of dinoflagellates, flagellates >20 µm and ciliates were converted to cell carbon following Strathmann (1967) for diatoms and dinoflagellates, Verity et al. (1992) for flagellates and Putt & Stoecker (1989) for ciliates. The fixation procedure did not allowed us to quantify the number of colonies of *Phaeocystis* sp., and consequently the carbon biomass calculated for this species does not include the carbon content of the colonial matrix (Rousseau et al. 1990, van Rijssel et al. 1997).

Primary production was measured as carbon assimilation after 24h *in situ* incubations. Samples were placed in tissue culture flasks (3 light flasks and 2 dark flasks) and immediately inoculated with ca. 10 µCi of $NaH^{14}CO_3$. Then, samples were placed in a rope with specially constructed holders. A weight kept the line vertical, whereas the surface of the line was maintained afloat by a large buoy. After 24h of incubation samples were recovered and filtered through 25 mm GF/F filters under low vacuum pressure. Filters were placed in scintillation vials and left in HCl fumes atmosphere for 12h to remove unassimilated ^{14}C. Radioisotope incorporation was determined with a liquid scintillation counter using the external standard and the channel ratio methods to correct for quenching. Integrated primary production (mg C m^{-2} d^{-1}) was calculated using the trapezoid rule.

5.2.3. Microzooplankton feeding impact

The feeding impact of microzooplankton on the several organisms of the plankton community was determined using the dilution technique (Landry & Hassett 1982) on 4, 6, 8, 10 and 13 May 2004. Water was collected from the surface with a Niskin bottle before dawn. All experimental containers, bottles and tubing were soaked in 10%HCl and rinsed with Milli Q water before each experiment. Filtered water was obtained by gravity filtration through a 0.8/0.2 µm Suporcap filter into a 25 l container. Sample water was transferred to another 25 l container, very gently through a tube. In the laboratory, dilutions were prepared directly in 2.3 l polycarbonate bottles (Nalgene). A total of 6 dilutions, in the proportions of 100, 80, 60, 40, 20 and 10% of sample relatively to filtered water were performed, with two replicates each. These bottles were incubated for 24h in running seawater and at *in situ* temperature and surface light conditions.

Samples to determine the biomasses of plankton organisms ≤200 µm were taken from the natural seawater at the beginning of the incubation and from each incubation bottle at the end of the incubation. Samples for microplankton biomass determinations were processed as described above. Pico- and nanoplankton were determined in subsamples of 10 ml and 40 ml respectively, 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 and 0.8 µm

black Millipore polycarbonate filters, respectively. 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. Bacterial biomass was estimated according to Lee & Fuhrman (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.

Changes in total plankton (≤200 µm) biomass and in the several plankton groups occurring between the beginning (C_0) and the end (C_t) of the incubation time (t = 1 day) were used to calculate the net growth rates (k, d^{-1}):

$$k = \frac{1}{t} \cdot \ln\left(\frac{C_t}{C_0}\right) \tag{5.1}$$

Mortality rates due to microzooplankton (m, d^{-1}) were calculated as the slope and the specific growth rates (μ, d^{-1}) as the y-axis intercept of the linear regression between the net growth rates and the fraction of unfiltered seawater (X) (Landry & Hassett 1982):

$$k = \mu - m \cdot X \tag{5.2}$$

In the cases of non-linear responses (saturated and saturated-increased), the specific growth rate (μ) was obtained by the y-axis intercept of the regression of the linear part of the response. The mortality rate (m) was obtained by equation 5.2, replacing the specific growth rate calculated and using the net growth rate obtained in the undiluted sample (see section 2).

The daily mortality impact on the standing stock (%SS, $\%d^{-1}$) and on production (%P, $\%d^{-1}$) were calculated as:

$$\%SS = 1 - e^{-m} \times 100 \tag{5.3}$$

$$\%P = \frac{m}{\mu} \times 100 \tag{5.4}$$

The quantity of carbon consumed (G, mg m^{-3} d^{-1}) and produced (P, mg m^{-3} d^{-1}) were estimated as:

$$G = m \times C_m \tag{5.5}$$

$$P = \mu \times C_m \tag{5.6}$$

where C_m (mg m^{-3}) is:

$$C_m = C_0 \left[e^{(\mu - m)t} - 1 \right] / (\mu - m)t \tag{5.7}$$

5.3. RESULTS

5.3.1. Hydrographic conditions

The water column (maximum depth = 6.5m) was well mixed from surface to bottom during all days (Figs. 5.2a & b). There was a slight increase in the salinity of the water column, which varied between 30.7 on the first 3 days and values higher than 31 on the last two days (Fig. 5.2a). Temperature did not show any tendency, varying between 12.6 and 13.3ºC (Fig. 5.2b).

The light attenuation coefficient in the water column (Fig. 5.2c) varied between 0.66 and 1.03 m^{-1}, with the highest value occurring on 8 May and the lowest on 13 May, at the end of the sampling. Relatively low values (0.71 m^{-1}) were also recorded at the beginning of the sampling, on 4 May (Fig. 5.2c).

5.3.2. Chl *a* and *Phaeocystis* sp.

Chl *a* concentration increased from 20.4 (± 1.0) to 26.8 (± 1.3) mg chl *a* m^{-3} during the first 5 days, falling to 11.1 (± 0.2) mg chl *a* m^{-3} on the last day (Fig. 5.3a). Differences in chl *a* concentrations between the 3 sampling depths were not significant ($0.40 < p < 0.88$; t-test for paired samples).

Chl *a* concentration was positively correlated with phytoplankton abundance ($r = 91$, $p < 0.01$) and carbon biomass ($r = 0.89$, $p < 0.01$), two variables that showed a similar evolution during the sampling period (Fig. 5.3b & c). The phytoplankton community was dominated by *Phaeocystis* sp., which accounted for ~98% of the total phytoplankton abundance and ~90% of the total phytoplankton biomass (Fig. 5.3 b & c). This species

reached an abundance of 3.4 x 10^4 (± 1.3 x 10^4) cells ml^{-1} and a biomass of 871 (± 343) mg C m^{-3} during the peak of the bloom on 8 May, and then dropped to 1.3 x 10^4 (± 2.5 x 10^3) cells ml^{-1} and 345 (± 63) mg C m^{-3} on the last day. Differences between depths in abundance and biomass of *Phaeocystis* sp. and total phytoplankton were not significant ($0.40 < p < 0.95$; t-test for paired samples). Despite *Phaeocystis* sp. was mainly forming colonies (as it was observed in fresh samples and deduced from the high accumulation of gelatinous structures on filters), the lugol's iodine that was added to preserve the phytoplankton samples destroyed these structures. This resulted in a much higher abundance of *Phaeocystis* sp. as solitary cells (99.4%) than as colonial forms (0.6%) in the counted samples.

