Vrije Universiteit Brussel
Laboratorium voor Ecologie en Systematiek

Responses of calanoid copepods to changes in phytoplankton dominance in the diatom - *Phaeocystis globosa* dominated Belgium coastal waters

by

Antajan Elvire

A thesis submitted in fulfilment for the degree of
Doctor of science
September 2004

Promotors: Profs. M.-H. Daro, M. Tackx and Dr S. Gasparini

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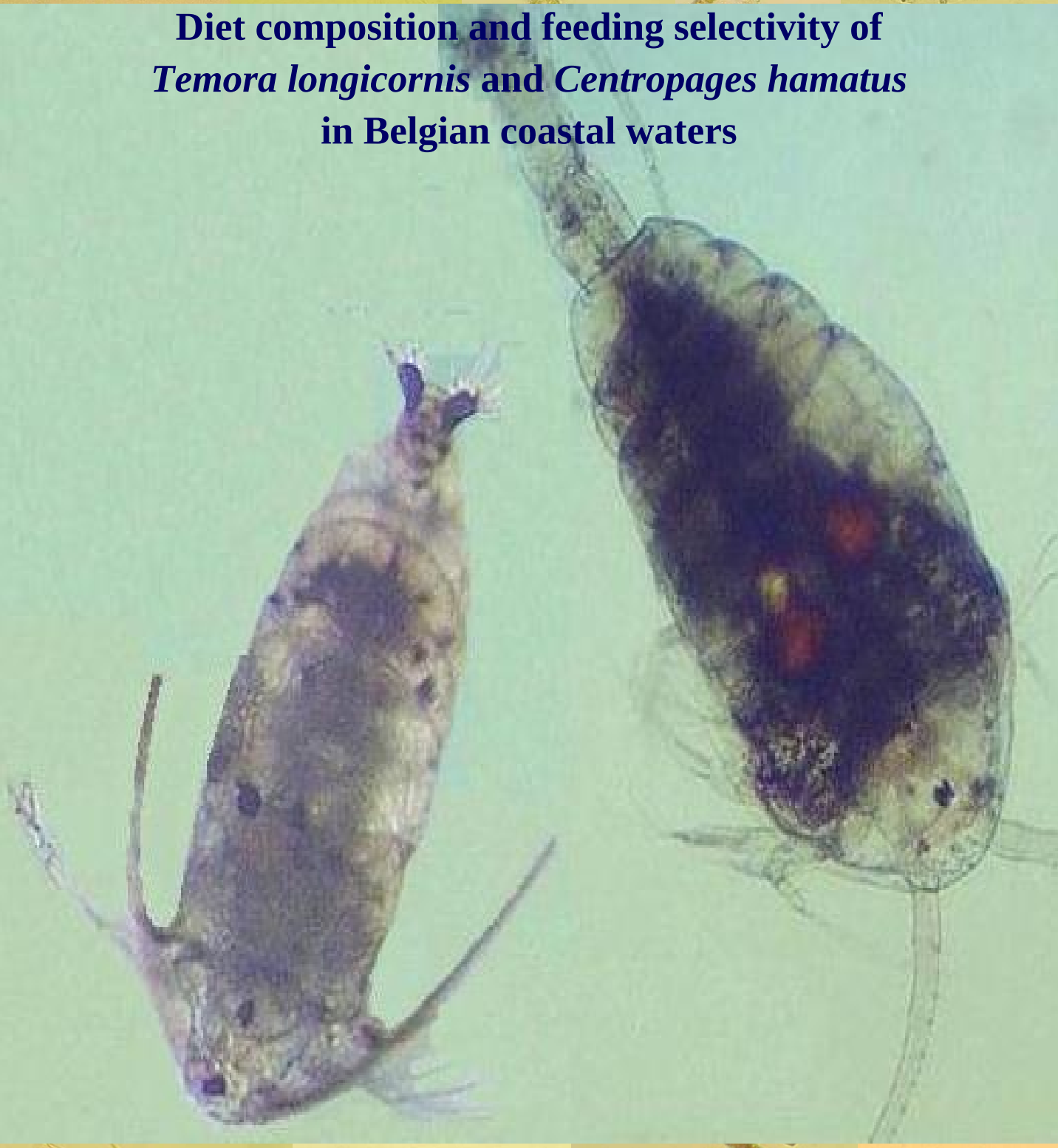

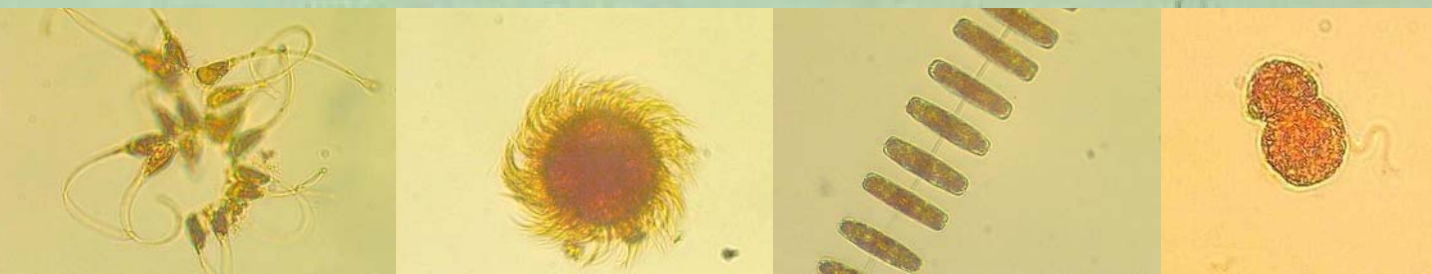

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If children took a moment to look at copepods, they would forget about dinosaurs!

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ABSTRACT

Copepods feeding behaviour according to changes in composition and availability of their potential preys in Belgian coastal waters were appraised through the combination of different field experiment approaches.

The gut fluorescence method was combined with egg production measurements to estimate the contribution of herbivory to the total ingestion required to sustain *Temora longicornis'* egg production. The results showed that during fall and winter herbivory was often sufficient to cover the nutritional needs of *T. longicornis*, while during spring and summer they needed heterotrophic food to meet their energetic demands for egg production. When *T. longicornis* carbon requirements for egg production increase the contribution of non-phytoplankton food sources to total carbon ingestion becomes more important. The phytoplankton spring bloom, either during diatom dominance or during *P. globosa* dominance, did not enhance the contribution of herbivory to the copepods' diet. HPLC gut pigment analysis showed that diatoms were the main phytoplankton group ingested, whereas no evidence for ingestion of *P. globosa* and nanoflagellates was found.

In situ feeding incubation experiments were also conducted with two dominant copepod species in Belgian coastal zone, *T. longicornis* and *Centropages hamatus*, designed to estimate diet composition and prey selectivity upon natural plankton assemblage. The results showed that copepods were not only omnivorous, but highly selective as well, consuming the diverse categories of prey in a different proportion than expected from prey availability *in situ*. Although diatoms were an important contributor to the copepod's diet in this study, they were only ingested according to their abundance or even slightly rejected. There was an overall positive selection for ciliates by both copepod species, with ciliates < 18 µm being the preferred prey category, whereas *P. globosa* and nanoflagellates were selected against. Dinoflagellates were diversely appreciated according to the copepod species considered. *Temora longicornis* showed a clear preference for small dinoflagellates and a more neutral selection for dinoflagellates > 18 µm. Prey-selectivity appeared to be not size-dependant, and other factors such as food quality and prey motility are assumed to be the driving factors of selective feeding in Belgian waters.

Total carbon ingestion estimated for *T. longicornis* from the field incubation experiments (phytoplankton + microzooplankton) appeared to be not sufficient to cover egg production carbon requirements estimated from the egg production measurements. This

supports the hypothesis that another possible source of carbon (e.g. detritus, copepod eggs) was not taken into account in the incubation experiments.

This study also critically examined the use of HPLC pigment analyses to trace the fate of phytoplankton in the food chain. In particular we demonstrated that 19'-hexanoyloxyfucoxanthin is not the appropriate pigment to estimate *Phaeocystis* abundance and trace its trophic fate in BCZ, and that the use of alloxanthin as biomarkers for copepod grazing on Cryptophyceae is not suitable both for quantitative and qualitative estimates.

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INTRODUCTION

Role of copepods in pelagic food webs

Copepods are the numerically dominant metazoan plankton in the sea, representing 55 to 95 % of organisms caught in plankton samples (Longhurst 1985). Within the zooplankton, they are among the smaller animals (0.25 - 18 mm) and consequently, their proportion in terms of biomass is lower, between 25 to 80 % depending on the area or the season (Mauchline 1998). So, their dominance in pelagic ecosystems has made copepods a thoroughly investigated group.

In the past copepods were considered mainly as herbivorous, feeding on phytoplankton and forming a direct link between it and higher trophic levels in a short and efficient food chain (classical food chain; Steele 1974). Since high production of picophytoplankton (Sahlsten et al. 1988), which is too small (< 2 μm) to be grazed on by copepods but readily available for ciliates and heterotrophic flagellates was observed, copepods are not any longer considered as the main consumers of the primary production (Lenz 1992, Banse 1995, Waterhouse and Welschmeyer 1995). This was reinforced by the work of Azam et al. (1983) on the 'microbial loop', showing that microzooplankton is an important grazer of both primary (phytoplankton) and secondary (heterotrophic bacteria) producers. It is now known that copepods are more omnivorous in their feeding habits and adapted to variable food conditions. Poulet (1983) points out that "Marine copepods can potentially obtain food from any known stock of organic matter, in either dissolved or particulate form". Thus, although copepods do consume phytoplankton to a very large extent, microzooplankton is also an important component of their diet (Gifford and Dagg 1988, Stoecker and Capuzzo 1990, Lenz 1992, Kleppel 1993). The production of the microbial food web is then available to higher trophic level through predation of microzooplankton by copepods, connecting the microbial food web to the classical food chain (Fig. 1). Copepods are themselves eaten by a vast variety of invertebrate species (e.g. shrimps and mysids) as well as by fish larvae and planktivorous fish among which some have economical interest such as herring, cod and sole (Sorbe 1983, Thompson and Harrop 1991, Aaser et al. 1995, Grioche 1998).

In return, by their excretion (mainly ammonia and phosphate) copepods provide nitrogen and phosphorus required for phytoplankton growth (Harris and Malej 1986). As the result of their "sloppy" feeding and faecal pellets egestion, organic matter is released into the

water column. This material, along with leakage of dissolved organic matter from phytoplankton cells (Lancelot 1979), is absorbed by the heterotrophic bacteria, recycling elements lost from the food web back into the food web through the microbial loop.

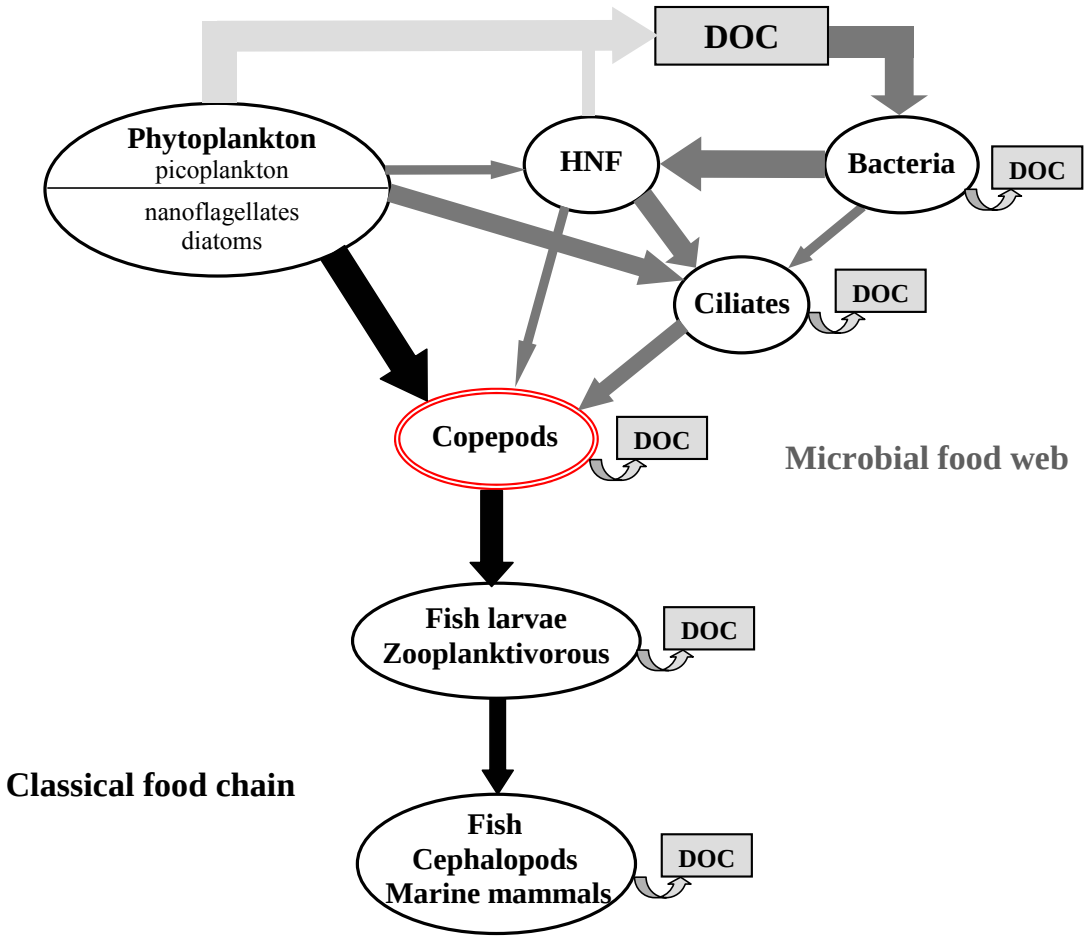


Figure 1: Simplified food web structure showing classical food chain and microbial food web. (DOC = dissolved organic carbon and HNF = heterotrophic nanoflagellates). The arrows indicate the relative importance of the different pathways.

Both food web types (the classical and the microbial) coexist in all areas of the ocean, but their relative significance changes with region and season. In nutrient-rich waters dominated generally by diatoms e.g. in upwelling areas or during the spring and autumn outbursts of temperate waters, the traditional food chain is predominant. On the contrary, in nutrient constrained environments where pico- and nanoplankton are dominant, e.g. oligotrophic oceans and in strongly stratified waters of the temperate summer, the microbial food web is of larger importance (Cushing 1989, Kiørboe 1993). In eutrophicated coastal ecosystems, however, nutrient enrichment induces a shift in the composition of phytoplankton, often characterised by the development of inedible or toxic phytoplankton

species which are not entering the food chain and may be harmful to other organisms (Hansen et al. 1995, Engström et al. 2001). Coastal copepods are frequently faced with such food environment during the season of their highest abundance (Fransz et al. 1992).

Marine coastal eutrophication: the case of the southern North Sea

Eutrophication is the process of nutrient enrichment (usually by nitrogen and phosphorus) in aquatic ecosystems and the concomitant production of undesirable biological effects such as bloom of algae (either micro- or macrophytes). Since nutrient inputs are required for the natural functioning of aquatic systems, eutrophication merely indicates that the system cannot cope with the available inputs. The symptoms of eutrophication as a response to nutrient enrichment differ greatly, due mainly to differences in the physical characteristics of the different systems receiving this 'excess' nutrients and organic matter. For example, the mixing state (stratification or not) of the receiving body of water and the residence time (or flushing time) of the freshwater inputs and its nutrients in the system determine the intensity of a particular symptom and thereby the sensitivity of systems to eutrophication events or symptoms. The longer the flushing time, the more vulnerable the system to nutrient enrichment, as excess nutrients are available to primary producers for a greater period of time.

Estuaries and shallow coastal waters can be considered as naturally organically enriched and represent some of the world's most productive environments. About 28 % of the total global primary production takes place there, while these systems cover only 8 % of the Earth’s surface (de Jonge and Elliott 2001). These systems require little additional material to become eutrophic. Anthropogenic activities can greatly accelerate eutrophication by increasing the rate at which nutrients and organic substances enter aquatic ecosystems from their surrounding watersheds. Agricultural and urban runoff, industrial effluents, sewage discharges can over-stimulate the growth of algae, creating conditions that can greatly affect the trophic structure and functioning of the ecosystem.