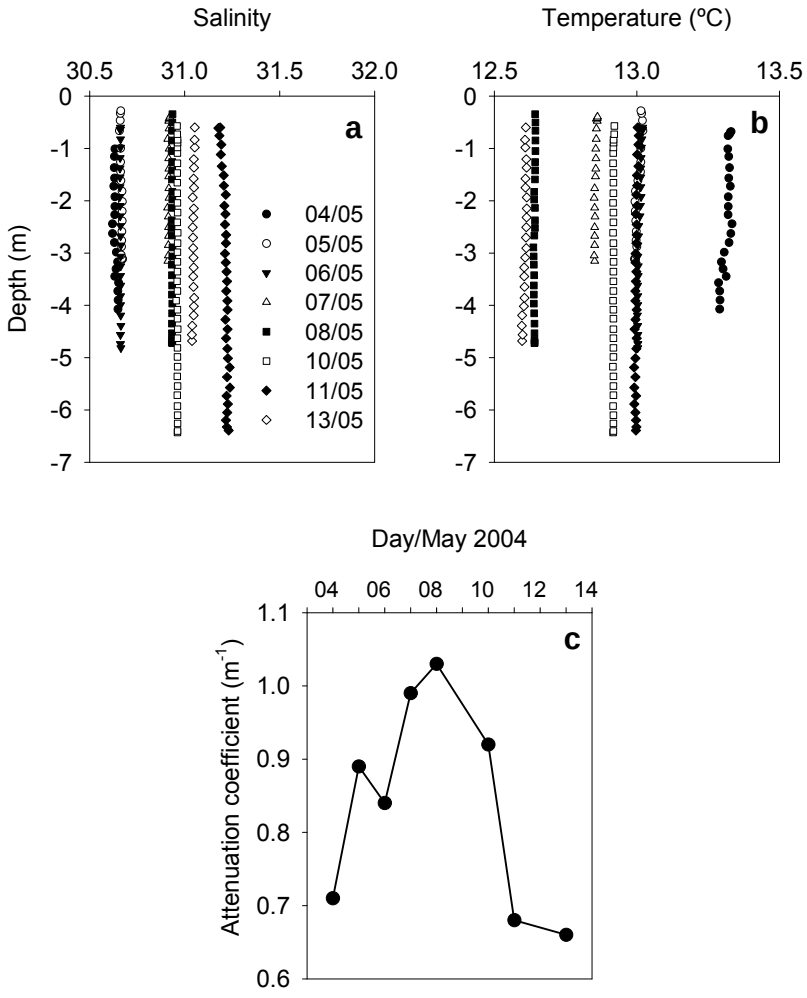

Fig. 5.2. Vertical profiles of (a) salinity (psu), (b) temperature (ºC), and (c) light attenuation coefficient (m^{-1}) in the water column.

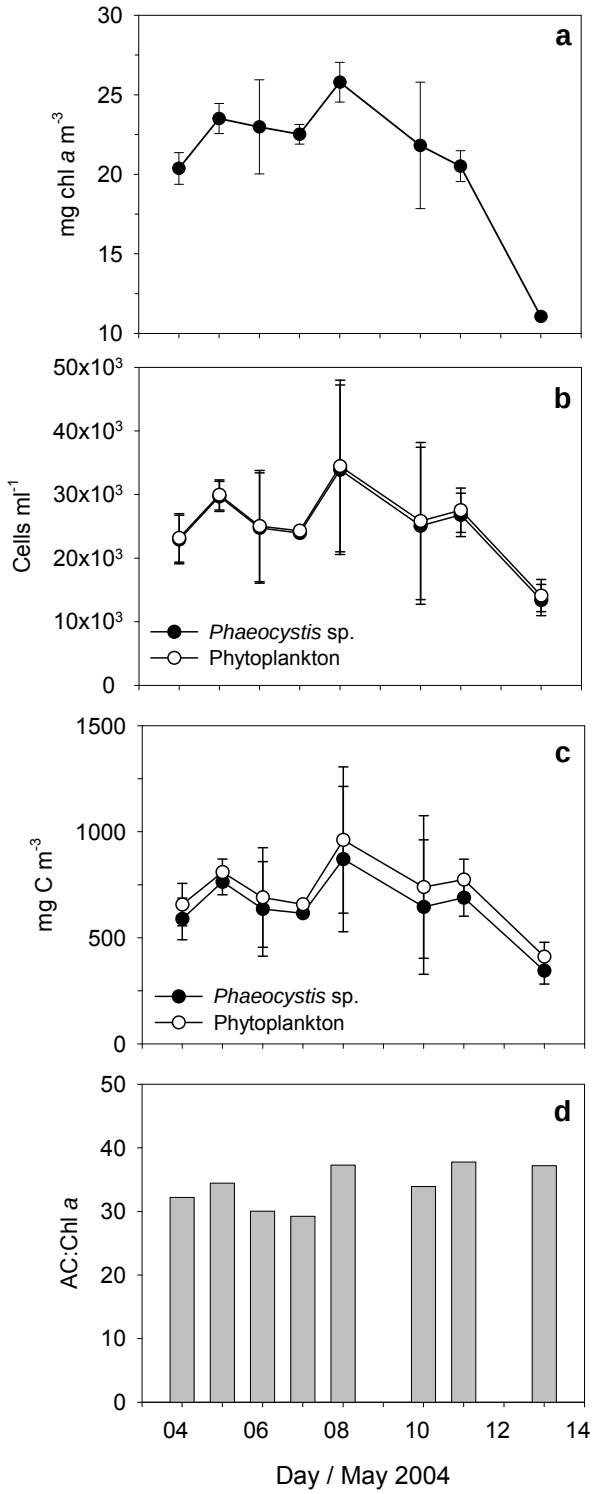

Fig. 5.3. Mean values in the water column of (a) chl a concentration (mg chl *a* m^{-3}), (b) cell abundance of *Phaeocystis* sp. and total phytoplankton (cells ml^{-1}), (c) carbon biomass of *Phaeocystis* sp. and total phytoplankton (mg C m^{-3}) and (d) autotrophic carbon to chl *a* ratio. Vertical bars in a, b, and c are standard deviations.

As a result of the correlation between phytoplankton carbon biomass and chl *a* concentration, the autotrophic carbon to chl *a* (AC:chl *a*) ratio (Fig. 5.3d) remained

relatively constant around a mean value of 34 ± 3 during the sampling period. Nevertheless, the AC:chl *a* ratio was slightly but significantly lower ($p < 0.05$, t-test for two samples) when chl *a* and phytoplankton biomass increased (31 ± 2, 4 to 7 May) than when these two variables decreased (37 ± 2, 8 to 13 May)

5.3.3. Primary production

Primary production decreased with depth (Fig. 5.4) in accordance with the high attenuation coefficient in the water column (Fig. 5.2c). Thus, primary production varied between 102 and 608 mg C m^{-3} d^{-1} at the surface, 76 and 444 mg C m^{-3} d^{-1} at the middle depth and between 0 and 89 mg C m^{-3} d^{-1} at the bottom layer (Fig. 5.4), being higher the differences between depths on 8 May. Thus, the nil value recorded at the bottom layer on 8 May coincided with the strongest light attenuation in the water column (Fig. 5.2c), which led to a photic layer shallower than the depth at the station. Depth integrated primary production varied between 353 and 1626 mg C m^{-2} d^{-1}, with lowest values at the beginning and the end of the sampling period (Fig. 5.4) and highest values in between, on 8 and 10 May. Integrated primary production was positively correlated with integrated phytoplankton carbon biomass ($r = 0.74$; $p < 0.05$).

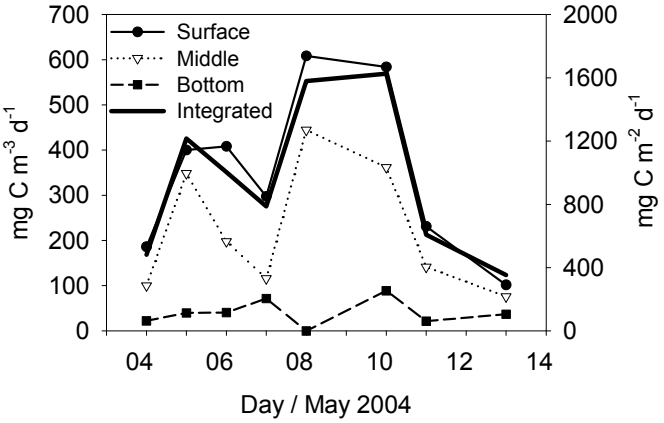

Fig. 5.4. Primary production rates at the surface and at middle and bottom depths (mg C m^{-3} d^{-1}). Values of integrated primary production (mg C m^{-2} d^{-1}) are also given.