The southern bight of the North Sea, extending from the straits of Dover to the German bight, and including the coastal waters of France, Belgium, the Netherlands and Germany constitutes one example of eutrophicated coastal ecosystem due to human activities (Fig. 2). This coastal area is under the influence of seven major west-European river discharges: the Seine, Scheldt, Meuse, Rhine, Ems, Weser and Elbe. The watersheds of these rivers, draining an area of about 580,000 km^2, are very densely populated, highly

industrialised and used for intensive agricultural practices (OSPAR 2000). As a consequence, the Atlantic waters flowing into the southern bight of the North Sea through the Dover straits along a southwest-northeast axis are progressively enriched by nutrients from river discharges. The riverine inputs are characterized by large amounts of anthropogenic nitrogen (N) and phosphorus (P). About 720 kt of inorganic N (of which 80 % is NO_3) and 50 kt of P (65 % PO_4) are discharged every year in the southern bight of the North Sea (Lancelot 1995).

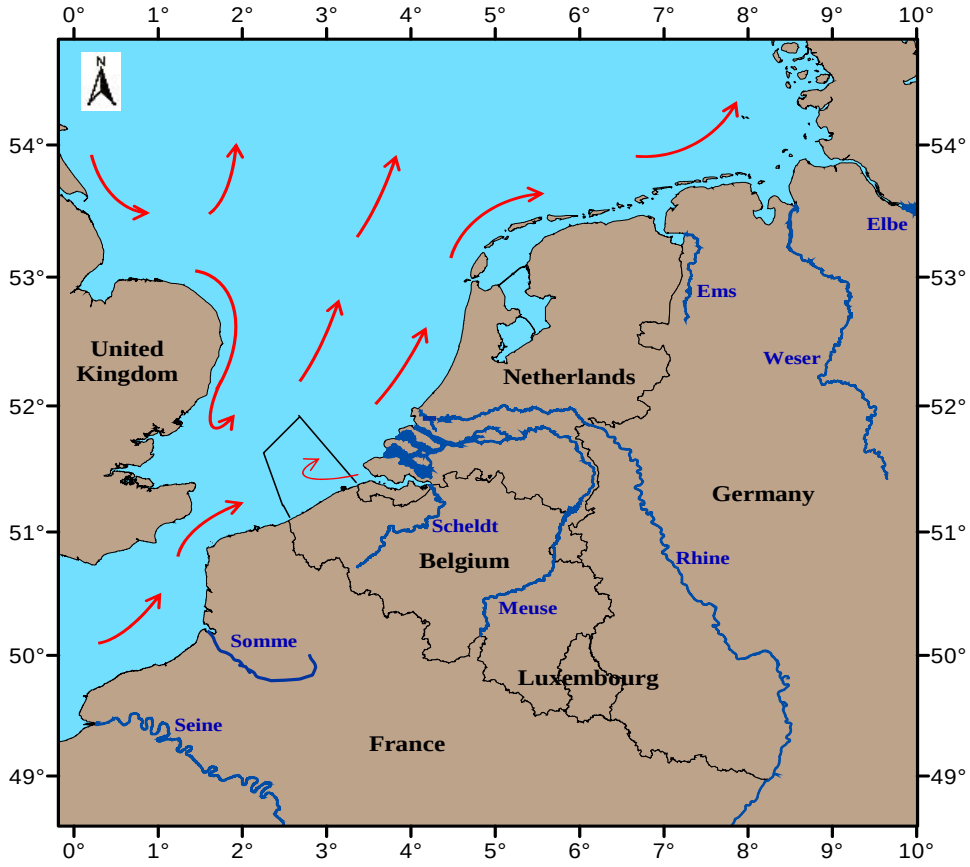


Figure 2: Map showing the watershed of the main rivers discharging in the southern bight of North Sea. Arrows indicate the general residual circulation of water masses flowing north-eastward.

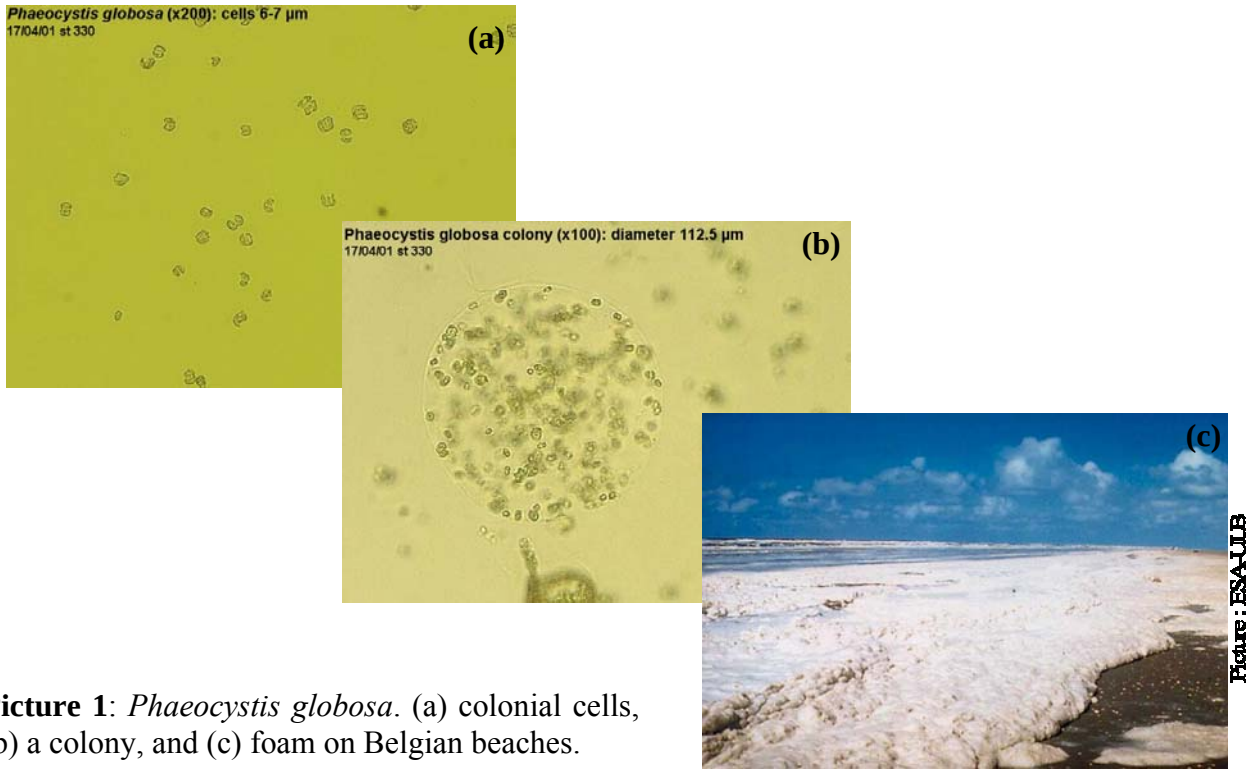
A manifest effect of anthropogenic nutrient enrichment of the southern bight of the North Sea is the recurrent appearance of massive blooms of the colony-forming haptophyte *Phaeocystis* sp. every year between April and May (Gieskes and Kraay 1975, Bätje and Michaelis 1986, Cadée and Hegeman 1986, Veldhuis et al. 1986, Lancelot et al. 1987, Cadée and Hegeman 2002). This *Phaeocystis* species is characterized by a heteromorphic life cycle which alternates between different types of free-living cells of 3 to 8 µm in diameter and mucilaginous colonies whose size vary from 10 µm to 2-3 mm (Rousseau et al. 1994; Picture

1a,b). This alga is responsible for the accumulation of large colonies which some years dominate the phytoplankton community at more than 90 % (C biomass and cell number; Rousseau et al. 1990). The favourable conditions allowing the development of the colonial form seem to be a warming up of waters with optimal light conditions in combination with high ambient nitrate but low phosphate, i.e. high N/P ratios (Lancelot et al. 1998). The link between the magnitude of *Phaeocystis* colony blooms and the general nitrate enrichment of the continental coastal waters of the North Sea is clearly evidenced by the significant relationship existing between *Phaeocystis* cell density and nitrate concentrations at the *Phaeocystis* onset (Lancelot 1995). The magnitude of the *Phaeocystis* blooms increase from the French to the German coastal waters, indicating the cumulative effect of the nutrient river discharges along the coast, as well as the general increase in industrial and farming activities year by year since the 70's (Cadée and Hegeman 1991, Lancelot et al. 1991, Lancelot 1995, Lancelot et al. 1998).

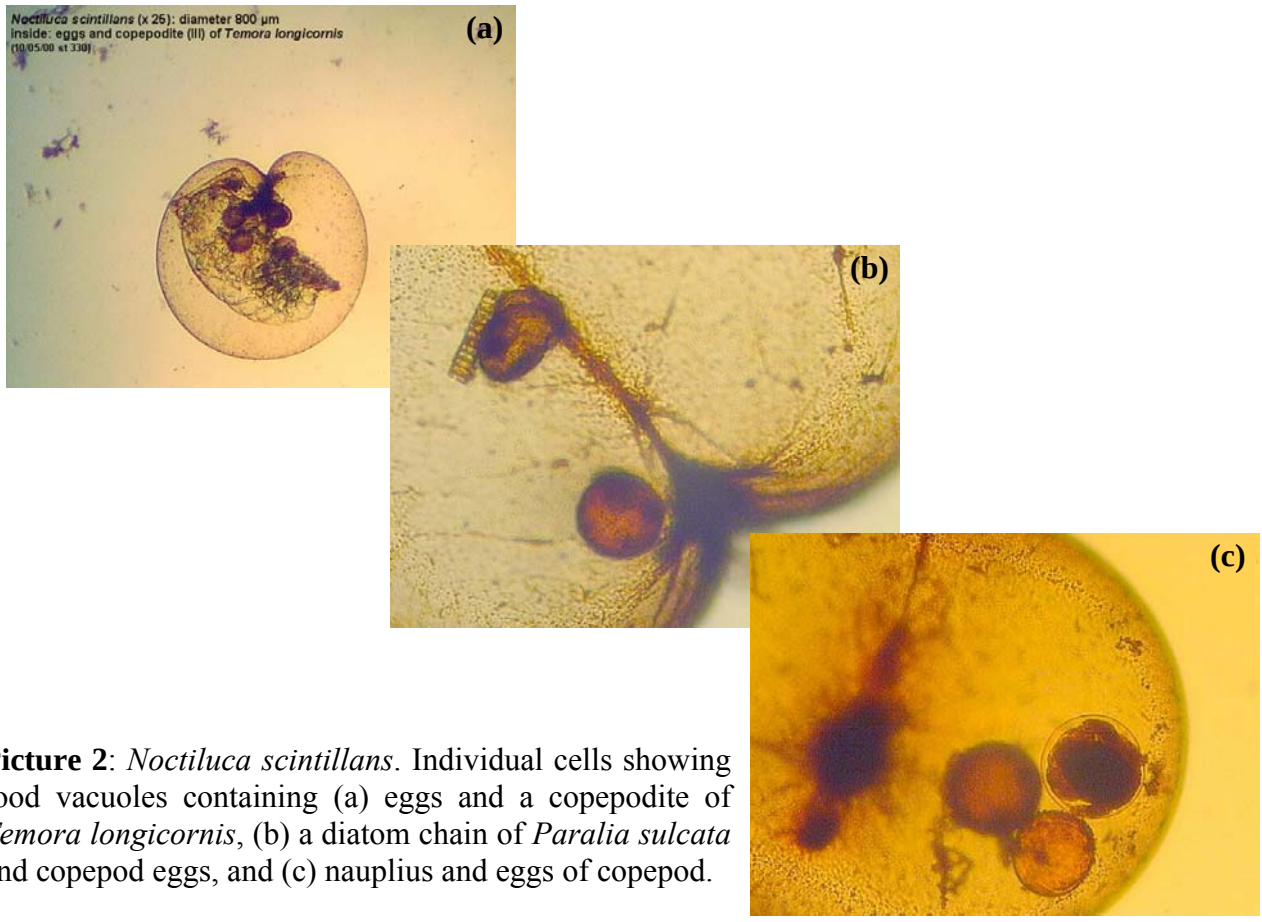
Another visual effect of eutrophication in the southern bight of the North Sea is the accumulation at the surface of the sea of a large heterotrophic dinoflagellates species, *Noctiluca scintillans* (Uhlig and Sahling 1990). This blooming-species is a strongly buoyant planktonic form with a very large cell (200-2000 μm), inflated (balloon-like), having a ventral groove containing the flagella, a tooth and tentacle. The presence of photosynthetic symbionts can cause the cytoplasm to appear pink in colour (Sweeney 1978). *Noctiluca scintillans* red tides frequently form in late June to July following the *Phaeocystis* bloom and often resulting in a strong pinkish red or orange discoloration of the water (tomato-soup). This species is phagotrophic (Picture 2), feeding on phytoplankton (mainly diatoms and other dinoflagellates), protozoans, detritus, and copepod and fish eggs (Steidinger and Tangen 1996, Kiørboe and Titelman 1998, Nakamura 1998, Quevedo et al. 1999). Its strong abundance is clearly related to eutrophication by the organic matter (Lacaze 1996).

Impact of harmful algal bloom

These changes in species composition resulting from nutrient enrichment are being seriously considered because of the nuisances they cause to the coastal ecosystem and to recreative and aquaculture activities as well as the uncertainty surrounding their impact on higher trophic levels and related biological harvestable resources. Species of *Phaeocystis* have historically not been regarded as a toxin-producer (Larsen and Moestrup 1989). However, as



Picture 1: *Phaeocystis globosa*. (a) colonial cells, (b) a colony, and (c) foam on Belgian beaches.



Picture 2: *Noctiluca scintillans*. Individual cells showing food vacuoles containing (a) eggs and a copepodite of *Temora longicornis*, (b) a diatom chain of *Paralia sulcata* and copepod eggs, and (c) nauplius and eggs of copepod.

early as 1930 *Phaeocystis* was suspected to cause avoidance of herring (Savage 1930), and copepods have been demonstrated to avoid grazing on healthy colonies (Daro 1985, Estep et al. 1990). *Phaeocystis* was listed as a nuisance species by the Intergovernmental Oceanographic Commission, IOC (Moestrup and Thomsen 1995), mainly due to clogging of fishing nets and accumulation of thick layers of odorous foam on beaches (Lancelot et al. 1987; Picture 1c), but also for causing anoxia during dense blooms (Rogers and Lockwood 1990). Recently it was observed that the northern polar *Phaeocystis* species, *P. pouchetii,* reduced appetite of Atlantic salmon cultivated in sea cages (Eilertsen and Raa 1995). It also releases some compounds to its surrounding that appear to hold anaesthetic or anti-mitotic properties (Stabell et al. 1999, Hansen et al. 2003) and are toxic to cod (*Gadus morhua*) larvae (Aanesen et al. 1998). Hansen et al. (2004) have recently isolated and characterised a cytotoxic polyunsaturated aldehyde produced and released by *P. pouchetti* along the coast of the northern Norway. As a consequence *P. pouchetii* has been introduced to the IOC Taxonomic Reference List of Toxic Plankton Algae (Moestrup 2004). Such toxicity has not been demonstrated for the southern North Sea *Phaeocystis* strain but is under study.

Likewise, toxic blooms of *N. scintillans* have been linked to massive fish and marine invertebrate kills. Although this species does not produce a toxin, it has been found to accumulate toxic levels of ammonia which is then excreted into the surrounding waters possibly acting as the killing agent during blooms (Okaichi and Nishio 1976).

Thesis outline

Due to the impressive development in spring of *Phaeocystis* colonies in the Belgian coastal zone (BCZ), several research programmes have focused on understanding the mechanisms through which a change in riverine nutrient supply induces modifications in the phytoplanktonic community structure and hence the functioning of the planktonic food-web. Progress achieved in the understanding of eutrophication mechanisms in the BCZ were integrated in the biogeochemical model MIRO (Lancelot et al. 1997), which synthesised current knowledge on the kinetics and the factors controlling the main auto- and heterotrophic processes in this coastal ecosystem. The MIRO model has been recently upgraded, based on process-level field studies in the context of the Belgian national research programme for sustainable development of the North Sea, AMORE (Advanced MOdelling and Research on Eutrophication). A three-dimensional version of MIRO (3D-MIRO&CO) has been

constructed based on the coupling of the 3D-COHSNS hydrodynamical model developed for describing water transport in BCZ (Lacroix et al. 2004) to an upgraded version of MIRO model (Lancelot et al. 2004). The coupled model calculates the seasonal vertical and horizontal transport of the ecosystem and biogeochemical state variables in the domain of the Southern Bight of the North Sea. Model simulations thus produce information on the seasonal and geographical spreading of nutrients and phytoplankton blooms in the investigated domain. A weak point of the model, however, is the underestimate of the observed spring-summer copepod biomass which was attributed to inappropriate parameterization of mesozooplankton feeding. Mesozooplankton in the BCZ representing considerable biomasses in spring (Rousseau et al. 2000), its interaction with the spring phytoplankton is an important factor in the MIRO model. In its current version, copepods graze on both diatoms and microzooplankton. Feeding is described by an unique sigmoid Holling-III function (Holling 1959) relating copepod ingestion rates to prey density, and the share between diatoms and microzooplankton ingestion is calculated based on the relative proportion of their biomass. Thus, no selective feeding (food preference) is presently considered in the MIRO model due to the lack of data and the current controversy about copepod preference for diatoms and microzooplankton (see Nejstgaard et al. 2001 and references therein). As explained above, the ability to eat a variety of foods allows copepods to modify their diet when faced with a variable food environment. Switching between herbivory and carnivory is an example of how copepods can respond to substantial changes in food composition and food abundance (e.g. Nejstgaard 1997) or to differential nutritional needs for metabolism (Kleppel and Burkart 1995, Klein Breteler et al. 1999). A different grazing pressure upon the different available prey (top-down control) could constitute an alternative explanation to inorganic nutrient unbalance (bottom-up control) for the development of blooming species in eutrophicated coastal ecosystem (Gasparini et al. 2000). Thus the feeding behaviour of copepods is of great importance in understanding the fate of phytoplankton production in BCZ and its relation to food web structure.

As a contribution to the AMORE project, the aim of this thesis was to further explore and quantify the interaction between the main copepods and their potential prey in the Belgian coastal waters. Considering the implications of a flexible feeding mode for copepods, the main strategy consisted of considering the entire protist potential prey community, and not only the dominant phytoplankton groups, diatoms and *Phaeocystis*. The feeding responses of copepods to variations in natural food composition and abundance were studied by a series of

sampling and field experiments during AMORE 1999-2001 campaigns in Belgian coastal zone:

- The temporal and spatial dynamics of the food environment of copepods in the Belgian coastal waters are presented in **chapter I**, with emphasis on diatoms and *Phaeocystis* communities but also looking at other nanoflagellates and microzooplankton as potential food resources. An additional goal of the chapter I was to obtain a dynamic picture of algal pigments, determined using high-performance liquid chromatography (HPLC), in the water column. The objective was to define which biomarker pigments can be used to distinguish between the main phytoplankton groups in copepod's diet.
- The relevance of using the accessory pigment 19'-hexanoyloxyfucoxanthin as biomarker to trace *Phaeocystis* fate in water column and in food chain is further discussed in **chapter II**.
- Also here a critical evaluation of the use of alloxanthin pigment as indicator of recent Cryptophyceae ingestion, by *Temora longicornis* and *Centropages hamatus*, is presented in **chapter III.**
- In **chapter IV** the contribution of herbivory to the diet of *Temora longicornis* and the degree of food limitation of its egg production were investigated in relation to protist availability on a seasonal base during field experiments. These experiments also allowed evaluating the use of gut pigment biomarkers for determination of copepod diet composition in natural plankton.
- Finally, in **chapter V** we examined changes, during the course of the year, in prey selection and prey preference of *Temora longicornis* and *Centropages hamatus* fed upon natural plankton assemblage during *in situ* incubation experiments.

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Protist community structure and algal pigments in Belgium coastal waters from 1999 to 2001

1. Introduction

1.1. General description of the Belgian coastal zone

The Belgian coastal zone (BCZ), situated between 51° and 52°N, forms part of the southern bight of the North Sea (Fig. I-1). Extending to about 40 km offshore it is a relatively shallow area with the deepest (40 m) water found in the northwest. The 10 meter isobath runs along the coast from southwest to northeast with a break at the channel of the Scheldt estuary mouth. The area is characterised by a complex system of sandbanks grouped in a parallel pattern. They are elongated sedimentary bodies, typically tens of kilometres long, a few kilometres wide and 20 to 25 m high (Otto et al. 1990). The banks are separated by channels with maximum depth of about 30 m. This is an area of considerable sand transport and, for example, bathymetric changes are observed at time scales from days (probably related to wind events) to years. The tidal currents are a dominant feature of the circulation dynamics of the BCZ. They are of order 0.6 to 1.1 $m.s^{-1}$ and are primarily alongshore. Wind is also an important factor in the physical oceanography of the BCZ. In addition to tidal current, wind-driven currents can be particularly strong in shallow water. The prevailing wind speed is between 2 and 6 $m.s^{-1}$ and the direction from the southwest (Yang 1998).

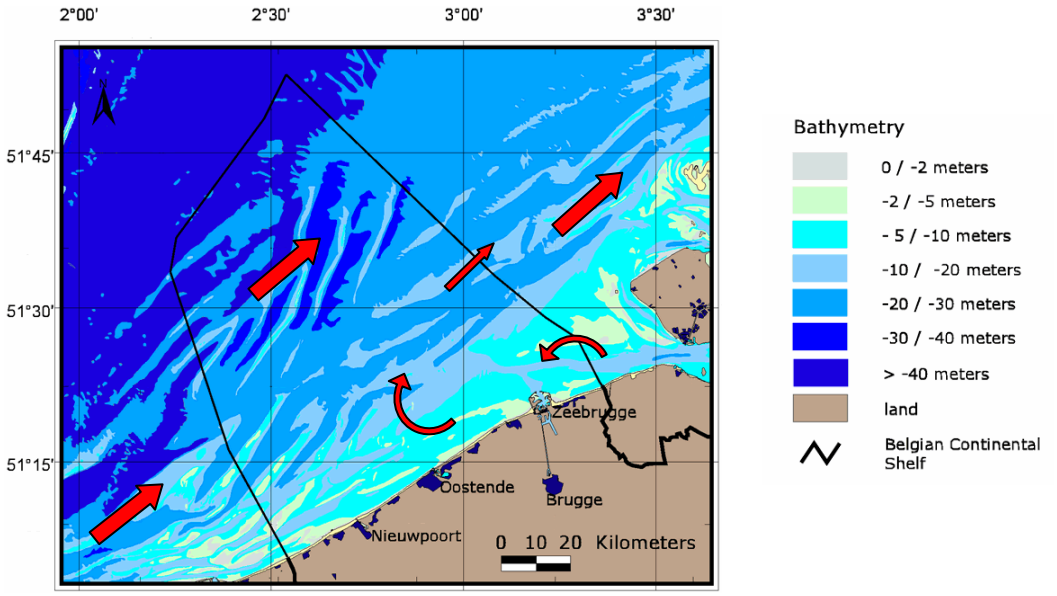

Figure I-1: Belgian continental shelf and bathymetry (source: MUMM*). Arrows represent the general circulation of Atlantic (thick) and Scheldt (thin) water masses.

*Management Unit of the Mathematical Models of the North Sea and Scheldt Estuary, Belgium

The Belgian coastal waters receive large quantities of anthropogenic nutrients via the nutrient-enriched Scheldt river and to some extend the rivers Rhine and Meuse (van Bennekon and Wetsteijn 1990). These main sources of nutrients are further added to the SW-NE flowing Atlantic waters already enriched in nutrients by the rivers Seine and Somme, local coastal effluents and atmosphere inputs (Lancelot et al. 1991). The Scheldt estuary induces a gyre in front of the Belgian coast in which the fresh water from the Scheldt resides for some time (Nihoul and Ronday, 1975). The average annual discharge from the Scheldt Estuary into the BCZ is about 100 $m^3.s^{-1}$ and is a source of suspended and dissolved material (Mommaerts 1991). This discharge is usually highest in winter and early spring. The discharge of continental suspended matter together with a quasi-permanent resuspension of sandy and silt sediments due to the high turbulent regime, supplies these coastal waters with large amounts of particulate matter. The strong tidal currents, combined with a shallow water column, ensure a complete vertical mixing of the water column (Simpson 1994). Consequently the BCZ is characterised as a homogenous body of water distinguishable from the adjacent open 'North Sea Water' (Joiris et al. 1982).

1.2. The spring diatom-Phaeocystis succession in the Belgian coastal waters

The BCZ is not only enriched, but also characterized by an unbalance in terms of N:P:Si ratio compared to diatoms (N:Si = 1; Brzezinski 1985) and phytoplankton (N:P = 16; Redfield et al. 1963) requirements (Lancelot et al. 1991, Lancelot 1995). Since 1988, the concentrations of nutrients and the succession of the various phytoplankton species have been monitored extensively to assess the quality of these eutrophicated coastal waters and its possible variation in relation to anthropogenic and natural impact (Lancelot 1995, Lancelot et al. 1998, Rousseau 2000, Rousseau et al. 2002). For the period 1988 to 1999, the winter concentrations of dissolved inorganic nutrients were in average: 3 µM, 33 µM, 1.2 µM and 18 µM for ammonium (NH_4), nitrate (NO_3), phosphate (PO_4) and silicate ($Si(OH)_4$) respectively (Rousseau 2000). The large excess of nitrogen (mostly nitrate) over phosphorus and silicon causes major algal blooms and shifts in phytoplankton abundance and succession, with an increasing relative importance of flagellates during the spring bloom (Lancelot et al. 1991, Lancelot 1995). Typically, a moderate diatom outburst initiates the vernal phytoplankton succession in early spring and is followed by a massive bloom of the non-siliceous colony-forming haptophyte *Phaeocystis*. The magnitude of *Phaeocystis* colonies is determined by

the nitrate excess left over at the end of the early spring silicate-controlled diatoms flowering (Lancelot 1995, Lancelot et al 1998).

1.3. The importance of pigments in phytoplankton studies

Because of chlorophyll's specificity to plants makes it an adequate indicator of phytoplankton biomass it is probably the most frequently measured biochemical parameter in oceanography. Since the 80's the development of automated high performance liquid chromatography (HPLC) methods allows separate quantification of phytoplankton chlorophylls, carotenoids and breakdown products and these can be used as marker pigments for specific phytoplankton taxa (Mantoura and Llewellyn 1983, Wright and Shearer 1984, Wright et al. 1991, Jeffrey et al. 1997a, Jeffrey et al. 1999, Zapata et al. 2000). For example, the pigments fucoxanthin (diatoms + golden-brown flagellates), 19'-hexanoyloxyfucoanthin (haptophytes), alloxanthin (cryptomonads), peridinin (dinoflagellates) and chlorophyll *b* (green algae) can indicate the presence of major algal types in the water column (see review in Jeffrey and Vesk 1997). Chlorophylls and associated carotenoid pigments are now being used to map the chemotaxonomic composition of phytoplankton in the oceans (Gieskes and Kraay 1983, Gieskes and Kraay 1986, Everitt et al. 1990, Bustillos-Guzman at al; 1995, Riegman and Kraay 2001), and also to track processes involved in the transformation, degradation and fate of phytoplankton populations (Head and Harris 1992, Head et al. 1994, Barlow et al. 1995).

This first chapter presents the temporal and spatial variations of the food environment for copepods in the Belgian coastal waters from 1999 to 2001 with special focus on the diatom and *Phaeocystis* communities, but also looking at other nanoflagellates (e.g. Prasinophyceae and Cryptophyceae), dinoflagellates and ciliates as target prey groups. Protist identification and enumeration were obtained through the classical method of cell counting by light microscopy. Algal chlorophyll and carotenoid concentrations were measured in parallel to (1) evaluate phytoplankton degradation through senescence/grazing processes, (2) determine the 'fingerprint' pigments that will be utilized as chemotaxonomic tracers of phytoplankton taxa in the copepod's diet (see chapter IV).

2. Materials and methods

2.1. Sampling stations and field campaigns

Samplings were carried out during AMORE 1999-2001 cruises on the R/V *Belgica* and *Zeeleeuw*. Five stations were chosen among stations of the Belgian monitoring network along a nearshore offshore transect crossing the plume of the Scheldt estuary (Fig. I-2). The characteristic parameters of the sampling stations are given in Table I-1. Station 330 constitutes since 1988 a reference station for monitoring seasonal and interannual changes in the Belgium coastal waters due to its average physico-chemical and biological characteristics (Ruddick et al. 1999, Rousseau et al. 2002). Stations 115 and 800 were sampled only during AMORE 1999 and 2000 cruises.

Table I-1: The geographical characteristics of sampling stations during AMORE 1999-2001 cruises in the Belgian coastal zone.

Station	Latitude	Longitude	Distance (km)	Depth (m)
115	51°09'30 N	2°36'20 E	4.26	15
230	51°18'50 N	2°51'00 E	9.63	13
330	51°26'00 N	2°48'50 E	25.56	24
435	51°35'00 N	2°47'40 E	42.60	32
800	51°51'00 N	2°52'00 E	72.23	35

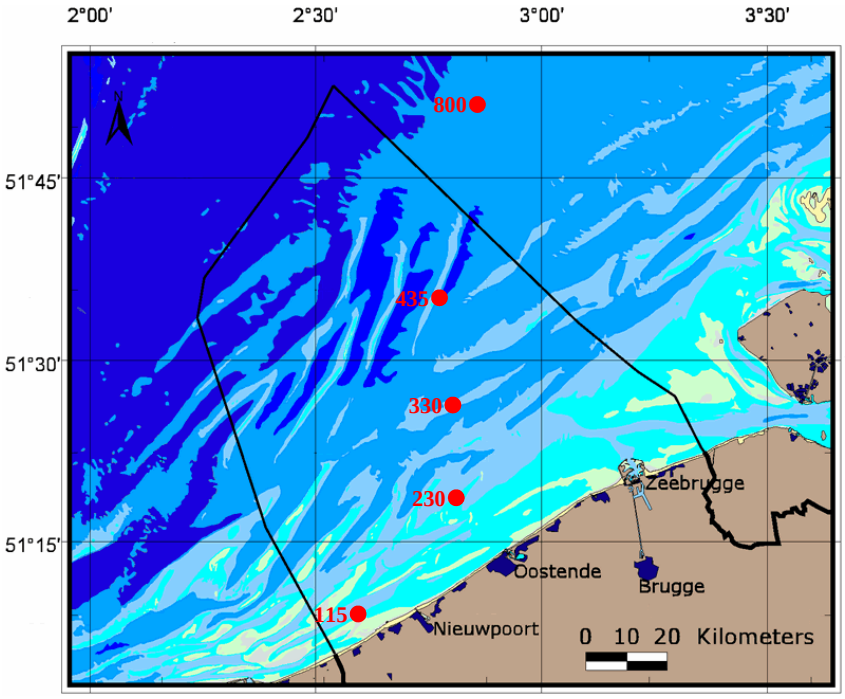

Figure I-2: Map of the Belgian coastal zone showing location of the sampling stations during AMORE 1999-2001 cruises.

2.2. Samples collection

Water samples were taken using a 10 l Niskin bottle, at subsurface, middle and near the bottom of the water column. Seawater samples (250-1500 ml) from the different depths were immediately filtered in triplicate using GF/F glass fibre filters (47 mm diameter, 0.7 µm nominal pore size) for HPLC pigment analysis. All filters were folded in an aluminium foil and immediately stored in liquid nitrogen throughout the cruise until the return to the lab where they were stored in an ultra-cold freezer at -85°C. Occasionally, additional water samples (250 ml) were taken in triplicate, after mixing of the three depths, and were fixed with 1% (final concentration) glutaraldehyde for algal cell counting.

At each station, CTD-profiles (Seabird) were made simultaneous with phytoplankton sampling to provide information on temperature, salinity, turbidity and oxygen.

2.3. HPLC pigment analyses

The frozen filters were cut into small pieces (several mm x 1 cm) and sonicated in centrifuge tubes (on crushed ice), with 2 ml of 100 % cold acetone for 2 x 15 seconds at 50 W using a Labsonic sonicator equipped with a 4 mm diameter probe inserted directly into the solvent. After sonication samples were kept for 2 hours at 4°C before centrifuged for 3 minutes at about 700 g. Supernatants were filtered onto 0.5 µm (Millex SR syringe equipped with 25 mm-filter of 0.5 µm-porosity), transferred to 1 ml vials and placed into the autosampler (kept at 4°c) prior injection through the HPLC column.

Reversed-phase HPLC analyses were conducted based on the slightly modified ternary gradient method of Wright et al. (1991) as described in Gasparini et al. (2000). The method of Wright et al. (1991) has been demonstrated to effectively separate over 50 carotenoids, chlorophylls and their derivatives. Our HPLC system consisted of a Waters Associates 600 Controller Pump, a 717 Autosampler and injector, coupled with dual-channel detection using a Waters 996 photodiode array detector, scanning from 350 to 700 nm and a Waters 470 scanning fluorescence detector set at excitation of 436 nm and emission at 676 nm, connected via a Waters system Interface Module to a micro-computer running Waters Millennium 32 software package (version 3.05, Waters Corporation, Inc.). The reversed-phased column used was a Spherisorb ODS2 (250 mm × 4.6 mm ID, 5 µm particle size), packed by Australian Government Analytical Laboratories, Melbourne.