5.3.4. Microzooplankton feeding impact

In addition to *Phaeocystis* sp., which represented ~90% of total phytoplankton biomass (Fig. 5.5a), diatoms were also present in the initial plankton populations of dilution experiments. Nevertheless, diatoms accounted for a minor fraction (3-7%) of the total autotrophic carbon (AC). The biomass of diatoms increased from ~25 mg C m^{-3} during the first days to ~40 mg C m^{-3} on the last days. Among this group, *Rhizosolenia imbricata*, *Guinardia delicatula*, *Cerataulina pelagica* and small and large *Pseudo-nitzschia* spp. were the most abundant species. The phototrophic ciliate *Mesodinium*

rubrum, autotrophic dinoflagellates and picoautotrophs (*Synechococcus*-like cyanobacteria and picoflagellates) were also found, but their contribution to phytoplankton biomass was very low (Fig. 5.5a). The evolution of heterotrophs was different to that of autotrophs (Fig. 5.5b), showing a peak on 6 May (155 mg C m^{-3}) due to the high biomass of heterotrophic nanoflagellates (HNF) on that day (121 mg C m^{-3}). HNF, which dominated within microzooplankton community at the beginning of the sampling period, accounting almost for 90% of the microzooplankton biomass, decreased from 6 May onwards. On the last day of sampling, HNF with a biomass of 12 mg C m^{-3} only represented 20% of microzooplankton carbon. Heterotrophic picoflagellates (HPF) showed also a decreasing tendency during the sampling period, but their biomass was much smaller, varying between 3.8 and 1.3 mg C m^{-3}. On the contrary, the importance of heterotrophic dinoflagellates (HDF) (mainly naked forms >20 µm), ciliates (mainly aloricate choreotrichs >50 µm) and heterotrophic bacteria (HB) increased through the sampling period (Fig. 5.5b); HDF from 3 to 26 mg C m^{-3}, ciliates from 7 to 23 mg C m^{-3} and HB from 19 to 37 mg C m^{-3}.

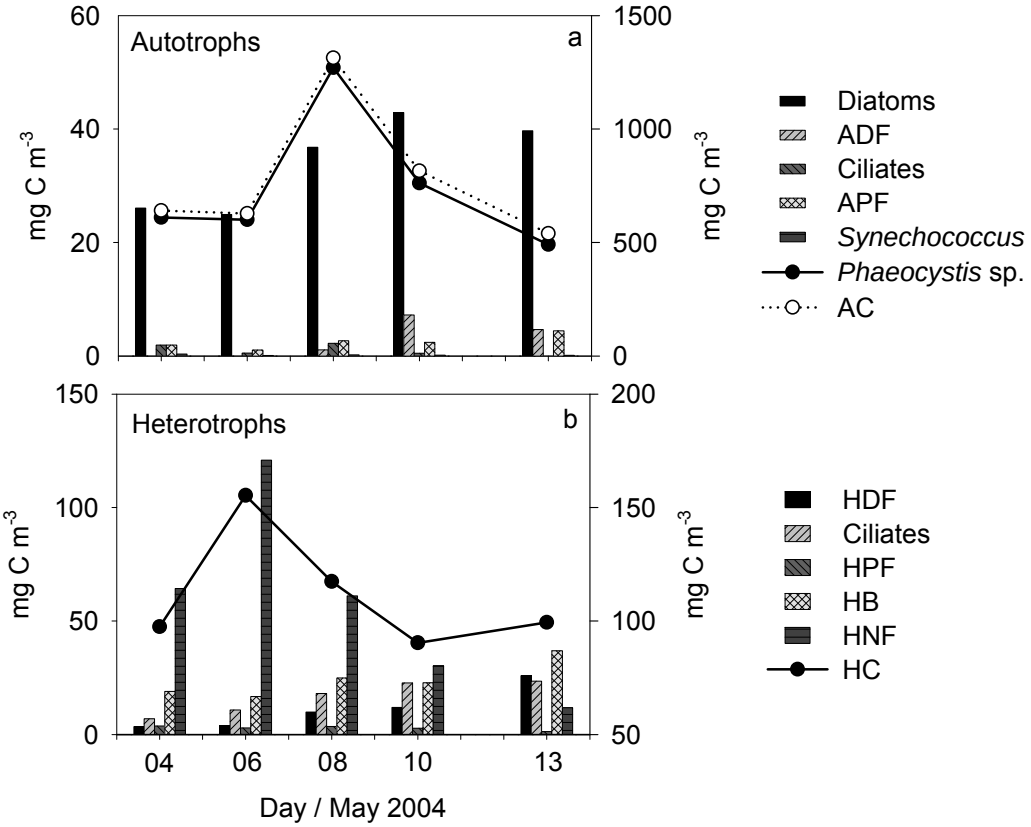

Fig. 5.5. Initial carbon biomass for each dilution experiment of (a) autotrophic and (b) heterotrophic plankton community. ADF: autotrophic dinoflagellates, APF: autotrophic picoflagellates, AC: autotrophic carbon, HDF: heterotrophic dinoflagellates, HPF: heterotrophic picoflagellates, HB: heterotrophic bacteria, HNF: heterotrophic nanoflagellates, HC: heterotrophic carbon. Left y-axis corresponds to bars and right y-axis corresponds to lines.

Table 5.1: Mortality (m, d^{-1}) and growth (μ, d^{-1}) rates obtained from the dilution experiments. AC: total autotrophic carbon, APF: autotrophic picoflagellates, HC: total heterotrophic carbon, HPF: heterotrophic picoflagellates, HNF: heterotrophic nanoflagellates, HB: heterotrophic bacteria, TC: total plankton carbon biomass (≤200µm). r^2 is the coefficient of determination for the linear regressions ($p < 0.05$) between the net growth rate and the fraction of unfiltered seawater. In cases of saturated or saturated-increased responses, r^2 was obtained for the linear part of the response (Section 2). When r^2 is not given, the growth rate was obtained only with the two highest diluted bottles (Gallegos 1989). a: saturated response. b: saturated-increased response. ns: not significant.

	04-05			06-05			08-05			10-05			13-05		
	m	***µ***	r^2	***m***	***µ***	r^2	***m***	***µ***	r^2	***m***	***µ***	r^2	***m***	***µ***	r^2
AC	0.39	0.66	0.84[b]	0.51	0.60	0.73[a]	0.23	0.36	b	0.39	0.91	0.76[a]	0.58	0.16	0.91
Diatoms		ns			ns			ns			ns		0.54	0.64	0.90[b]
Synechococcus	0.43	0.70	0.99[a]	0.21	1.26	0.93[a]	0.35	0.30	0.84	1.02	1.10	0.90	1.33	1.33	0.95[b]
APF	0.36	0.49	0.93		ns		1.02	1.04	0.71	0.87	1.67	0.70		ns	
***Phaeocystis* sp.**	0.43	0.70	0.86[b]	0.57	0.61	0.74[a]	0.26	0.38	b	0.43	0.96	0.76[a]	0.62	0.15	0.89
HC	0.69	1.24	0.89	0.97	1.03	0.80	1.36	1.37	0.97	1.25	1.48	0.87[a]	1.48	1.31	0.95
HPF	0.78	1.48	0.84[a]	0.80	1.49	0.80[a]	1.42	1.09	0.85	2.42	2.45	0.88[a]	2.64	2.74	0.91
HNF	0.00	1.09	0.95[b]	1.20	1.00	0.93	1.15	1.59	0.91	1.60	1.70	0.72	2.36	3.08	0.85
HB	1.73	1.42	0.93	1.45	1.78	0.86[a]	1.15	1.20	0.81	1.52	1.86	0.96[a]	1.20	1.23	0.91[a]
TC	0.44	0.77	0.96[b]	0.52	0.53	0.67	0.48	0.53	b	0.51	1.00	0.75[a]	0.65	0.25	0.90

According to the outcome of the dilution experiments (Table 5.1), heterotrophic pico- and nanoplankton (HB, HPF and HNF), *Synechococcus* and *Phaeocystis* sp. were consumed during all the sampling period. Diatoms were also consumed, but only on May 13. Growth and mortality rates were highly variable amongst the several organisms, with growth rates varying between 0.15 and 3.08 d^{-1} and mortality rates between 0 and 2.64 d^{-1}. The rates were generally higher for total heterotrophic carbon (HC) ($1.03 \leq \mu \leq 1.48$ and $0.69 \leq m \leq 1.48$) than for total AC ($0.16 \leq \mu \leq 0.91$ and $0.23 \leq m \leq 0.58$). Growth and grazing rates of AC were very similar to those obtained for *Phaeocystis* sp. ($0.15 \leq \mu \leq 0.96$ and $0.26 \leq m \leq 0.62$).