Our standard procedure consisted of the injection of 200 µl of extract run through a 30 min gradient at a flow rate of 1 ml.min^{-1} (Table I-2). The eluant was linearly changed from solvent A (80:20 methanol:0.5 M ammonium acetate, aq.; pH 7.2 v/v) to B (90:10 acetonitrile 210 nm UV cut-off grade: water; v/v) in 4 min, which improved the separation of polar compounds, and then changed to 20% B and 80% C (100% ethyl acetate HPLC grade) in 14 min. This was followed by a return to 100% B in 3 min with final ramping to 100% A in 3 min. The program returned to initial conditions and re-equilibrated for 5 min before the next sample injection.

Table I-2: HPLC solvent system program

Time (min)	Flow rate (ml.min-1)	% A	% B	% C	Conditions
0	1.0	100	0	0	injection
4	1.0	0	100	0	linear gradient
18	1.0	0	20	80	linear gradient
21	1.0	0	100	0	linear gradient
24	1.0	100	0	0	linear gradient
29	1.0	100	0	0	equilibration

A: 80:20 methanol:0.5 M ammonium acetate (aq.; pH 7.2)
B: 90:10 acetonitrile 210 nm UV cut-off grade: water (v/v)
C: ethyl acetate HPLC grade

Pigment were detected by absorption at 436 nm and identified based on comparison of their retention time and spectra with standards. High-purity HPLC standards for chlorophyll *a*, pheophytin *a*, chlorophyll c_3, fucoxanthin, alloxanthin, peridinin and 19'-hexanoyloxyfucoxanthin were obtained from the International Agency for ^{14}C determination (Hørsholm, Denmark). Pheophorbide *a* was obtained from the ICN Biomedicals, Inc. Other pigments were identified using retention time and spectra of well characterised pigments (Jeffrey et al. 1997b). Our HPLC technique did not allow us to separate chlorophylls c_1 and c_2.

2.4. Protist enumeration and biomass estimate

Microscopic observations of phytoplankton and microzooplankton organisms were carried out on preserved material with an inverted microscope provided with phase contrast (ZEISS Sedival) in 5 ml sedimentation chambers after pre-concentration following the method of Colijn et al. (1990). Depending on cell density random fields (60-100) or selected

transects of the entire sedimentation chamber were counted for microplankton (x 200) and small flagellates (x 400). When possible, the cells were identified to species level, but some of the observed forms had to be placed into taxonomic categories such as dinoflagellates or 'unidentified nanoflagellates'. In the latter were included nanoplankton sized flagellates which could not be identified with certainty.

The C-biomass of diatoms was calculated on the basis of cell density and biometry determined for each species. A specific average biovolume was measured on a cell population throughout the period of its development (Rousseau et al. 2002, and pers. com.). After application of the correction equation for fixation-induced volume change (Menden-Deuer et al. 2001), the biovolumes were converted into C-biomass using the size-dependant density relationship as recommended for diatoms by Menden-Deuer and Lessard (2000). Dinoflagellates, nanoflagellates and ciliates biomass were calculated by using carbon to volume relationships recommended by Menden-Deuer and Lessard (2000). *Phaeocystis* colonies disintegrated under the fixation and concentration procedures, and therefore they were counted by the total number of single cell. A conversion factor of 14.2 pgC per cell for colonial cells and of 10.8 pg C per cell for flagellated cells (microzoospores) was used to estimate *Phaeocystis* carbon biomass as recommended by Rousseau et al. (1990).

3. Results

3.1. Environmental variables

Temperature, salinity, turbidity and oxygen profiles revealed vertical homogeneity at all stations. Thus the results reported in Figure I-3 are average values of the water column measured at the different stations during AMORE cruises in 1999-2001. The seasonal variations of temperature (Fig. I-3a) show a winter minimum of about 5°C and a summer maximum of about 20°C during the investigated period. The spatial changes of temperature show an increase along the nearshore-offshore transect from September to March and a decrease in spring and summer. However, if we consider all the values over the studied period temperature was not significantly different among stations ($F = 0.8$, n = 92, $p > 0.05$, 1-way ANOVA).

The average of salinity recorded for each station over the studied period is shown in Table I-3. Salinity was significantly different among stations ($F = 12.5$, n = 92, $p < 0.001$, 1-way ANOVA). Physical factors determining salinity distribution are tidal dispersion, river discharges, wind stress and advection. Except for station 800, the salinity variations observed

during the studied period is much more important than that observed during a tidal cycle (maximum amplitude of 0.53) as revealed by a 24-cycle measurement done every three hours at station 330 in May 2000. Thus, the salinity variations observed during the year (Fig. I-3b) reflect changing hydrographic conditions due to the varying mixing of freshwater discharges from river Scheldt with Atlantic waters. Average salinity at station 230 was significantly lower than at the other stations ($p < 0.05$ Tuckey's test) and seems to be the most influenced by the freshwater discharge from the Scheldt estuary, as shown by the amplitude of salinity variations. Average salinity at station 330 was also significantly different than at stations 435 and 800 ($p < 0.05$, Tuckey's test). Station 800 showed homogenous salinity over the study of more than 34 PSU characteristic of the Atlantic waters entering the BCZ through the Dover straits.

Table I-3: Average, minima, maxima and amplitude of variation of salinity at the sampling stations of the BCZ during AMORE 1999-2001 cruises.

Stations	115	230	330	435	800
Average	33.61	32.24	33.15	33.98	34.60
Minimum	31.34	29.42	32.00	32.95	34.37
Maximum	34.79	34.39	34.28	34.87	34.75
Amplitude	3.45	4.97	2.28	1.92	0.38

The discharge of suspended matter from the Scheldt estuary together with bottom erosion and resuspension due to the high turbulent regime supply this shallow area with large amounts of particulate matter. Turbidity values measured at the sampling stations varied between 2 and 19 NTU (Fig.I-3c) and were significantly different among stations ($F = 10.4$, $n = 62$, $p < 0.001$, 1-way ANOVA). The nearshore stations 115 and 230 presented significantly higher turbidity than the offshore and deeper stations 330, 435 and 800 ($p < 0.05$, Tuckey's test).

Fig. I.3d presents the oxygen values expressed as concentration (mg l^{-1}) and as percentage of oxygen saturation. Oxygen concentrations were similar among stations ($F = 1.1$, $n = 57$, $p > 0.05$, 1-way ANOVA). Belgian waters were very well oxygenated, often even oversaturated or near saturation (> 80 %) over the entire water column due to the high turbulent regime in this coastal area. Highest concentrations (> 9 mg l^{-1}) were observed in early spring during phytoplankton bloom. Oxygen concentrations decreased rapidly after the spring bloom with the increase of temperatures and zooplankton activity (mainly bacterial activity) reaching the lowest values in summer and fall.

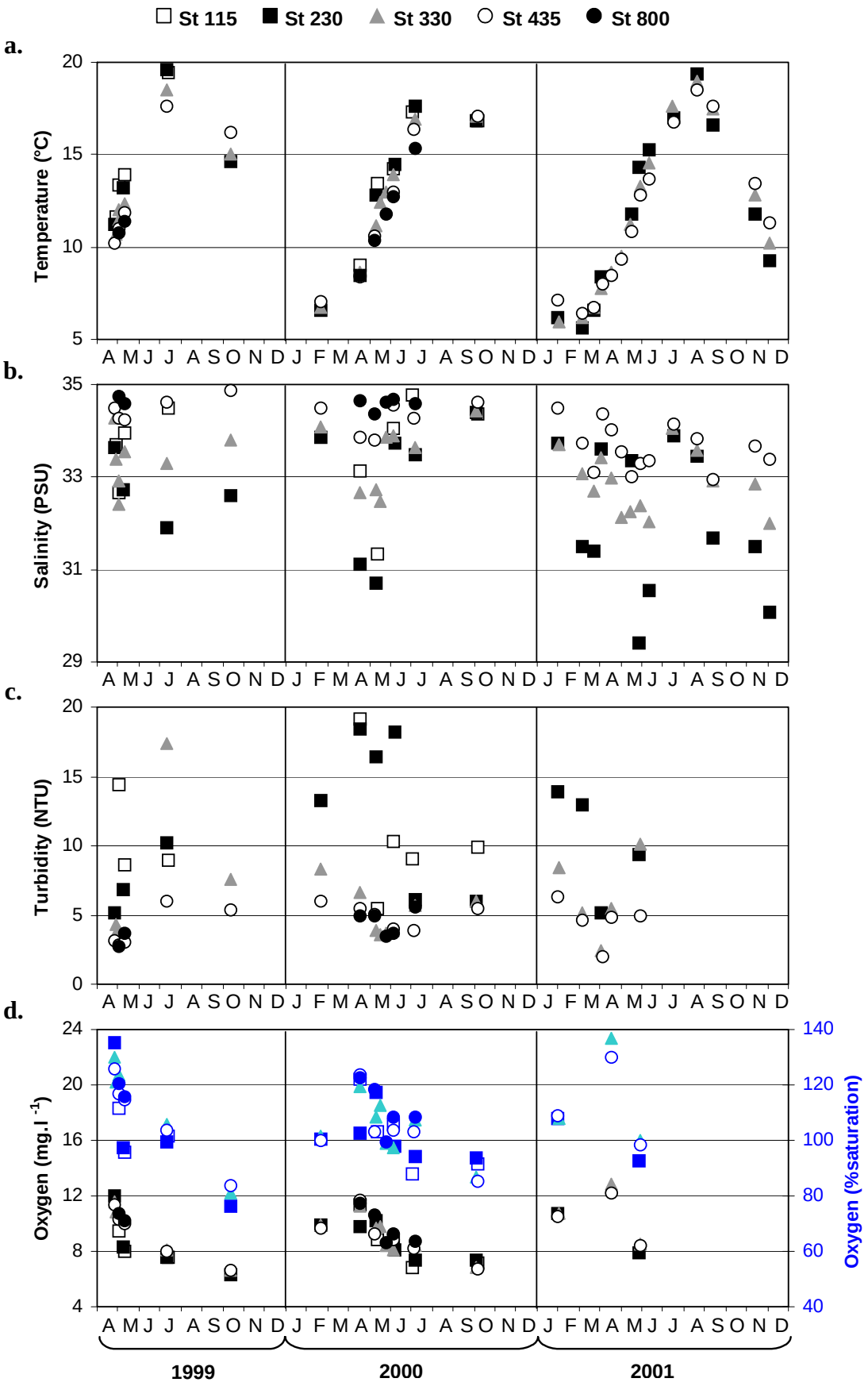

Figure I-3: Environmental parameters measured at the sampling stations of the Belgian coastal zone during AMORE 1999-2001 cruises.

3.2. Protist composition and seasonal pattern

All the phytoplankton and microzooplankton taxa which were identified in the samples are listed in Table I-4. Benthic species were rarely found, most of the phytoplankton species were typical neritic and pelagic species. Two fresh water species, *Scenedesmus acuminatus and S. quadricauda,* were only found at station 115 from samples collected the 12th May 2000, corresponding to the lowest salinity recorded to this station (31.34 PSU) and showing the influence of freshwater input on station closest to land.

Fig. I-4 and I-5 show seasonal variation of respectively abundance and biomass for diatoms, *Phaeocystis globosa*, nanoflagellates (other than *P. globosa*), dinoflagellates and ciliates at the different stations studied from 1999 to 2001. As shown by the monthly sampling in 2001, diatoms were present throughout the year reaching a maximum of abundance in March (ca. 1700 cell ml^{-1}). In April and early-May *P. globosa* dominated diatoms and reached its maximum abundance each year around mid-April (more than 20,000 cell ml^{-1}). At this time *P. globosa* C-biomass (up to 380 µgC l^{-1}) was two to three time higher than diatom C-biomass. A second but rather modest *P. globosa* growth was observed in September 2001 (ca. 1450 cell ml^{-1}). A bloom of nanoflagellates composed of Cryptophyceae, Prasinophyceae (*Pyramimonas* spp. and *Tetraselmis* spp.) and other unidentified nanoplankton-sized flagellates occurred at the end of the *Phaeocystis* bloom in May 2001 and was particularly impressive at station 230 (2300 cell ml^{-1}; 311 µgC l^{-1}). The abundances of these nanoflagellates, other than *P. globosa*, were significantly correlated with each other (Table I-5). Both dinoflagellate and ciliate abundances increased strongly at the wax and wane of *P. globosa* bloom (maximum values: 67 and 31 cell ml^{-1} respectively). The dinoflagellates were dominated by unidentified species < 20 µm (approximately equivalent spherical diameter). Dinoflagellates > 20 µm were mainly the phototrophic *Prorocentrum* spp. and the heterotrophic *Protoperidinium* spp. and *Noctiluca scintillans*. In general there was no obvious geographical trend in phytoplankton abundance and/or biomass along the nearshore offshore transect.

Table I-4: List of protist observed

Diatoms	
Division: Chromophyta	
Class: Bacillariophyceae	
Order: Centrales	
	Actinoptychus senarius (Ehrenberg) Ehrenberg 1843
	Actinoptychus splendens (Shradbolt) Ralfs 1861
	Bacteriastrum hyalinum Lauder 1864
	Bellerochea malleus (Brightwellii) Van Heurk 1885
	Brockmanniella brockmannii (Hustedt) Hasle 1983
	Cerataulina pelagica (Cleve) Hendley 1937
	Chaetoceros curvisetus Cleve 1989
	Chaetoceros danicus Cleve 1889
	Chaetoceros socialis Lauder 1864
	Chaetoceros densus (Cleve) Cleve 1901
	Chaetoceros spp.Ehrenberg 1844
	Coscinodiscus concinnus Wm. Smith 1856
	Coscinodiscus radiatus Ehrenberg 1841
	Coscinodiscus wailesii Gran & Angst 1931
	Coscinodiscus spp. Kützing 1844
	Dactyliosolen fragilissimus (Bergon) Hasle 1996
	Ditylum brightwellii (T. West) Grunow in Van Heurck 1883
	Eucampia zodiacus Ehrenberg 1839
	Guinardia delicatula (Cleve) Hasle 1996
	Guinardia flaccida (Castracane) H. Peragallo 1892
	Guinardia striata (Stolterfoth) Hasle 1975
	Helicotheca tamesis (Shrubsole) Ricard 1987
	Lauderia annulata Cleve 1873
	Leptocylindrus danicus Cleve 1889
	Leptocylindrus minimus Gran 1915
	Lithodesmium undulatum Ehrenberg 1841
	Melosira spp.C.A. Agardh 1824
	Odontella aurita (Lyngbye) Agardh 1832
	Odontella mobiliensis (Bailey) Grunow 1884
	Odontella regia (Schulze) Simonsen 1974
	Odontella rhombus (Ehrenberg) Kützing
	Odontella sinensis (Greville) Grunow 1884
	Paralia sulcata (Ehrenberg) Cleve 1873
	Rhizosolenia imbricata Brightwell 1858
	Rhizosolenia robusta Norman in Pritchard 1861
	Rhizosolenia setigera Brightwell 1858
	Skeletonema costatum (Greville) Cleve 1873
	Stephanopyxis turris (Arnott in Greville) Ralfs in Pritchard 1861
	Thalassiosira levanderi (Goor) Hasle 1983
	Thalassiosira nordenskioeldii Cleve 1873
	Thalassiosira punctigera Fryxel, Simonsen & Hasle 1974
	Thalassiosira rotula Meunier 1910
	Thalassiosira spp. Cleve 1873
	Triceratium alternans Bailey 1851
	Triceratium favus Ehrenberg 1839
Order: Pennales	
	Asterionellopsis glacialis (Castracane) Round 1990
	Asteroplanus karianus (Grunow) Garner et Crawford 1997

Table I-4: continued

	Bacillaria paxillifera (Muller) Hendley 1951
	Delphineis surirella (Ehrenberg) G.W. Andrews 1981
	Diploneis spp. Cleve 1894
	Ephemera planamembranacea (Hendley) Paddock 1988
	Meuneria membranacea (Cleve) P.C. Silva 1996
	Navicula transitans Cleve var. *derasa* Grunow f. *delicatula* Heimdal 1970
	Nitzschia longissima (Brébisson) Ralfs 1861
	cf. Nitzschia spp.
	Pleurosigma spp. W. Smith 1852
	Pseudo-nitzschia delicatissima (Cleve) Heiden & Kolbe 1928
	Pseudo-nitzschia seriata (Cleve) Pergallo 1900
	Raphoneis amphiceros Ehrenberg 1844
	Thalassionema frauenfeldii (Grunow) Hallegraeff 1986
	Thalassionema nitzschioides (Grunow) Mereschkowsky 1902
Nanoflagellates	
Division: Chromophyta	
Class: Haptophyceae	
	Phaeocystis globosa Scherffel 1899
Class: Cryptophyceae	
	Leucocryptos marina (Braarud) Butcher 1967
Division: Chlorophyta	
Class: Prasinophyceae	
	Pyramimonas spp. Schmarda 1850
	Tetraselmis spp. Stein 1878
Class: Euglenophyceae	
	Eutreptiella sp. de Cunha 1914
Class: Chlorophyceae	
	Scenedesmus acuminatus (Lagerheim) Chodat
	Scenedesmus quadricauda (Turpin) Brébisson 1913
Dinoflagellates	
Division: Chromophyta	
Class: Noctiluciphyceae	
	Noctiluca scintillans Kofoid & Swezy 1921
Class: Dinophyceae	
	Ceratium fusus (Ehrenberg) Dujardin 1841
	Gyrodinium lachryma (Meunier) Kofoid & Swezy 1921
	Prorocentrum micans Ehrenberg 1833
	Prorocentrum triestinum Schiller 1918
	Protoperidinium spp. Bergh 1881
	Pyrocystis lunula (Schütt) Schütt 1896
Ciliates	
Division: Ciliophora	
Class: Litostomatea	
	Mesodinium rubrum Lohman 1908
Class: Phyllopharyngea	
	Acineta tuberosa Ehrenberg 1838
	Acineta spp. Ehrenberg 1833
Class: Prostomatea	
	Tiarina fusus Claparède & Lachman 1857
Class: Spirotrichea	
	Favella spp. Jørgensen 1924
	Tintinnopsis spp. Stein 1867

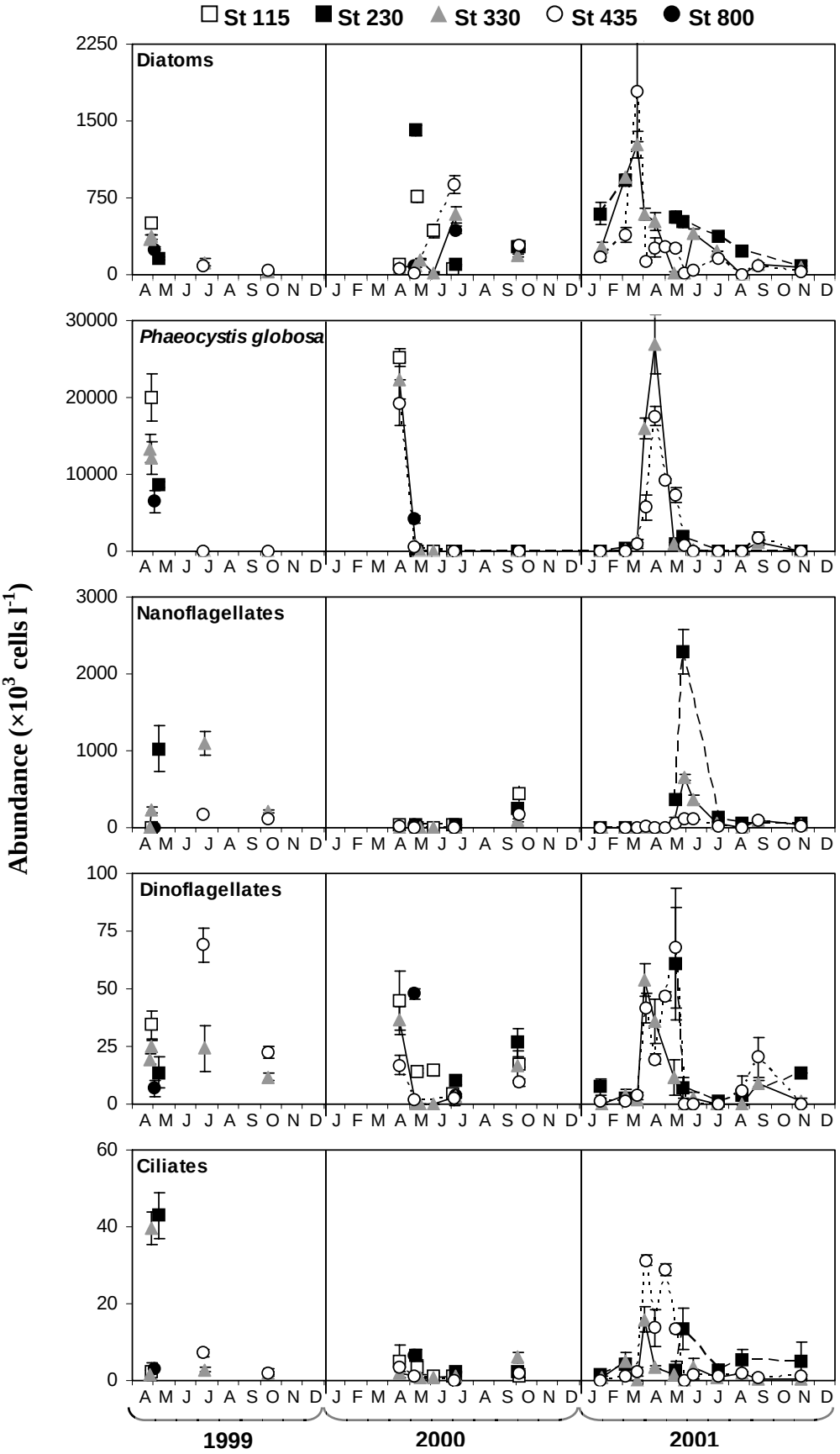

Figure I-4: Seasonal variation of diatom, *Phaeocystis globosa*, nanoflagellate (other than *P. globosa*), dinoflagellate and ciliate abundances ($\times 10^3$ cell l^{-1} ± SE) at stations of the Belgian coastal zone during AMORE 1999-2001 cruises. Note the vertical scales.

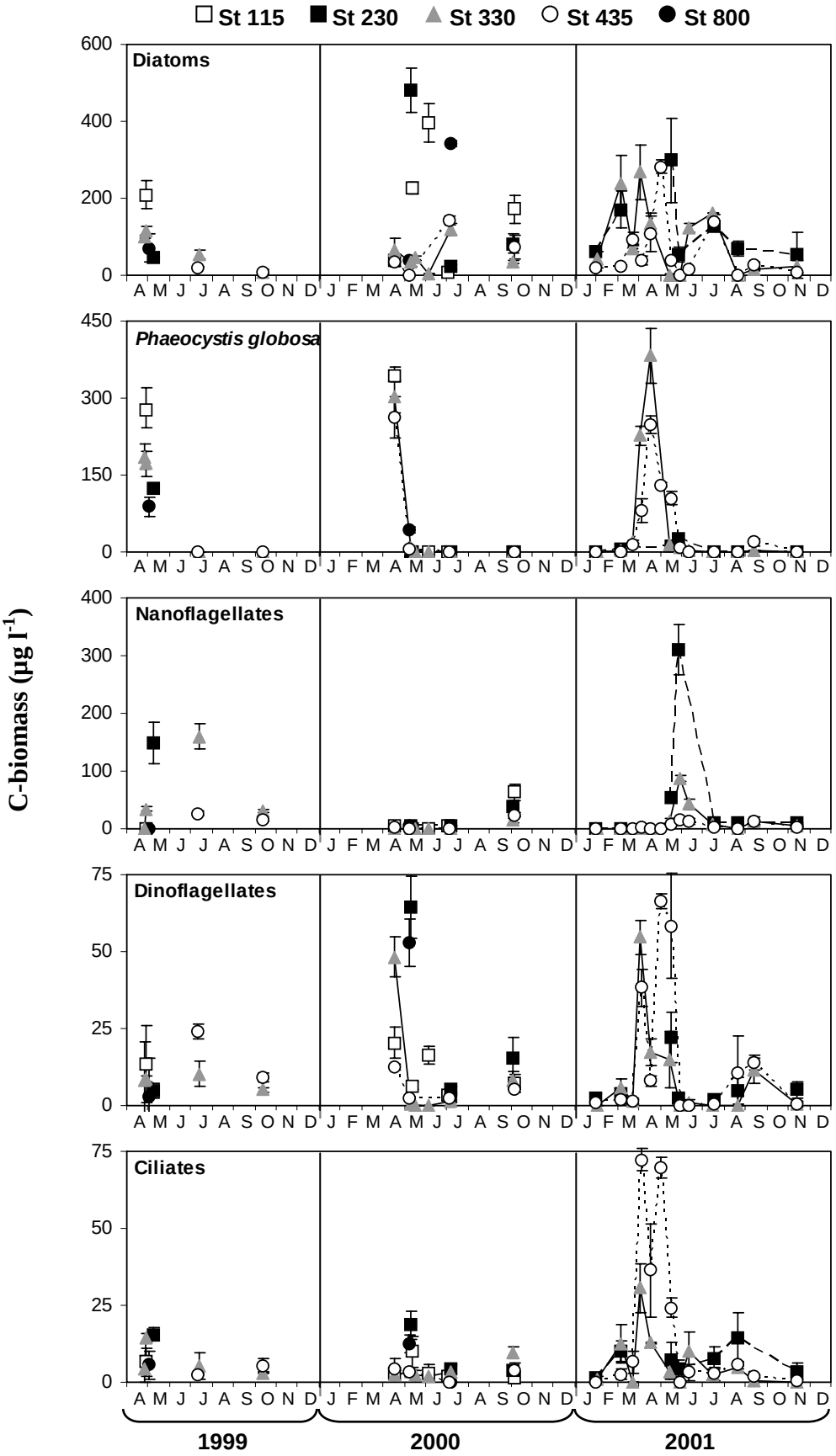

Figure I-5: Seasonal variation of diatom, *Phaeocystis globosa*, nanoflagellate (other than *P. globosa*), dinoflagellate and ciliate C-biomass (µgC l^{-1} ± SE) at stations of the Belgian coastal zone during AMORE 1999-2001 cruises. Note the vertical scales.

Table I-5: Correlations among protist biomass. The top number represents the Spearman correlation coefficient and the number in parenthesis is the significance value (*P*-value). Sample size is 60.

	Diatoms	***P. globosa***	**Dinofg.**	**Crypto.**	**Prasino.**	**Unidentified nanofg.**
P. globosa	0.1564 (0.2329)					
Dinoflagellates	**0.2769 (0.0322)**	**0.4209 (0.0008)**				
Cryptophyceae	-0.0024 (0.4996)	-0.2342 (0.0717)	-0.2514 (0.0526)			
Prasinophyceae	-0.1944 (0.1367)	-0.1385 (0.2914)	-0.2566 (0.0478)	**0.7919 (0.0000)**		
Unidentified nanofagellates	-0.1095 (0.4050)	-0.0591 (0.6535)	0.1706 (0.1926)	**0.3915 (0.0020)**	**0.4861 (0.0000)**	
Ciliates	**0.4009 (0.0015)**	**0.3924 (0.0019)**	**0.5117 (0.0000)**	-0.1734 (0.1852)	-0.2566 (0.0478)	0.0467 (0.7230)

Due to seasonal changes in species composition of the diatom community and the large size spectrum of this class, diatom C-biomass (Fig. I-5) showed much more variability than diatom abundance. The succession of the most abundant diatom species in 2001 is presented in Fig. I-6. During late-winter early-spring period the diatom community was characterized by small chain-forming species. *Thalassiosira levanderi* initiated the diatom succession in February with *Nitzcshia longissima* and *Thalassionema nitzshioides*. In March small species of the genus *Chaetoceros* largely dominated by *C. socialis* became dominant, representing until 90 % of the diatom community. A fusiform nitzschioid diatom was observed as an occasional epiphyte on *C. socialis* colonies, but its proper identification and taxonomic placement are uncertain. These two species partly co-occurred with *P. globosa* during the outburst of *Phaeocystis* bloom, but were rapidly replaced by a diatom assemblage dominated by *Rhizosolenia imbricata* and *Guinardia delicatula* in April, and by *Pseudo-nitzschia delicatissima* and *Cerataulina pelagica* in May. High abundances of *Asterionellopsis glacialis* and *Chaetoceros curvisetus* were also observed in May at a nearshore station only (st. 230). These co-occurrences of diatoms with *Phaeocystis* colonies bloom was also observed in spring 1999 and 2000. During summer, the diatoms community was once again dominated by *R. imbricata* and *G. delicatula* in the presence of some *Leptocylindrus danicus* and *Guinardia striata*. In autumn the low diatom abundances (< 100 cell ml^{-1}) were mainly dominated by *Paralia sulcata* and occasionally by *Eucampia zodiacus*.

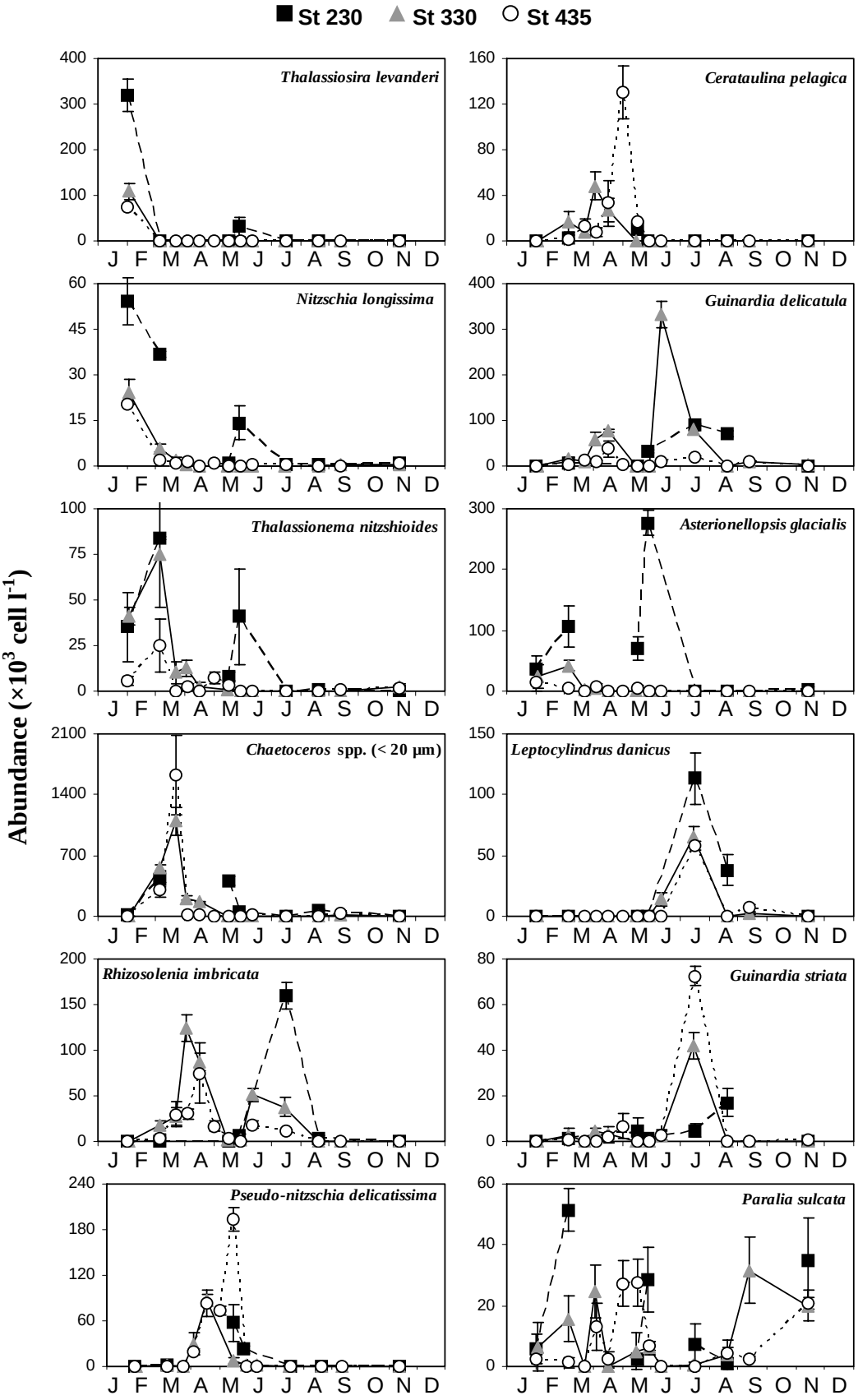

Figure I-6: Abundance of the 12 most abundant diatom species ($\times 10^3$ cell l^{-1} ± SE) at 3 stations of the Belgian coastal zoone during AMORE 2001 cruises. Note the vertical scales.

3.3. Phytoplankton pigments in the water column

Absorbance chromatograms obtained in four representative situations in 2001 are presented in Fig. I-7, showing the elution pattern of a range of chlorophyll and carotenoid pigments detected in the Belgian coastal waters. These situations correspond to the bloom of *C. socialis* (22nd March), the *P. globosa* maximum (17th April), the occurrence of Cryptophyceae and Prasinophyceae (28th May) and the modest bloom of *P. globosa* in fall (11th September). The pigment concentration ranges are given in Table I-6.