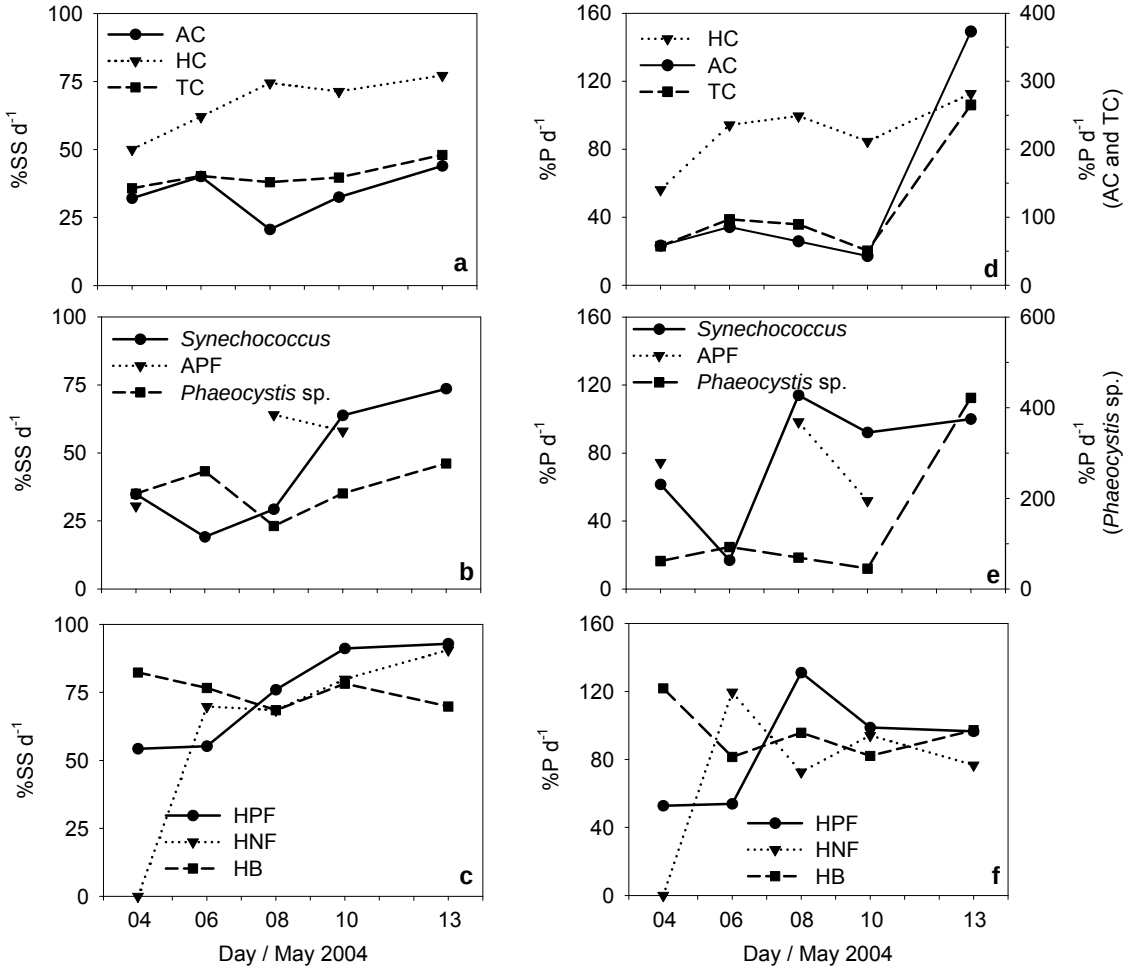

Fig. 5.6. Percentages of standing stock (a-c) and production (d-f) consumed daily by microzooplankton of the bulk plankton groups (a & d), autotrophic plankton groups (b & e) and heterotrophic plankton groups (c & f). AC, autotrophic carbon; HC, heterotrophic carbon; TC, total carbon; APF, autotrophic picoflagellates, HPF, heterotrophic picoflagellates; HNF, heterotrophic nanoflagellates; HB, heterotrophic bacteria.

In terms of percentages of the standing stock consumed by microzooplankton (Figs. 5.6a-c), HC was more impacted (Fig. 5.6a, 67 ± 11%) than AC (Fig. 5.6a, 34 ± 9%). Moreover, there was an increase in the consumption of the standing stock of HC from 50% being ingested at the beginning of the sampling to almost 80% at the end (Fig. 5.6a). Among autotrophs, the lowest grazing impact (21%) was found on 8 May (Fig 5.6a), coinciding with the maximum peak in the abundance of *Phaeocystis* sp. (Fig. 5.3b, c). Regarding the several plankton groups, the highest impact occurred on the standing stock of picoheterotrophs (75 ± 6% for HB and 74 ± 19% for HPF), followed by HNF (62 ± 36%), though HNF were not consumed on 4 May (Fig. 5.6c). The impact on the standing stock of *Phaeocystis* sp. (37 ± 9%) was relatively low, always below 50%. On average, 40 ± 5% of the whole plankton standing stock (≤200µm) was consumed by microzooplankton (Fig. 5.6a).

Concerning the microzooplankton impact on production, a sudden increase was observed for AC and TC on the last day of sampling (Fig. 5.6d), when consumption shifted from 63 ± 18% of AC and 74 ± 23% of TC produced during the previous days to 373% of the AC and 265% of the TC produced on 13 May. This evolution closely resembled that observed for the impact on the production of *Phaeocystis* sp. (Fig. 5.6e), which derived from a consumption of 67 ± 20% of the production between 4 and 10 May to a value of 422% on 13 May. The impact on the production of the other two phytoplankton groups with significant responses in the dilution experiments was highly variable, ranging from 17 to 100% for *Synechococcus* and from 52 to 98% for APF (Fig. 5.6e). In contrast, the microzooplankton impact on the production of HC gradually increased over the sampling period, varying from 56% on the first day to 113% on the last day (Fig. 5.6d), showing a relatively constant and significant impact (96 ± 16%) on the production of HB (Fig. 5.6f). The other two heterotrophic groups, HPF and HNF, also experienced a high impact (87 ± 33% and 73± 45%, respectively) on their production, although it was highly variable (Fig. 5.6f).

In spite of these percentages, consumption of autotrophic biomass (255–420 mg C m^{-3} d^{-1}) was always higher than consumption of heterotrophic biomass (90–161 mg C m^{-3} d^{-1}) (Fig. 5.7a). *Phaeocystis* sp. was virtually the only phytoplankton species consumed from 4 to 10 May and represented 95% of the total AC removed on 13 May, when diatoms were also consumed (Fig. 5.7b). Among heterotrophs (Fig. 5.7c), HNF, which were not consumed on the first day of sampling, however were highly consumed on 6 May (131 mg C m^{-3} d^{-1}), showing then a decreasing tendency. In contrast, the HB biomass daily removed by microzooplankton increased over the sampling period, from 28 mg C m^{-3} d^{-1} on the first day to 45 mg C m^{-3} d^{-1} in the last dilution experiment. The autotrophic biomass produced (68-980 mg C m^{-3} d^{-1}) during the sampling period showed higher variations than

the biomass consumed (Figs. 5.7a & d). Again, *Phaeocystis* sp. was the phytoplankton species with the highest production (Figs. 5.7b & e), representing >96% of the total autotrophic carbon produced. In contrast, consumption (90-161 mg C m^{-3} d^{-1}) and production (120-164 mg C m^{-3} d^{-1}) of heterotrophic biomass were more tightly coupled (Figs. 5.7a,d,c,f), with HNF showing higher consumption during the first 3 days of sampling and HB acquiring more importance at the end.