Table I-6: Peak Number, identification, abbreviation, retention time, concentration range and month of occurrence of phytoplankton pigments detected in seawater samples from cruises AMORE 2000 and 2001.

No.	Pigment	Abbreviation	Retention time (min)	Range ($\mu g\ l^{-1}$)	Occurrence (month)
1	Chlorophyllide *a*	Chlide *a*	9.40		all
2	Chlorophyll c_3	Chl c_3	9.64	0.00 - 1.90	all except 11, 12
3	Chlorophyll c_1+c_2	Chl c_1+c_2	10.69		all
4	Peridinin	Perid	12.80	0.00 - 0.12	1, 2, 8, 9, 11
5	Fucoxanthin	Fuco	14.10	0.08 - 8.97	all
6	19'-Hexanoyloyfucoxanthin	Hex-fuco	14.65	0.00 - 0.11	1, 2, 3, 4, 5, 8, 9, 10
7	Pheophorbide *a*	Phide *a*	15.86	0.00 - 0.62	6, 7, 10
8	Diadinoxanthin	Diadino	16.20		all
9	Alloxanthin	Allo	17.00	0.00 - 0.44	all
10	Diatoxanthin	Diato	17.50		all
11	Zeaxanthin	Zea	18.44		5, 6
12	Chlorophyll *b*	Chl *b*	19.80		all exept 3, 4
13	Phytylated chlorophyll *c*-like	Phytyl-chl *c*	20.10		4, 5
14	Chlorophyll *a* allomer	Chl *a* allomer	20.54		all
15	Chlorophyll *a*	Chl *a*	20.83	0.15 - 28.35	all
16	Chlorophyll *a* epimer	Chl *a* epimer	20.97		all
17	Pheophytin *a*	Phytin *a*	23.00	0.00 - 0.45	all
18	*β*-*β*cartene	*β*-*β*car	23.57		all

Chlorophyll *a* concentration integrated on the water column varied between 0.15 and 28.35 $\mu g\ l^{-1}$ (Fig. I-8). Increases in chlorophyll *a* concentrations were associated with increases in phytoplankton containing fucoxanthin (Spearman rank $r_s = 0.948$, $n = 64$, $p < 0.001$) and coincided with diatom (March and June) and *P. globosa* (April) blooms. The chlorophyll *a* degradation products that occurred in all samples were pheophytin *a*, chlorophyllide *a*, and chlorophyll *a* allomer and epimer, the latter being present at trace level. Distribution of pheophytin *a* concentrations differed significantly among stations (Kruskal-wallis test, $n = 64$, $p = 0.0018$), stations near the coast (115 and 230) having usually the highest concentrations. Surprisingly, pheophorbide *a* was only detected in samples collected in June, July and October 2000, but never in 2001.

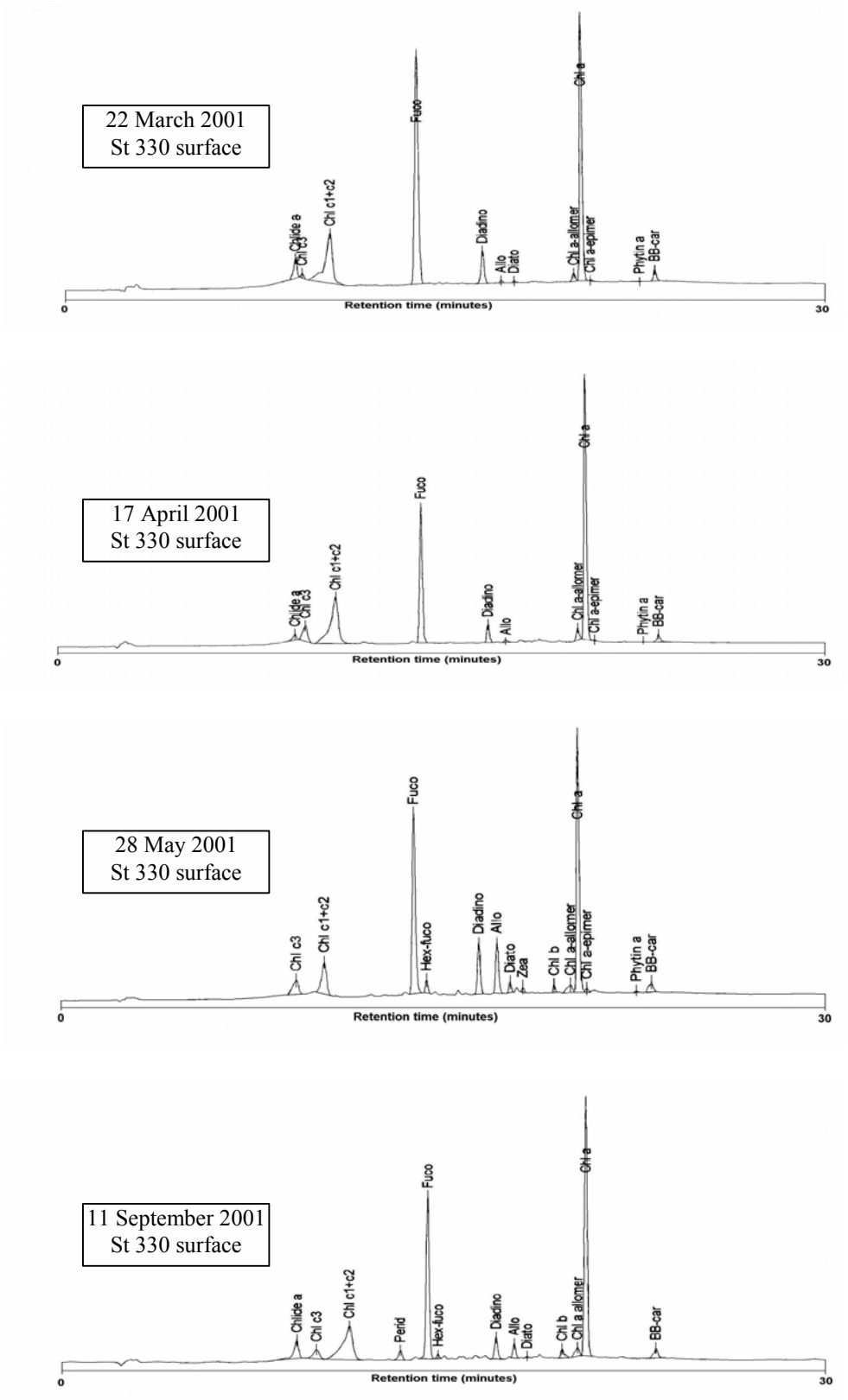

Figure I-7: Absorbance chromatograms (detection at 436 nm) of samples taken at four representative periods at station 330 of the Belgian coastal zone in 2001. Abbreviations refer to the pigment name in Table I-6.

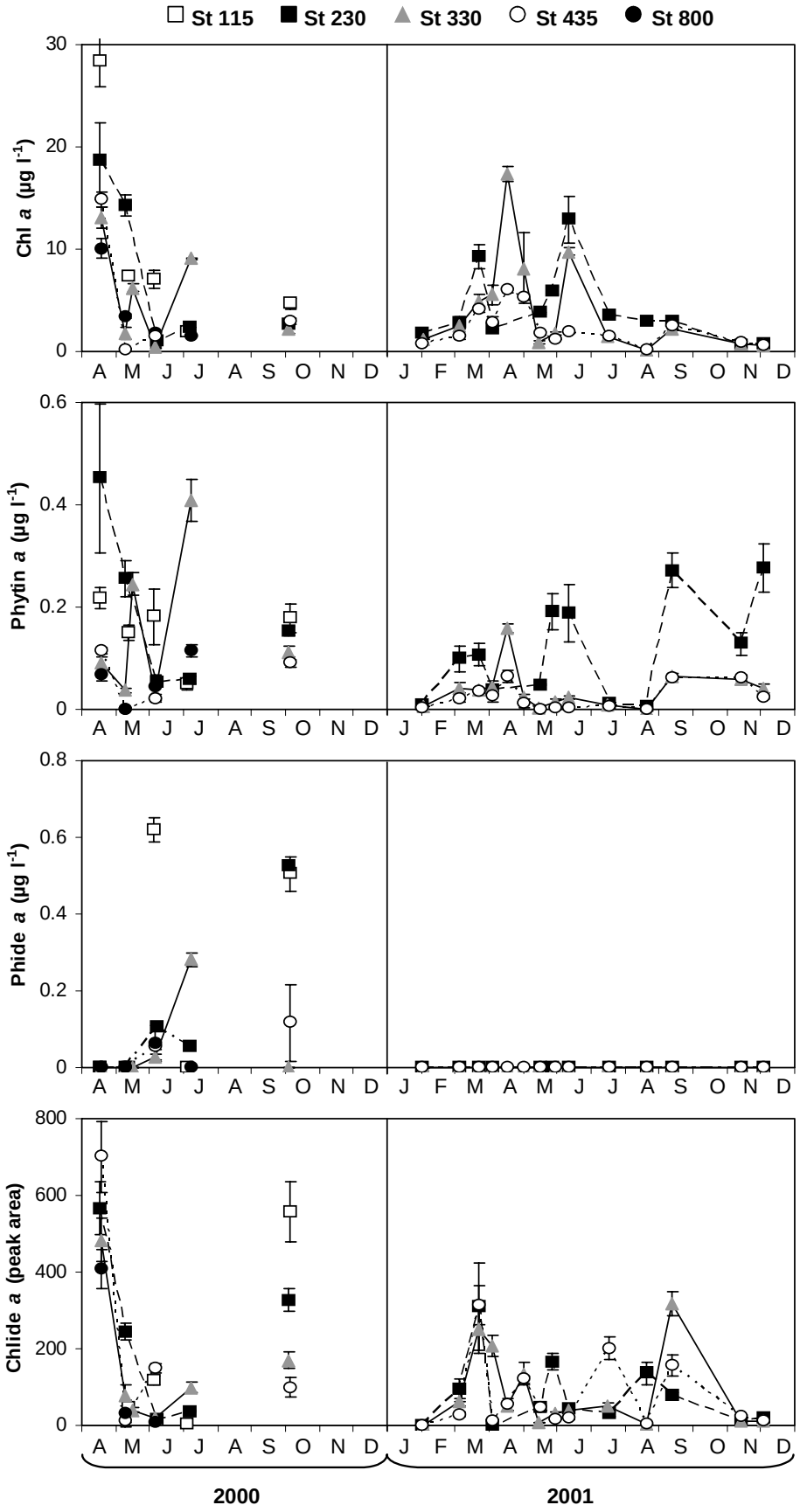

Figure I-8: Seasonal variation of chlorophyll *a* concentrations (µg l^{-1} ± SE) and of its degradation products at stations of the Belgian coastal zone during AMORE 2000-2001 cruises. See Table I-6 for pigment abbreviations. Note the vertical scales.

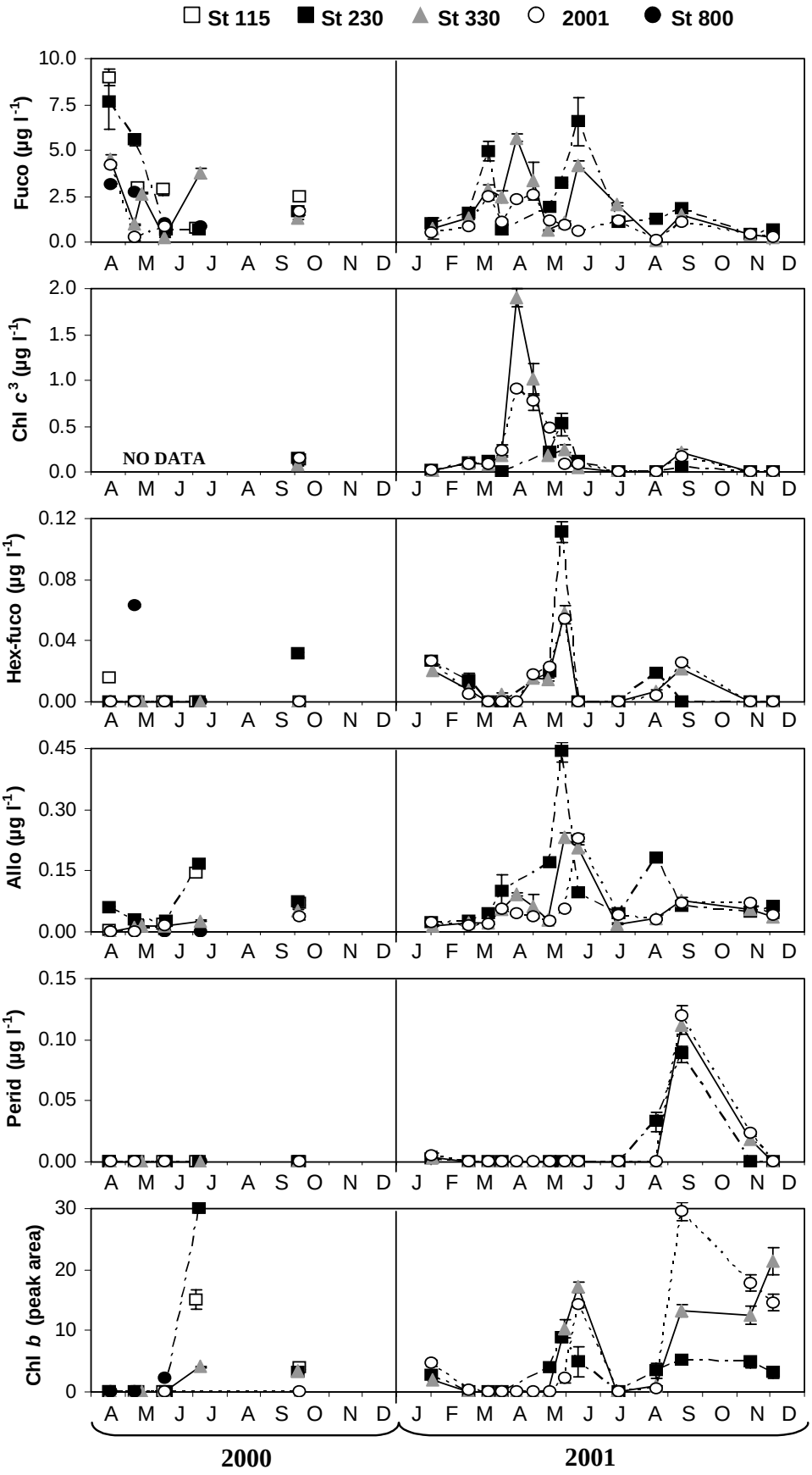

Figure I-9: Seasonal variation of taxonomic chlorophyll and carotenoid concentrations (µg l^{-1} ± SE) at stations of the Belgian coastal zone during AMORE 2000-2001 cruises. See Table I-6 for pigment abbreviations. Note the vertical scales.

Numerous chlorophyll and carotenoid pigments were detected by HPLC. Our interest, however, was in those carotenoids that can be utilized as taxonomic markers of classes or groups of classes of phytoplankton. Seasonal variations of the main chemotaxonomic carotenoids found in the water column are presented in figure I-9. The major carotenoid, fucoxanthin, had concentration ranging between 0.08 and 8.97 µg l^{-1} and was significantly correlated with the golden-brown algae biomass, diatoms and *P. globosa* ($r_s = 0.780$, n = 51, $p < 0.001$) which contributed to the greatest part of the phytoplankton in Belgian coastal waters. Chlorophyll c_3 which was highly correlated with *P. globosa* biomass ($r_s = 0.812$, n = 51, $p < 0.001$) increased from a background level of less than 0.25 µg l^{-1} up to 1.90 µg l^{-1} in April 2001, corresponding with maximum abundance of *P. globosa*. Interestingly 19'-hexanoyloyfucoxanthin, frequently used as key marker of haptophytes, was not correlated with *P. globosa* and showed maximum concentrations (up to 0.11 µg l^{-1}) at the end of the *Phaeocystis* bloom. Alloxanthin was commonly found in small amounts with highest values observed in May at the end of *P. globosa* bloom, corresponding to an increase in cyptomonad biomass ($r_s = 0.661$, n = 51, $p < 0.001$). Traces of peridinin were only found in 2001 with the highest concentration, 0.12 µg l^{-1}, observed in September, but it was not correlated with any of the protist groups we looked at. As no chlorophyll *b* standard was available we used chromatographic peak area to compare relative abundance of this pigment on a temporal scale. Chlorophyll *b* showed the most important chromatographic peak areas in June and in autumn and was significantly correlated with the prasinophytes and 'unidentified nanoflagellates' group ($r_s = 0.650$ and $r_s = 0.474$, respectively, n = 51, $p < 0.001$).

4. Discussion

4.1. Protist dynamics in Belgian coastal waters

Diatoms were the main phytoplankton community component during the sampling period and constituted, with the haptophyte *Phaeocystis globosa,* the bulk of phytoplankton biomass during the spring bloom. The succession of the different diatom communities and of *P. globosa* recorded from 1999 to 2001 was similar to that observed in Belgian coastal waters since 1988 (Lancelot et al. 1998, Rousseau 2000). According to Rousseau et al. 2002, the seasonal pattern of diatoms in Belgian coastal waters could be partly due to their silica requirement. In the early phase of the growing season 2001, the algal crop consisted primarily of small colony-forming diatoms. The growth of this diatom community, dominated by *Thalassiosira levanderi* and *Thalassionema nitzchioides* seems controlled by the winter

concentration of silicate (Lancelot 1995, Rousseau et al. 2002). The wane of this diatom bloom was preceded by a change in diatom dominance in favour of *Chaetoceros socialis*, a species assumed to have a low silicate requirement according to Rytler et al. (1971, cited in Gieskes and Kraay 1975). At this time silicate exhaustion is generally observed in water column (Rousseau 2002). *Chaetoceros socialis* was associated with the onset of *P. globosa*, a colonial haptophyte that does not require silicate for its growth, and which became the dominant primary producer in April both in 2000 and 2001. The magnitude of this recurrent *Phaeocystis* bloom is determined by the nitrate excess left over at the end of the early spring silicate-controlled diatoms flowering (Lancelot 1995, Lancelot et al 1998). In May senescent *Phaeocystis* colonies were colonised by high abundance of *Pseudo-nitzschia delicatissima*. Recently the genus *Pseudo-nitzschia* has attracted more scientific attention than do many diatoms because of the discovery that the metabolite domoic acid is produced by at least five species in this genus (Hallegraeff et al. 1995) of which *P. delicatissima* and *P. seriata* found in our samples. Domoic acid is a neurotoxin causing gastrointestinal and neurological illness (sometimes fatal) in humans, termed ASP (amnesic shellfish poisoning), and mortalities among a variety of marine invertebrates. An assemblage of larger thin-walled diatoms characterized by *Rhizosolenia imbricata*, *Guinardia* spp and *Cerataulina pelagica* co-occurred with *P. globosa* and constituted the bulk of phytoplankton biomass in summer. This diatom assemblage was found to have a lower silica content (Si:C) compared to the diatom assemblage occurring in early spring (Rousseau et al. 2002). A smaller peak of *P. globosa* colonies similar to those occurring in spring was observed in September 2001. Moderate blooms of *Phaeocystis* have already been reported in adjacent coastal areas of the Southern Bight of the North Sea in fall and in winter (Hamel 1930, Cadée and Hegeman 1986, Cadée 1991). Overwintering colonies of *Phaeocystis* are assumed to release flagellate forms in spring which in turn produce the colonies of the spring bloom (Cadée 1991).

Apart from these two main phytoplankton components, cryptomonads, prasinophytes, dinoflagellates and ciliates were occasionally important contributors of protist biomass in Belgian coastal waters. Dinoflagellates and ciliates were significantly associated with the bloom of *Phaeocystis*. Among the heterotrophic dinoflagellates, blooms of *Noctiluca scintillans* (up to several hundred cells per litre) were observed particularly at the nearshore stations 115 and 230 from mid spring to summer in 2000 and 2001. High abundance of *N. scintillans* has already been reported during and after *Phaeocystis* blooms in the southern North Sea (Weisse and Scheffel-Möser 1990, Frangoulis et al 2001). Microscopic observations confirmed that *N. scintillans* is able to ingest small *Phaeocystis* colonies

(Jakobsen and Tang 2002). Apart for *N. scintillans*, no attempts have been made to distinguish between autotrophic and heterotrophic dinoflagellates in this study.

4.2. Assignment of signature pigments to phytoplankton senescence/predation

Earlier studies raised the possibility that occurrence of chlorophyll degradation products can give clues to the physiological state of the phytoplankton and grazing activity by zooplankton. Chlorophyllide *a* and pheophytin *a* were the major decomposition products of chlorophyll *a* in Belgian coastal waters. Degradation of chlorophyll *a* into chlorophyllide *a* and pheophytin *a* (fig. I-10) follows two different chemical reaction pathways (Shuman and Lorenzen 1975).

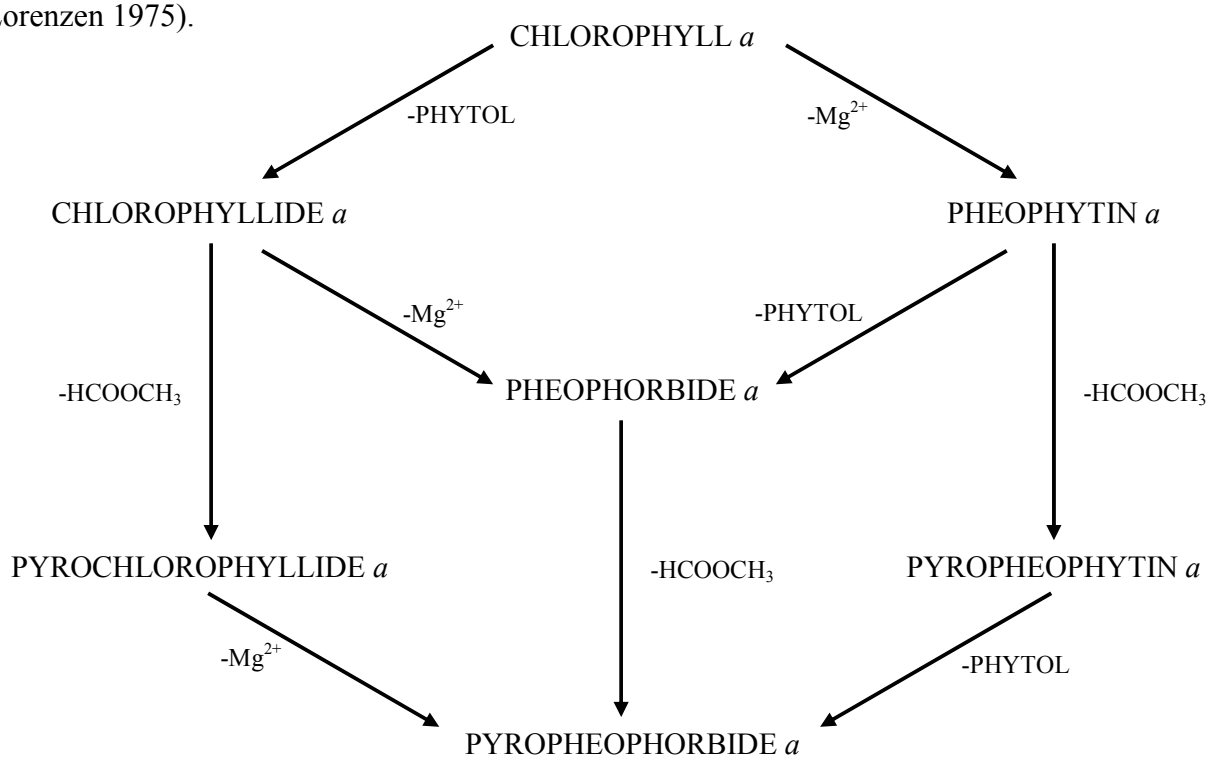

Fig. I-10: Degradation pathways which chlorophyll *a* may follow in senescent and/or grazed phytoplankton (from Louda et al. 1998). Loss of phytol chain is due to the enzyme chlorophyllase, while loss of Mg^{2+} occurs chemically when exposed to an acidic environment or enzymatically by the action of magnesium dechelatase. Pyropheopigments correspond to the loss of methylated carboxyl group at C-13 position by pyrolyse.

Chlorophyllide *a* is formed by dephytylation (loss of phytol chain) of chlorophyll *a* by the enzyme chlorophyllase. This chemical reaction occurred when the disintegration of cells and chloroplast structures makes chlorophyll accessible to this thylakoid-bound enzyme (Holden 1961). The presence of chlorophyllide *a* in phytoplankton field samples was then used as an indication of senescent algae especially during diatom blooms (Jeffrey 1974,

Hallegraeff 1981, Jeffrey and Hallegraeff 1987). More recently however, it has been shown that sample workup could also mediate initial chlorophyll *a* degradation. Cellular disruption during harvesting (filtration), extraction procedure and long term storage can lead to the generation of chlorophyllide *a* by the action of chlorophyllase (Mantoura et al. 1997, Louda et al. 1998). Thus, although chlorophyllide *a* was significantly correlated to phytoplankton biomass, senescent processes in Belgian coastal waters cannot be clearly demonstrated since it was impossible to separate senescent from handling alteration in the samples studied.

Pheophytin *a*, is formed by the loss of the Mg atom that is present in the tetra-pyrrole ring of the chlorophyll molecule (Fig. I-10). This reaction occurs chemically when chlorophyll *a* is exposed to an acidic environment (Vernet and Lorenzen 1987, Porra et al. 1997) or enzymatically by the action of magnesium dechelatase (Louda et al. 1998). Further degradation of pheophytin *a* occurs with the loss of the phytol chain (dephytylation) under lower pH or mediated by chlorophyllase to generate pheophorbide *a* (Jeffrey and Hallegraeff 1987). The widespread presence of pheophytin *a* and pheophorbide *a* in copepod faecal pellets seems to indicate that processes of this kind seem to happen in copepod guts (Hallegraeff 1981, Vernet and Lorenzen 1987, Head and Harris 1992, Head and Horne 1993). Further degradation of pheophorbide *a* occurs inside faecal pellets after they are released to produce pyropheophorbide *a* (Head and Harris 1992, 1996). Surprisingly, pheophorbide *a* was sporadically found in samples in 2000 and never in 2001, contrary to pheophytin *a* which was always present in Belgian coastal waters. Pheophorbide is normally the most abundant of the pheopigments in marine temperate waters (Vernet and Lorenzen 1987). According to Mantoura et al. (1997), pheophytin *a* and pheophorbide *a* are not formed during long-term storage of filtered samples and if present they must have been in the original samples. Since pheophytin *a* is an intermediate product of degradation of chlorophyll *a* to pheophorbide *a*, pheophytin-rich samples could be considered less degraded than pheophorbide-rich samples. Reduced degradation may be the result of faecal pellets produced at high food concentrations that result in shorter gut passage time and thus reduced time of exposure to degradation in the gut (Vernet and Lorenzen 1987). Another explanation of pheophytin *a* dominance in Belgian coastal waters is a possible higher rate of destruction for pheophorbide *a* (in colourless products), thus leaving pheophytin *a* as the surviving molecular species (Louda et al 1998). In the present work, pheophytin *a* was particularly dominant at the stations closest to the coast (115 and 230) where the turbidity was the most important due to the input of large quantities of organic material by the Scheldt estuary. High turbidities favour the installation of a large population of particle-attached bacteria (Crump et al. 1998) which are known to produce

pheophytin *a* and pyropheophytin *a* through their exoenzymatic activities (Bidigare et al. 1986, Lemaire et al. 2002). Additionally, there is increasing evidence of pheophytin *a* and pyropheophytin *a* generation during senescent-depth processes of diatoms and dinoflagellates species (Louda et al. 1998, 2002). Thus, consideration of these pigments as a strict marker for grazing activities by herbivorous zooplankton, such as copepods needs to be moderated.

From these results it appears that the story of chlorophyll *a* derivatives in marine environment is a great deal more complex than chlorophyllide *a* simply reflecting senescence and pheophytin *a*/pheophorbide *a* indicating predation.

4.3. Assignment of signature pigments to phytoplankton taxa

Fucoxanthin was the major accessory pigment in agreement with the dominance of golden-brown algae, represented overall by diatoms and *P. globosa*. The distinction between diatoms and haptophytes can be difficult since these algae often contain the same pigments. Stauber and Jeffrey (1988) analysed 51 species of diatoms and found that all but one contained fucoxanthin. The pigment composition of a *P. globosa* strain isolated from Belgian coastal waters in April 2001 revealed that fucoxanthin was the main carotenoid pigment of that species and that fucoxanthin to chlorophyll *a* ratio was comparable to values reported for diatoms (see chapter 2). Therefore in samples in which both diatoms and *P. globosa* were abundant fucoxanthin did not allow separation of these two taxonomic groups. Moreover, other fucoxanthin-containing golden-brown flagellates (e.g. Chrysophytes or other Haptophyte species) might have been placed into the taxonomic category 'unknown nanoflagellates' and might have also contributed to a substantial part of the fucoxanthin concentration in Belgian coastal waters. Jeffrey and Wright (1994) analysed 50 strains of 29 haptophyte species and reported that 19'-hexanoyloxyfucoxanthin and chlorophyll c_3 were frequently present (68% and 56% of the strains analysed respectively). The former was not correlated with *P. globosa* and the sampling of 2001 clearly shows a time lag between *P. globosa* spring bloom and 19'-hexanoyloxyfucoxanthin concentration. The presence of this pigment can therefore not be used as indicative of *P. globosa* occurrence in Belgian coastal waters. Chlorophytll c_3 on the other hand was significantly correlated with *P. globosa* and seems to be an interesting biomarker of *P. globosa*. However, small amount of chlorophyll c_3 were also detected when *P. globosa* was clearly not present in water column (e.g. summer period) and therefore we could not attribute this pigment to the presence of *P. globosa* alone, since other haptophytes and some diatoms (Stauber and Jeffrey 1988) are known to contain

this pigment as well. More details about pigment composition of *P. globosa* and possibility of using its pigments to trace *P. globosa* in the food web are given in chapter II.

The significant correlation between alloxanthin concentration and Cryptophyceae abundance confirms the chemotaxonomic marker quality of this pigment in natural sea water samples as reported in the literature (Gieskes and Kraay 1983, Jeffrey and Vesk 1997, Breton et al. 2000). However, alloxanthin was detected throughout the year whereas Cryptophyceae were not always observed. This apparent discrepancy may be due to the damages caused by fixative which render identification of 'fragile' flagellates somewhat difficult. On the other hand endosymbiotic Cryptophyceae are found in planktonic ciliates such as *Mesodinium rubrum* (Parsons and Blackbourn 1968; Hibberd 1977; Gieskes and Kraay 1983), and in dinoflagellates such as *Gymnodinium acidotum* (Wilcox and Wedemayer 1984) and some *Dinophysis* spp. (Meyer-Harms and Pollehne 1998; Hackett et al. 2003). It is thus possible that part of the Cryptophyceae were overlooked partly because they were damaged by fixative, partly because they occurred as endosymbiont in other protists.

Peridinin is found only in certain Dinophyceae, and so it is an unequivocal marker for this class (Mackey et al. 1996, Jeffrey and Vesk 1997, Breton et al 2000). Peridinin was only detected from August to November 2001 in Belgian coastal waters. Although dinoflagellates were actually observed at that time, major blooms of dinoflagellates occurred in April and May both in 2000 and 2001. The discrepancy could be explained by the dominance of (1) peridinin-lacking autotrophic dinoflagellates such as some *Gymnodinium* species (Johnsen and Sakshaug 1993, Zapata et al 1998) and/or (2) non-phototrophic dinoflagellates since as many as 50% of dinoflagellate species are now known or presumed to be heterotrophic or mixotrophic (Larsen and Sournia 1991). Then, the absence of peridinin, even in samples where dinoflagellates were detected by microscopy, precluded the evaluation of the contribution of dinoflagellates in copepod diet base on the presence of this pigment in copepod's gut.

Chlorophyll *b* is considered to be a good marker for green algae, which include the Chlorophyceae, Prasinophyceae and Euglenophyceae (Jeffrey and Vesk 1997). Euglenoids were very scarcely observed in microscopic observations and were not considered as a significant contributor of chlorophyll *b* in this study. The small flagellates of the Chlorophyceae are not easily identified in field samples and may have been included into the 'unknown flagellate' group. However, the absence of detectable concentrations of violaxanthin and lutein, the major pigments in Chlorophyceae, suggests that they were relatively rare in Belgian coastal waters. Prasinophyceae were regularly observed, representing up to 40% of

the nanoflagellates (other than *P. globosa*) in June 2001, and were significantly correlated with chlorophyll *b*. However chlorophyll *b* was also correlated with the group of 'unknown flagellates', indicating that some Prasinophyceae (and/or Chlorophyceae) would be included into this group and contributed to the chlorophyll *b* pattern.