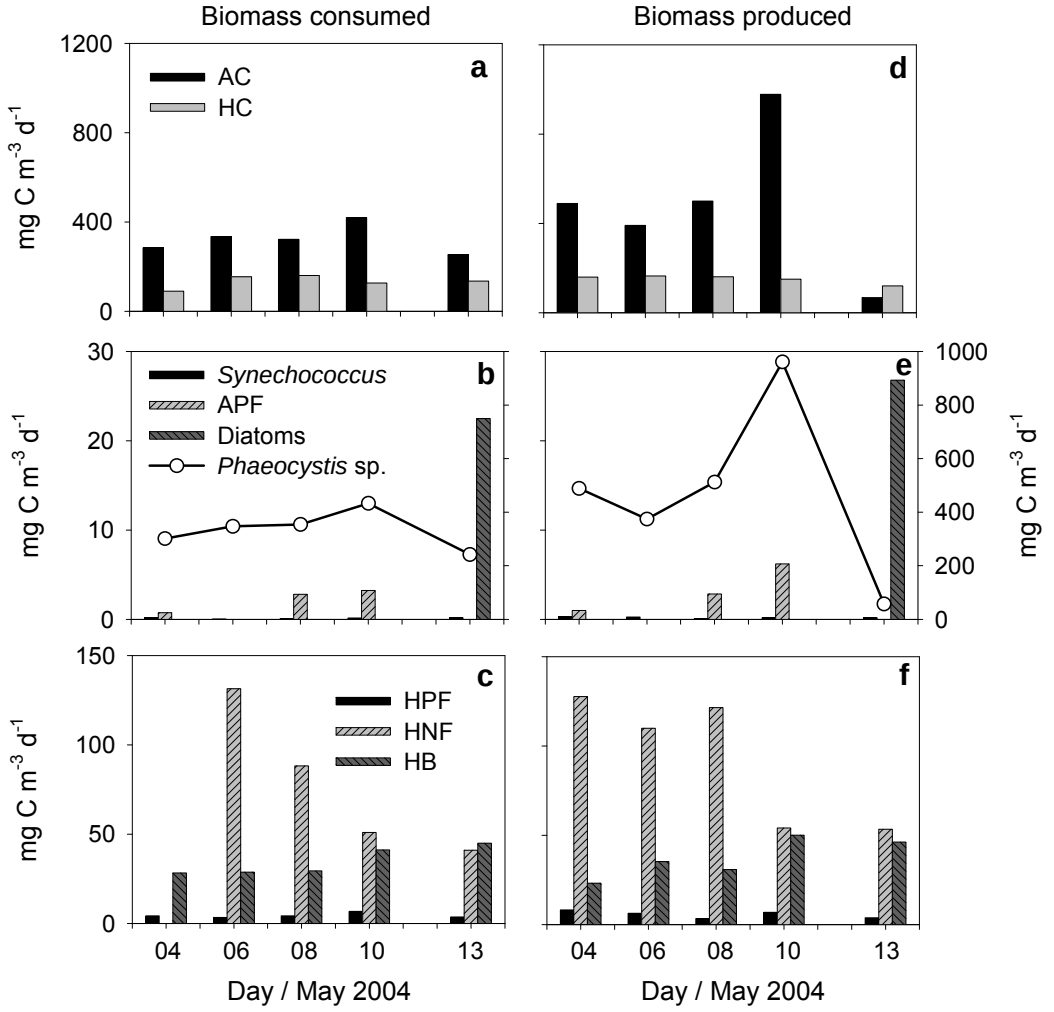

Fig. 5.7. Plankton carbon biomass daily ingested by microzooplankton (left panels) and daily produced (right panels). (a & d) total autotrophic heterotrophic plankton, (b & e), autotrophic plankton groups, (c & f) heterotrophic plankton groups. AC, autotrophic carbon; HC, heterotrophic carbon; APF, autotrophic picoflagellates, HPF, heterotrophic picoflagellates; HNF, heterotrophic nanoflagellates; HB, heterotrophic bacteria. Right y-axis in b & e corresponds to lines.

5.4. DISCUSSION

5.4.1. The *Phaeocystis* sp. bloom

Blooms of *Phaeocystis* spp. in the Oosterschelde (Laanbroek et al. 1985, Bakker et al. 1990) and in the adjacent North Sea (e.g. Veldhuis et al. 1986, Weisse & Scheffel-Möser 1990, Rousseau et al. 2000) are frequent in spring. Here, we followed an almost monospecific *Phaeocystis* sp. bloom in the Oosterschelde well reflected in the evolution of chl *a* concentration and cell abundance (Fig. 5.3a, b). The bloom was also discernible in the evolution of primary production (Fig. 5.4), which was correlated with *Phaeocystis* sp. biomass, and in the evolution of the light attenuation coefficient (Fig. 5.2c). Even though the progress of this bloom was observed inside the Oosterschelde, its advection from the North Sea cannot be disregarded, because the increase in salinity recorded during the sampling period (Fig. 5.2a) points to the possible input of water from the North Sea, saltier than the water in the Oosterschelde (Laanbroek et al. 1985).

The high chl *a* concentration (20 mg chl *a* m^{-3}) and the high cell abundance of almost 23 x 10^6 cells l^{-1} *Phaeocystis* sp. (Fig. 5.3a, b) recorded on the first day of sampling, above the threshold reported for the formation of colonies (Schoemann et al. 2005), indicate that our sampling started after the onset of the bloom, when *Phaeocystis* sp. was already on the colonial form. The bloom rapidly evolved to reach its maximum 4 days later, and then declined. Changes in temperature and/or nutrient limitation have been assigned as triggering factors for the beginning of bloom collapse (Veldhuis et al. 1986, Verity et al. 1988b). In this case temperature did not show any significant change (Fig. 5.2b) and unfortunately nutrients were not determined during this study. However, the changes observed in the AC:chl *a* ratios (Fig. 5.3d), with slightly but significant higher ratios during the demise phase, point to nutrient limitation as the possible factor triggering the bloom decline. Other loss factors, as sedimentation, have also been reported, but sinking seems unusual in this region where tidal currents are strong enough to prevent it (van Boeckel et al. 1992, Wassmann 1994, Brussaard et al. 1995). In fact, in this work we did not observed accumulation of phytoplankton in bottom layers at the end of sampling (Fig. 5.3a-c).

5.4.2. Microzooplankton feeding impact

Our results showed that microzooplankton organisms were significant consumers in the pelagic system, but their impact varied during the bloom period (Fig. 5.6). The highest quantity of carbon biomass consumed by microzooplankton corresponded to *Phaeocystis* sp. cells (Figs. 5.7a & b). However, this pattern was forced by the dominance of this species during the sampling period; resulting in higher quantities consumed even though

mortality rates were lower than those of the other plankton groups (Table 5.1). Despite these high quantities consumed, microzooplankton was unable to control the bloom development, consuming only a fraction of the *Phaeocystis* sp. standing stock and production (Figs. 5.6b & e). However, on the last sampling day, when the bloom decayed, microzooplankton consumption exceeded 4 times the production of this haptophyte (Fig. 5.6e). These variations in consumption must be related to the structural changes occurring in *Phaeocystis* sp. through the sampling period. With exception of few dinoflagellate species, such as *Noctiluca scintillans* (Jakobsen & Tang 2002) or *Gyrodinium spirale* (Stelfox-Widdicombe et al. 2004), microzooplankton is not an efficient consumer either of the whole colonial structure or the colonial single cells protected by the colony skin (Hamm et al. 1999). However, when *Phaeocystis* sp. is in the form of single cells in the water column, microzooplankton organisms are able to ingest them (Admiraal & Venekamp 1986, Weisse & Scheffel-Möser 1990, Tang et al. 2001). Here we found a higher microzooplankton grazing on *Phaeocystis* sp. on the last day (Figs. 5.6b & e), when colonies presumably had collapsed and cells were free-living. As on the previous days *Phaeocystis* sp. was probably forming colonies, the impact of microzooplankton would have occurred on the less abundant solitary cells that usually are present in the water column coexisting with colonial structures (Rousseau et al. 1994). Consumption of some colonial forms could also have occurred, because large naked forms predominated among heterotrophic dinoflagellates and these organisms could feed on *Phaeocystis* sp. colonies as *Gyrodinium spirale* do it (Stelfox-Widdicombe et al. 2004). It must be noted however that the accentuated impact on *Phaeocystis* sp. production on May 13 was not due to a higher grazing mortality, but to the low growth rate of *Phaeocystis* sp. on this day, several times lower than the mortality rate (Table 5.1). This low growth rate can indicate that microzooplankton was not the only mortality factor for *Phaeocystis* sp. on this day. When viral lysis is significant, the conventional dilution protocol can under-estimate the specific growth rates (Evans et al. 2003, Baudoux et al. 2006). Cell lysis, mediated by viral attack, has been shown to be important in the termination of the *Phaeocystis* spp. blooms and, together with microzooplankton grazing, have been assigned as significant factors for the decrease in biomass (Rousseau et al. 2000, Brussaard et al. 2005a, Baudoux et al. 2006). Thus, the high microzooplankton grazing on *Phaeocystis* sp. production at the end of the bloom must be taken carefully, as probably both viral lysis and grazing by micro-heterotrophs were acting on this alga simultaneously.

Consumption by microzooplankton during the *Phaeocystis* sp. bloom was not restricted to this alga. Picoautotrophs were also grazed (Table 5.1), although the quantities of carbon consumed of these organisms were insignificant due to their low biomasses (Fig. 5.7b). Grazing on diatoms, which only occurred on the last day (Table

5.1), could be related to the presence of *Pseudo-nitzschia* spp., species that are known to be attached to *Phaeocystis* sp. colonies (Sazhin et al. 2007). Thus, *Pseudo-nitzschia* spp. could have been attached to the colonial forms during the bloom and hence be protected against grazing. On the last day, with the disintegration of colonies, *Pseudo-nitzschia* spp. could became free-living, and as *Phaeocystis* sp. free cells be more susceptible to pelagic consumption.

In addition to phytoplankton, heterotrophs were also an important nutritional source for microzooplankton (Fig. 5.7a & c). In relative terms of biomass and abundance, microzooplankton impact on heterotrophs was actually higher than on phytoplankton (Figs. 5.6a). Among this group, picoheterotrophs were highly impacted by microzooplankton, with ~75% of their standing stock and ~90% of their production being channelled to the microbial food web (Fig. 5.6c & f). The microzooplankton consumption was not enough to fully control HB, which showed slight increases in biomass (Fig. 5.5b) and production (Fig. 5.7f) over the sampling period, reaching maximum values during the bloom decay. This increase can be associated with the response of HB to the increase in dissolved organic matter concentrations due to colony disintegration (Laanbroek et al. 1985, Veldhuis et al. 1986, Noordkamp et al. 2000, Verity et al. 1988b, Rousseau et al. 2000, Brussaard et al. 2005b). HNF were also consumed (Fig. 5.7c), in agreement with other studies indicating consumption of these organisms by microzooplankton during *Phaeocystis* sp. blooms (Brussaard et al. 1995, 1996, Rousseau et al. 2000).

Noteworthy, microzooplankton impact increased towards the end of the sampling period (Fig. 5.6). This was not only observed for grazing on phytoplankton, but also for predation on heterotrophic components, and this indicates an increasing importance of the microbial food web in the transfer of biomass with the progress of the bloom. These results adds new evidences to previous reports (Admiraal & Venekamp 1986, Weisse & Scheffel-Möser 1990, Brussaard et al. 1995, Peperzak et al. 1998, Rousseau et al. 2000), supporting the view that *Phaeocystis* sp. blooms favour the dominance of the microbial food web.

5.4.3. Concluding remarks

These results indicate that a significant fraction of the *Phaeocystis* sp. bloom was channelled through the microbial food web, with two pathways in which microzooplankton is a direct intervenient. Firstly, direct consumption of *Phaeocystis* sp. cells by microzooplankton occurred in all experiments and increased during the bloom decay. Secondly, microzooplankton consumption of small heterotrophs allowed the return to the food web of the high amounts of organic matter released during the collapse of the bloom (Alderkamp et al. 2007): HB taking advantage of the released organic matter, and HNF

being favoured by the increase in bacterial production. Thus, microzooplankton, which is consumed by copepods during *Phaeocystis* blooms (Hansen et al. 1993, Gasparini et al. 2000), transfers the photosynthesised organic matter to higher trophic levels, linking the microbial and classical food webs and therefore enhancing the efficiency of the pelagic food web.

6.
GENERAL DISCUSSION

6.1. METHODOLOGICAL CONSIDERATIONS

The dilution technique (Landry & Hassett 1982) revealed as a useful and easy tool to estimate the microzooplankton feeding impact in the three coastal systems studied here. Comparing with the other methods reported in literature (e.g. Gifford 1988, Landry 1994), the simplicity of the protocol, the little manipulation required and the fact that growth and mortality rates of preys can be obtained simultaneously, convert the dilution technique in an advantageous procedure to estimate microzooplankton herbivory, and in a lesser extent bacterivory, worldwide (e.g. Landry et al. 1984, Tremaine & Mills 1987, Calbet & Landry 2004). However, the concurrent use of this method with microscopy to determine the microzooplankton feeding impact on the several plankton groups, such as was performed in this work, is not commonly explored (Landry et al. 1984, Paranjape 1990, Weisse & Scheffel-Möser 1990, Fonda Umani & Beran 2003). Three main disadvantages are inherent to the use of microscopy, when compared with the other techniques normally applied together with the dilution method: (i) it is laborious; (ii) it is time-consuming and (iii) it provides some results with high inaccuracy, such as those coming from counting errors of very low abundant organisms. Nevertheless, microscopic analyses in dilution experiments supply valuable information regarding the selective impact of microzooplankton on the several plankton species and groups present in each experiment. Thus, the information is not limited to the autotrophic component, as given by chl *a* and pigment analysis, or to the small-sized organisms, when cell enumeration by flow cytometry is performed. Moreover, cell enumeration and identification by microscopy does not include the problems that habitually are associated with pigment analyses. Chl *a* determinations or pigment analysis by HPLC can provide erroneous estimates of growth and grazing rates due to the presence of intact pigments inside predators at the beginning or the end of the incubation (Barlow et al. 1988, Waterhouse & Welschmeyer 1995, Mostajir et al. 1998, Strom et al. 1998) or to changes in pigment concentrations caused by light acclimation during incubation (McManus 1995). Therefore, microscopy should be considered as an important complement to the dilution technique that gives a comprehensive insight of the trophic relationships existing in pelagic microbial food webs, despite the labour and time that it requires.

The analysis of all components of the plankton community was also useful in this work to validate the efficiency of the dilution technique to estimate growth and mortality rates. Regardless of its widespread application, several critics connected to the correct accomplishment of the basic assumptions of the method shade its strength (see section 1, sub-section 1.5). Several concerns about the violation of the basic assumptions of this methodology emerged in this work when two types of non-linear responses were obtained in the three coastal systems. While saturated feeding responses are very common and

well-accepted (Gallegos 1989, McManus & Ederington-Cantrell 1992, Strom et al. 2001), the saturated-increased response was only reported in two works (Gallegos 1989, Elser & Frees 1995). The internal checking of the basic assumptions of the dilution method performed with the results from the Ría de Vigo showed that non-linear responses can result from non-linear feeding behaviour of microzooplankton in response to food availability. Although this conclusion implies that the assumption according to which microzooplankton feeding is linearly related to prey density was not realized, the use of the technique is still valid, once mathematical calculations of the growth and mortality rates are adapted to go beyond this difficulty (Gallegos 1989, section 2). Applying the same reasoning to the other two coastal systems, the same type of feeding behaviour of the microzooplankton could explain the occurrence of non-linear responses. Both Limfjord (section 4) and Oosterschelde (section 5) were systems with high plankton biomass, where microzooplankton could choose different preys depending on their concentration.

Based on the approach used to calculate mortality and growth rates whenever non-linear responses were obtained, some modifications in the dilution protocol are proposed to optimize the use of the dilution technique (section 2). Namely, it is considered that the intermediate dilution levels are not needed and hence the dilution series can be limited to three levels: two high dilution levels, which could allow estimating the specific growth rate, and the unfiltered sample from where the mortality rate can be deduced. This change implies the reduction in the water volumes needed as well as the reduction in the time required to filter water and prepare the dilution levels. Currently, a similar approach is being used by Landry et al. (Landry et al. 1984, 2008), who performs the dilution technique with only two dilution levels that enable him to estimate both rates assuming a linear relationship of the response. To relax the linear assumption, once non-linear relationships were obtained here, another high dilution level is necessary for the correct estimation of the specific growth rate. The use of only three dilution levels, with the consequent reduction in time and labour, enable the organization of more complex experiments, such as those that allow estimating microzooplankton impact at different depths in the water column with *in situ* incubations (Landry et al. 2008).

In addition to these modifications, other challenges in the application of the dilution method can be perceived in recent bibliographic reports. Evans et al. (2003) and Baudoux et al. (2006) used the dilution technique to separate the impact of microzooplankton from that of viruses on phytoplankton. This procedure would have been very useful in our experiments in the Oosterschelde, when viral lysis must have been an important mortality factor in the last experiment (section 5). Another innovation of the technique was recently presented by Calbet et al. (2008) and Hirose et al. (2008), who used the dilution technique

combined with size-fractionation, which enable them to assign different feeding impacts to several microzooplankton groups.

In spite of the numerous improvements and new utilities of the dilution method, it must be regarded that several issues probably will never be solved with the experimental techniques presently available. First of all, the dilution method provides the predation impact of the whole microzooplankton community, without enabling the exact determination of the individual clearance rates. Although this problem is partially solved by the use of the dilution method and size-fractionation as described above (Calbet et al. 2008, Hirose et al. 2008), it is impossible to clarify this topic undoubtedly. This issue is also complicated by the uncertainties in the trophic mode of numerous organisms, which leads to an unclear definition of microzooplankton. Mixotrophy is a common feature among protists (e.g. Sanders 1991, Bockstahler & Coats 1993, Stoecker 1999). Depending on environmental conditions (nutrients, prey availability), mixotrophic organisms can change between autotrophy and heterotrophy, which can also lead to the appearance of non-linear responses in dilution experiments as discussed in section 2. Finally, this method has also limitations inherent to all *in vitro* incubations (bottle effects). The consequences of confinement and isolation from the natural environment can be more or less adverse depending on the incubation time and the extent to which incubations reproduce *in situ* conditions.

Lastly, it must be noted that the sampling scheme was distinct for the three coastal systems studied. In the Ria de Vigo, a seasonal study was performed, with eight experiments widespread in four different months, while in the Limfjord and Oosterschelde, sampling only comprised nine successive (or almost successive) days, Thus, in the Ría de Vigo, it was obtained a wider perspective on the functioning of the system over the year, while in the Limfjord and Oosterschelde, the variability in biological parameters was only determined during that short-time period.

6.2. THE MICROZOOPLANKTON FEEDING IMPACT IN COASTAL SYSTEMS

This work adds further information to the important role that microzooplankton plays as consumer of other plankton organisms in coastal regions, namely in three coastal systems where data of this type are scarce. The results showed that a significant fraction of the plankton biomass, including autotrophic and heterotrophic organisms, was consumed by microzooplankton (Figure 6.1).

The average values for the impact of microzooplankton on phytoplankton standing stock in the three systems (~34-64%) compare well with the average values given by Calbet & Landry (2004) for coastal areas (~47%). However, the estimates obtained for the

impact on primary production in the Limfjord (95 ± 17%) and Oosterschelde (125 ± 140%) are well above the mean values obtained by Calbet & Landry (2004) for coastal systems (~60%). This could be a consequence of the short sampling period in those systems, which did not include a wider data set in which variability could be higher. Also the high average value obtained for the impact of microzooplankton on primary production in Oosterschelde is forced by the extremely high impact on *Phaeocystis* sp. on the last sampling day (see discussion in section 5).

The high impact on the standing stock and production of heterotrophs highlights the importance of microzooplankton as omnivorous, a role which is not habitually assessed. It must also be noted that these alternative heterotrophic food sources can be translated in a low impact on primary production, sometimes denoted in dilution experiments. This not necessarily means a lower importance of microzooplankton in marine systems, it essentially indicates that microzooplankton can feed on organisms other than phytoplankton.

Behind these general average values, several common patterns regarding the selective impact of microzooplankton can also be drawn for the three coastal systems studied here. Pico-heterotrophs (HB and HPF) were always highly consumed, indicating a very efficient top-down control of picoheterotrophic plankton by microzooplankton under all circumstances. Among microzooplankton, only HNF showed significant feeding responses in some experiments, indicating their consumption by other microzooplankton groups. In contrast, neither ciliates nor dinoflagellates showed significant feeding responses, demonstrating that their consumption by other microzooplankton, if occurring, must be occasional and not detectable in dilution experiments. Rather, main predators for these organisms must be larger consumers (Stoecker & Capuzzo 1990, Calbet & Saiz 2005), habitually not included in incubation bottles. Among phytoplankton, picoautotrophs (*Synechococcus* and APF) were also consumed by microzooplankton in the three systems, although feeding on these organisms was occasionally not significant. Besides, the biomass of these organisms was always very low, which led to very small quantities consumed when compared with other plankton groups. In contrast to heterotrophs and small phytoplankton, distinctive features were found for grazing on nano- and microphytoplankton in the three systems. Consumption of ANF occurred in the three systems, but showed higher importance in the Oosterschelde where this group, mainly *Phaeocystis* sp., dominated plankton biomass. Consumption of diatoms was especially important in the Limfjord and in the Ría de Vigo.

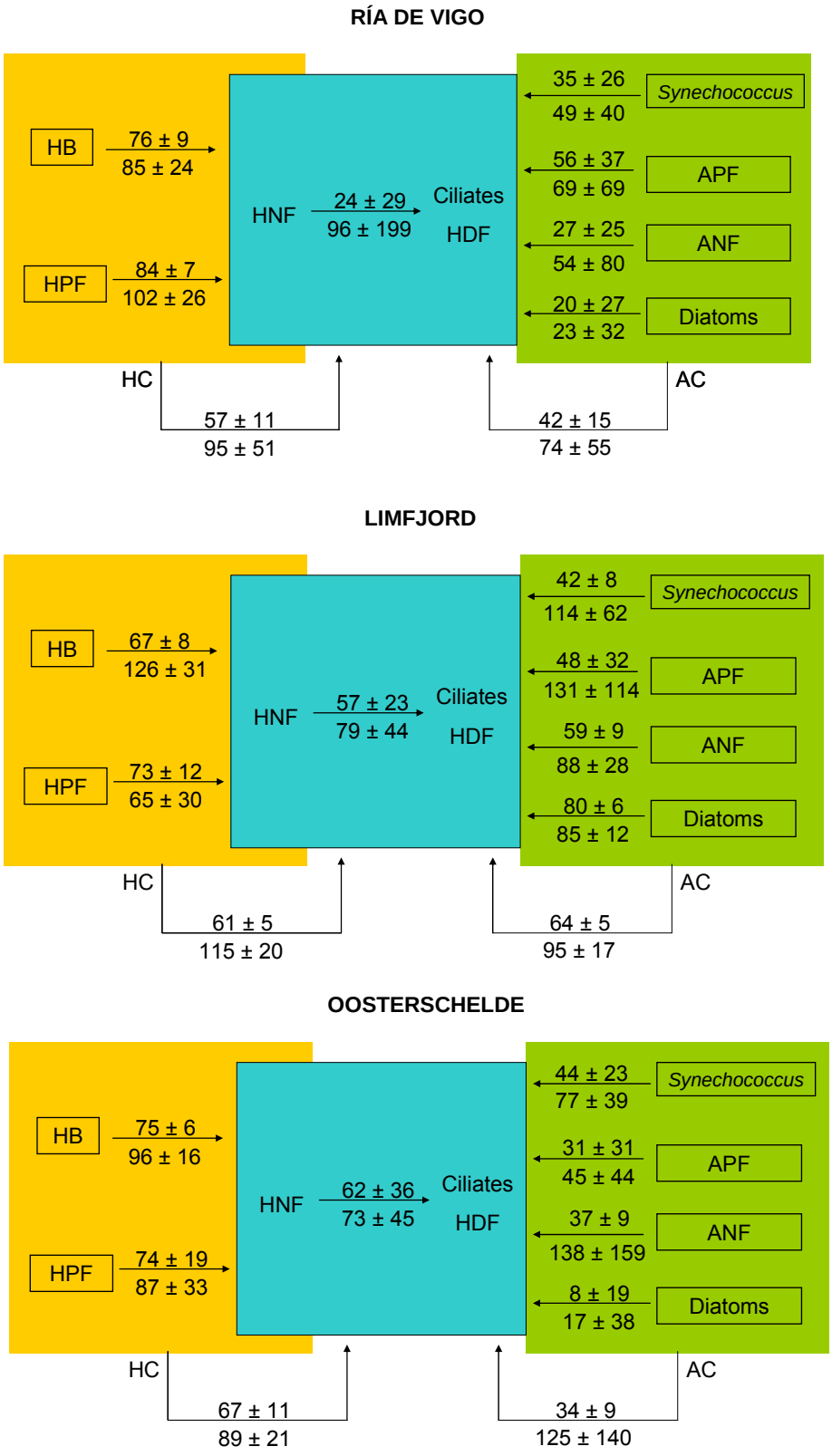

Fig. 6.1. Mean ± SD values (%) of the microzooplankton daily impact on the standing stock (numbers above arrows) and production (numbers below arrows) of the several plankton groups in each coastal system. Arrows leaving brown and green boxes denote microzooplankton impact on bulk heterotrophic (HC) and autotrophic carbon (AC), respectively. For average estimates, not significant responses within dilution experiments were considered to correspond to 0%.

Based on these trophic relationships, a general view of the microbial food web in these systems can be extracted (Fig. 6.1). It can be concluded that an efficient microbial loop, where a significant part of the production by small heterotrophs passes directly or indirectly (through HNF) to ciliates and dinoflagellates, was detected in the three systems. Among primary producers, all groups were grazed but there was a higher variability in the proportions impacted by microzooplankton depending on the system.

Definitely, microzooplankton must be considered nowadays a fundamental and integrating part of the pelagic food webs, where it is responsible for channelling a significant fraction of the plankton organisms to higher trophic levels. Microzooplankton not only impacts on communities dominated by pico- and nano-sized phytoplankton (e.g. Putland & Iverson 2007, this work), it also shows a high impact in highly productive coastal and estuarine systems, even during bloom periods when phytoplankton is dominated by diatoms (Strom et al. 2001, Olson & Strom 2002, Leising et al. 2005, Kim et al. 2007, this work). The importance of microzooplankton in coastal and productive systems has changed (or is changing) the ancient views of pelagic food webs. A complex food web, with numerous possibilities of trophic interactions mediated by microzooplankton, must be considered in future studies and models describing pelagic fluxes of matter and energy in any marine system.

CONCLUSIONS

1. The dilution technique is a very useful tool to estimate the microzooplankton feeding impact in marine systems. The use of microscopy in dilution experiments to identify changes in all plankton groups due to production and microzooplankton consumption provides a complete insight into the trophic relationships occurring in the microbial food web of marine systems.

2. Non-linear feeding behaviour of the microzooplankton induces non-linear feeding responses in dilution experiments. This non-linear feeding behaviour can occur in response to high food availability, which allows microzooplankton to select the more suitable prey when these organisms are feeding at their maximum ingestion rate.

3. The performance of dilution experiments using only three dilution levels -two high dilutions and the unfiltered sample- enables the correct estimation of growth and mortality rates even when non-linear responses occur and also allows saving time and effort in the preparation of the experiments.

4. In the coastal upwelling system of the Ría de Vigo, microzooplankton impact varied over the year. The microbial loop, occurring as a permanent background in the system, was relatively more important during downwelling conditions, when consumption of phytoplankton was low. During upwelling, major consumption occurred on phytoplankton, mainly diatoms.

5. Stratification-mixing cycles, which characterized the hydrodynamics in the shallow Limfjord, whenever occurring periodically are fundamental for the equilibrium of the biological communities in this system. Stratification, which favoured the transfer of plankton biomass through the microbial food web, contrasted with mixing events that provided food for the benthic organisms, nutrients for the pelagic system and affected adversely microzooplankton.

6. The microbial food web was an important route for the plankton community in the shallow Oosterschelde during a bloom of the colonial haptophyte *Phaeocystis* sp. The biomass of this species was channelled through microzooplankton by two ways: (i) direct consumption of free-living cells and perhaps some colonial forms

and (ii) consumption of heterotrophs, which were favoured by the high amounts of dissolved organic components released during the bloom decay.

7. Microzooplankton can have a significant impact in coastal and productive systems, challenging the ancient views of pelagic food webs in these areas. Microzooplankton can be a significant consumer of pico-, nano- and microphytoplankton, as well as exert a tight control on small heterotrophs. The complexity of pelagic food webs must be taken into account in models and subsequent studies, in order to provide more realistic estimates of matter and energy fluxes in marine systems. A more precise knowledge of the functioning of marine systems will increase our capacity to predict future changes and its consequences in the environment.

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In this work, the microzooplankton feeding impact on the plankton community has been assessed in three systems along the European coast: an embayment in Spain (Ría de Vigo), a shallow fjord in Denmark (Limfjord) and a shallow basin in the Netherlands (Oosterschelde). This was achieved by performing the dilution method (Landry & Hassett 1982) associated with microscopic enumeration and identification, which allowed determining the production of the several components of the plankton community, both autotrophic and heterotrophic, and their consumption by microzooplankton. The dilution method, albeit being the most widely used experimental technique to estimate the microzooplankton feeding impact in marine systems, can present several problems regarding the correct accomplishment of the basic assumptions on which it relies. In the first part of this work (section 2), the violation of these basic assumptions was analysed in relation to the observed deviations from linearity in a relationship that theoretically should be linear. Non-linear responses, which are also frequently reported in the literature but rarely analysed, were related to the non-linear feeding behaviour of the microzooplankton, which at high food availability reaches the maximum ingestion rate and enhances their selectivity for certain preys. These findings, which do not invalidate the use of this technique, provide further evidence for the introduction of some changes in the protocol in order to optimize its use.

In the following three sections (3, 4 and 5), the estimation of the microzooplankton feeding impact in the three coastal systems referred above, provided an insight into the impact of microzooplankton on different communities and under contrasting environmental conditions. In the seasonal study performed in the coastal upwelling system of the Ría de Vigo (section 3), the microzooplankton feeding impact on the several plankton groups varied over the year. Predation on heterotrophs was very constant and relatively more important during downwelling conditions, when consumption of phytoplankton was low. During upwelling, the main food source for microzooplankton was phytoplankton, mainly diatoms. In the Limfjord (section 4), the hydrodynamics of the system, characterized by stratification-mixing cycles during 9 successive sampling days, was very important for the coupling between the benthic and pelagic systems. Mixing events enhanced this coupling, favouring a short food chain where the impact of microzooplankton was lower. In contrast, stratification periods favoured the microbial food web and the decoupling between benthic and pelagic communities. In the Oosterschelde (section 5), microzooplankton was observed to have an important role during a bloom of the colonial haptophyte *Phaeocystis* sp., either from direct consumption of this algae, or from consumption of heterotrophs which were favoured by the dissolved organic matter released during the collapse of the bloom.

Major findings from this work are related to the validity of the use of the dilution technique, even when non-linear responses are obtained, and to the determination of the important role that microzooplankton plays in coastal systems as consumers of the several autotrophic and heterotrophic plankton groups. The understanding of the importance of microzooplankton in marine systems aids to a better knowledge of their functioning, enabling to develop more accurate models and predictions under the several environmental conditions.