4.4. Concluding remarks

The method based on microscopy suffers from the fact that a proportion of the algal crop, particularly flagellates, is damaged during fixation or not recognized by the microscopist for other reasons. Identification of phytoplankton can be assisted by analysis of taxon-specific pigments in suspended matter. In this way, the occurrence of nanoplanktonic microflagellates (Cryptophyceae, green algae, and Prymnesiophyceae) has been documented in the central and northern North Sea by Gieskes and Kraay (1983, 1984). HPLC-based chlorophyll and carotenoid signatures are now routinely used as a taxonomic complement to cell identification and quantification (e.g., Andersen et al. 1996, Mackey et al. 1996, Roy et al. 1996, Breton 2000). Nevertheless, it should be noted that a phytoplanktonic species can have a different pigment pattern than the taxonomic group it belong to and that a pigment suite may be shared by more than one algal group (e.g. some haptophytes and diatoms) as will see in the next chapter. HPLC pigment analysis cannot generally be used to make taxonomic distinctions within class whereas the microscopy method allows reliable quantification of single phytoplanktonic species and of those protists which lak marker pigments (e.g. ciliates and some dinoflagellates). Therefore, no single technique or methodology is ideal for resolving all the information relevant to the structure and dynamics of protist community and HPLC pigment analysis should be considered complementary to, but not exclusively a replacement for, microscopic enumeration (Tester et al. 1995).

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19'-hexanoyloxyfucoxanthin may not be the appropriate pigment to trace occurrence and fate of *Phaeocystis*: the case of *P. globosa* in Belgian coastal waters

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Abstract

Two haptophycean strains were isolated from field samples collected in 2001 in Belgian coastal waters (southern North Sea) during the *Phaeocystis* monitoring program of the AMORE Project. The morphology and pigment composition of these two strains, one identified as *Phaeocystis globosa* and the other as *Imantonia rotunda*, were carefully examined. The comparative analysis of their pigment signature revealed the presence of two fucoxanthin derivatives, 19'-butanoyloxyfucoxanthin and 19'-hexanoyloxyfucoxanthin (but-fuco and hex-fuco) in *I. rotunda*, which were undetectable in *P. globosa*. A further comparison of pigments and phytoplankton from field samples showed no significant correlation between hex-fuco concentration and *P. globosa* biomass in the water column. Low concentrations of this pigment were however detectable before and at the end of the *Phaeocystis* bloom. The presence of *I. rotunda* in the area, overlooked by light microscopy, but isolated in pure culture from field samples, might explain the presence of this pigment. We conclude that hex-fuco is not the appropriate pigment to estimate *Phaeocystis* abundance and trace its trophic fate in Belgian coastal waters. These results also indicate that pigment analysis should be coupled with a precise identification of phytoplankton taxa present in field samples.

1. Introduction

The colony-forming haptophyte *Phaeocystis* recurrently forms, during spring, massive blooms in the eastern Channel and southern bight of the North Sea (e.g. Bätje and Michaelis 1986, Cadée and Hegeman 1986, Veldhuis et al. 1986, Lancelot et al. 1987, Lancelot 1995, Breton et al. 2000, Cadée and Hegeman 2002). The uniqueness of *Phaeocystis* rests not only in the fabulous carbon biomass reached by its blooms (up to 10 mgC l^{-1}) but mostly in its exceptional physiology which impacts the food-web structures and hence global biogeochemical cycles (Lancelot et al. 1994). Of particular importance is the existence of a complex polymorphic life cycle exhibiting phase alternation between different types of free-living cells (vegetative non-motile, vegetative flagellate and microzoospore) of 3-8 µm in diameter and colonies usually reaching several mm (Rousseau et al. 1994, Peperzak et al. 2000). This feature associated with a taxonomic controversy on the species status (Sournia 1988, Baumann et al. 1994) may explain the confusion existing in the literature concerning the taxonomy of *Phaeocystis* in the North Sea. For some years, most researchers referred to *P. pouchetii* (Bätje and Michaelis 1986, Cadée and Hegeman 1986, Veldhuis et al. 1986, Lancelot et al. 1987). However, based on temperature tolerance and colony morphology, Baumann et al. (1994) reappraised the *Phaeocystis* literature dealing with the species status and concluded that *P. globosa* was the dominant blooming species in temperate waters. Apart from the colonies, *Phaeocystis* flagellated cells are difficult to identify with the light microscope. In addition to being small (3-5 µm), they lack distinctive morphological features since the haptonema, a characteristic organelle of the class, is not easy to detect under the light microscope. Moreover, the various fixatives used for preservation may damage the cells, rendering their enumeration somewhat imprecise. An accurate identification of the motile stage requires transmission or scanning electron microscopy for an examination of the scale covering (Puigserver et al. 2003) or RNA gene analysis (Zingone et al. 1999). In this direction Lange and Medlin (2002) recommended the development of oligonucleotide probes for a rapid and accurate identification of single cell stage. On the other hand there have been some attempts to quantify *Phaeocystis* from its pigment signature (Wright et al. 1996). The accessory pigment 19'-hexanoyloxyfucoxanthin (hex-fuco) has been reported as typical for *Phaeocystis* and its content relative to the more common fucoxanthin has been suggested as a proxy to estimate *Phaeocystis* abundance (Wright and Jeffrey 1987, Bjørnland et al. 1988, Jeffrey and Wright 1994, Jeffrey et al. 1997b, Llewellyn and Gibb 2000). This approach has been challenged by observations pointing out the apparent lack or extremely low

concentration of hex-fuco in some strains (Jeffrey and Wright 1994, Vaulot et al. 1994) as well as a distinct variation in pigment pattern for colony and free-living cells (Buma et al. 1991). In general, the hex-fuco content of *P. globosa* cells is much lower, if not absent, compared to the two cold species *P. antarctica* and *P. pouchetii* (Buma et al. 1991, Vaulot et al. 1994). Furthermore, recent studies are reporting significant variation in the relative abundance of hex-fuco and fucoxanthin to chlorophyll *a* in response to changing environmental factors (van Leeuwe and Stefels 1998, Llewellyn and Gibb 2000, Schlüter et al. 2000). Altogether these data suggest that the use of marker pigments as 'fingerprint' of the *Phaeocystis* genus in field samples requires a prior examination of the pigment composition of the species occurring in a given area.

In this study we compare the pigment composition of 2 strains of haptophyte species, *P. globosa* and *Imantonia rotunda*, isolated from Belgian coastal waters during a spring phytoplankton bloom. The opportunity of using pigments as a tool to detect *P. globosa* occurrence in the Belgian coastal waters is further discussed based on a crossed comparison of pigment and taxonomic dominance observed in field samples with the pigment signature of cultivated species.

2. Materials and methods

2.1. Sample collection

Sampling was carried out at station 330 (51°26'N, 2°48'50E; depth 20 m) and station 435 (51°35'N, 2°47'50E; depth 35 m) in the Belgian coastal waters during AMORE 2001 cruises aboard the R/V *Belgica* and *Zeeleeuw* (Fig. II-1). These stations were sampled fortnightly during spring and monthly for the rest of the year, weather permitting. Samples were collected using a 10 l Niskin bottle, at subsurface, middle and near the bottom of the water column. Seawater samples (250-1500 ml) from the different depths were immediately filtered in triplicate using GF/F glass fibre filters (47 mm diameter, 0.7 µm nominal pore size) for HPLC pigment analysis. All filters were folded in an aluminium foil and immediately stored in liquid nitrogen throughout the cruise until the return to the lab where they were stored at -85°C. Additional water samples (250 ml) were taken in triplicate, after mixing of the three depths, and were fixed with 1% (final concentration) glutaraldehyde for algal cell counting.

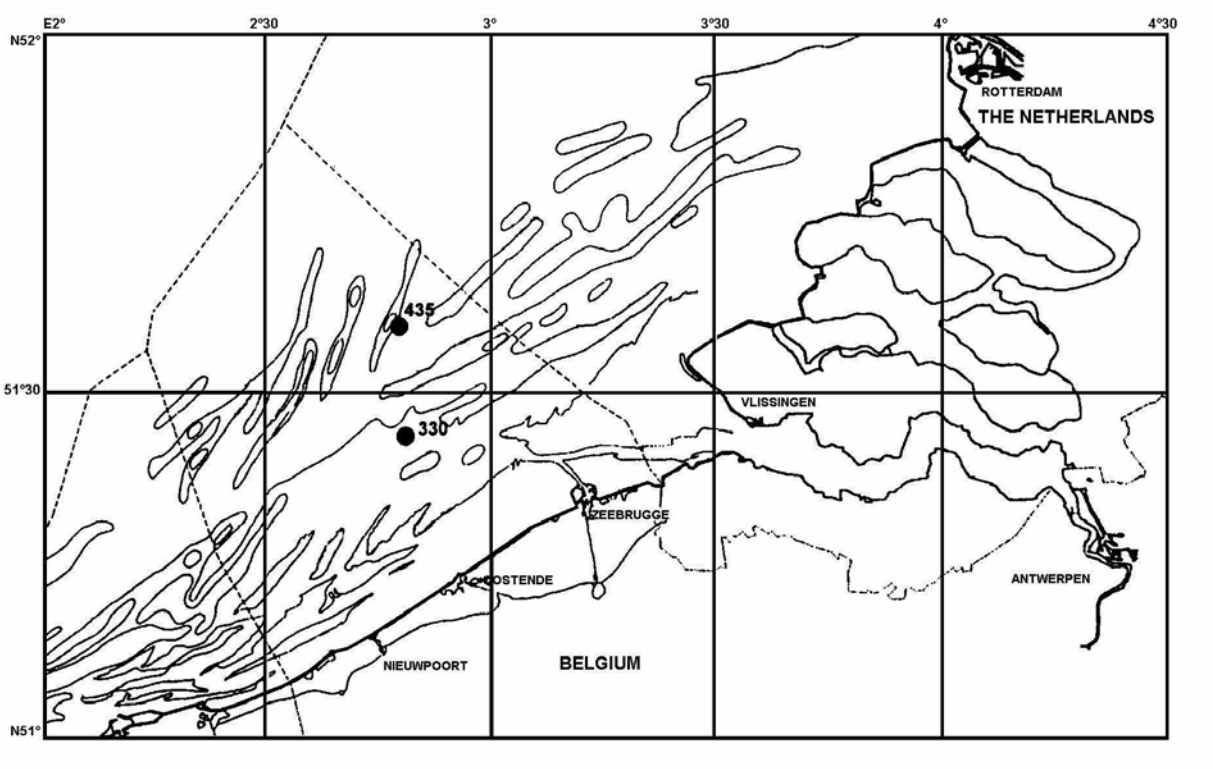

Figure II-1: Map of the Belgian coastal zone showing location of the two stations sampled during AMORE 2001 cruises.

2.2. Isolation and maintenance of algal cultures

Cultures of *Phaeocystis* and *Imantonia* were prepared from surface seawater samples collected at station 330 during the *Phaeocystis* bloom in April 2001 (17^{th} and 25^{th} April). *Phaeocystis* colonies were collected under a binocular microscope with an open-end Pasteur pipette. They were inoculated in culture medium F20, prepared with sterile filtered seawater enriched as in Veldhuis and Admiraal (1987) and supplemented with antibiotics (sulfated streptomycin and penicillin) at a final concentration each of 35 mg per litre of culture medium. Cultures were grown at 10°C under a 12h light:12h dark cycle at 100 µmole quanta m^{-2} s^{-1}. Culture maintenance was secured by weekly diluting an aliquot of the culture in fresh medium. At the same time another aliquot was carefully filtered under sterile conditions, on a Nuclepore filter (10 µm nominal porosity) before transfer in fresh medium. After three repetitions of this purification procedure (one per week), the axenic cultures were completely dominated by small flagellated cells that turned out to be *Imantonia* after SEM examination. Samples of *Phaeocystis* colonies and *Imantonia* cells were collected for taxonomic identification (epifluorescence and scanning electron microscopy) and pigment analysis (HPLC). Samples for epifluorescence microscopy were fixed with 1% (final concentration)

glutaraldehyde and stained with DAPI (Porter and Feig 1980) at a final concentration of 2.5 µg ml^{-1}. Samples for scanning electron microscopy were preserved with 1% (final concentration) lugol-glutaraldehyde solution and stored at 4°C in dark until analysis. Aliquot of each culture was filtered on Whatman GF/F (0.7 µm) glass-fibre filters and stored in an ultra-cold freezer at -85 °C for 2 days prior to HPLC analyses.

2.3. Scanning electron microscope (SEM) analysis

Preserved sub-cultures were centrifuged on a plastic cell culture coverslip (Thermanox) coated with L-polylysine for a better adhesion of material before being critical point dried, mounted on stubs and coated with gold. Observations of cell morphology and scales were made on a Hitachi S 4500 scanning electron microscope in Perpignan, France.

2.4. High Performance Liquid Chromotography (HPLC) pigment analysis

The frozen filters were cut into small pieces (several mm x 1 cm) and sonicated in centrifuge tubes (on crushed ice), with 2 ml of 100% cold acetone for 2 x 15 seconds at 50 W using a Labsonic sonicator equipped with a 4 mm diameter probe inserted directly into the solvent. After sonication samples were kept for 2 hours at 4°C before centrifuged for 3 minutes at about 700 g. Supernatants were filtered onto 0.5 µm (Millex SR syringe equipped with 25 mm-filter of 0.5 µm-porosity), transferred to 1 ml vials and placed into the autosampler (kept at 4°C) prior to injection in the HPLC. Reversed-phase HPLC analyses were conducted based on the ternary gradient method of Wright et al. (1991) as described in Gasparini et al. (2000). Pigments were detected by absorption at 436 nm and identified based on comparison of their retention time and spectra with standards. High-purity HPLC standards for chlorophyll *a*, pheophytin *a*, chlorophyll c_3, fucoxanthin, alloxanthin, peridinin and 19'-hexanoyloxyfucoxanthin were obtained from the International Agency for ^{14}C determination (Hørsholm, Denmark). Pheophorbide *a* was obtained from the ICN Biomedicals, Inc. Other carotenoids were identified using retention time and spectra of well characterised pigments (Jeffrey et al. 1997a). Our HPLC technique did not allow us to separate chlorophylls c_1 and c_2.

2.5. Phytoplankton enumeration and biomass estimate

Phytoplankton was analysed by an inverted microscope provided with phase contrast (ZEISS Sedival) in 5 ml sedimentation chambers after pre-concentration following the

method of Colijn et al. (1990). Depending on cell density optical fields or selected transects of the entire sedimentation chamber were counted at 200x and 400x magnification. Colonial *Phaeocystis* cells were free in our samples due to the dissolution of the colony matrix by the preserving agent. A conversion factor of 14.2 pgC per cell for colonial cells and of 10.8 pgC per cell for flagellated cells (microzoospores) was used to estimate *Phaeocystis* carbon biomass as recommended by Rousseau et al. (1990).

3. Results

3.1. Species identification: optical microscopy

Fig. II-2 shows photographs of a *Phaeocystis* colony from a field sample (Fig. II-2A), the *Phaeocystis* culture, with colonial forms and free-living cells (Fig. II-2B,C,D) and the culture containing microflagellates, found later to be *Imantonia* (Fig. II-2E,F), obtained under inverted and epifluorescence microscopy. Clearly the *Phaeocystis* colonies isolated from field samples (Fig. II-2A; bright field, inverted microscopy) hold characteristics of *P. globosa* i.e. spherical forms with cells evenly distributed (Baumann et al. 1994, Rousseau et al. 1994). Epifluorescence microscopy of the *Phaeocystis* colonial culture (Fig. II-2B,C,D) shows typical colony forms of *P. globosa* as well (Fig. II-2B) and flagellated and non-flagellated free-living cells of 3-4 μm in diameter (Fig. II-2C,D). The microflagellate culture was dominated by very small flagellated cells of 2-3 μm in diameter (Fig. II-2E,F) which need further scanning electron microscopy for identification.

3.2. Species identification: scanning electron microscopy

SEM revealed two different monospecific cultures. One was clearly of the *Phaeocystis*-type, with five ray star-like structures in the close surrounding of the cells (Fig. II-3A). The stars and their attached filaments are made of chitin (Chrétiennot-Dinet et al. 1997) and are characteristic for the genus. The small rounded cells observed in the *Phaeocystis* culture have two smooth flagella and a short haptonema (Fig. II-3A,B,C). They are 2.5 to 4.2 μm in diameter and correspond to the so-called microzoospores cited by Kornmann (1955). Their scale morphology has been described in details by Parke et al. (1971) based on transmission electron microscopy. In our culture, the flagella length was 4-5 μm (Fig. II-3B), in accordance with the $1^{1/2}$ times the cell diameter mentioned in Parke et al. (1971). The haptonema (Fig. II-3A,B,C) seems to be somewhat shorter (0.5 μm) than in the

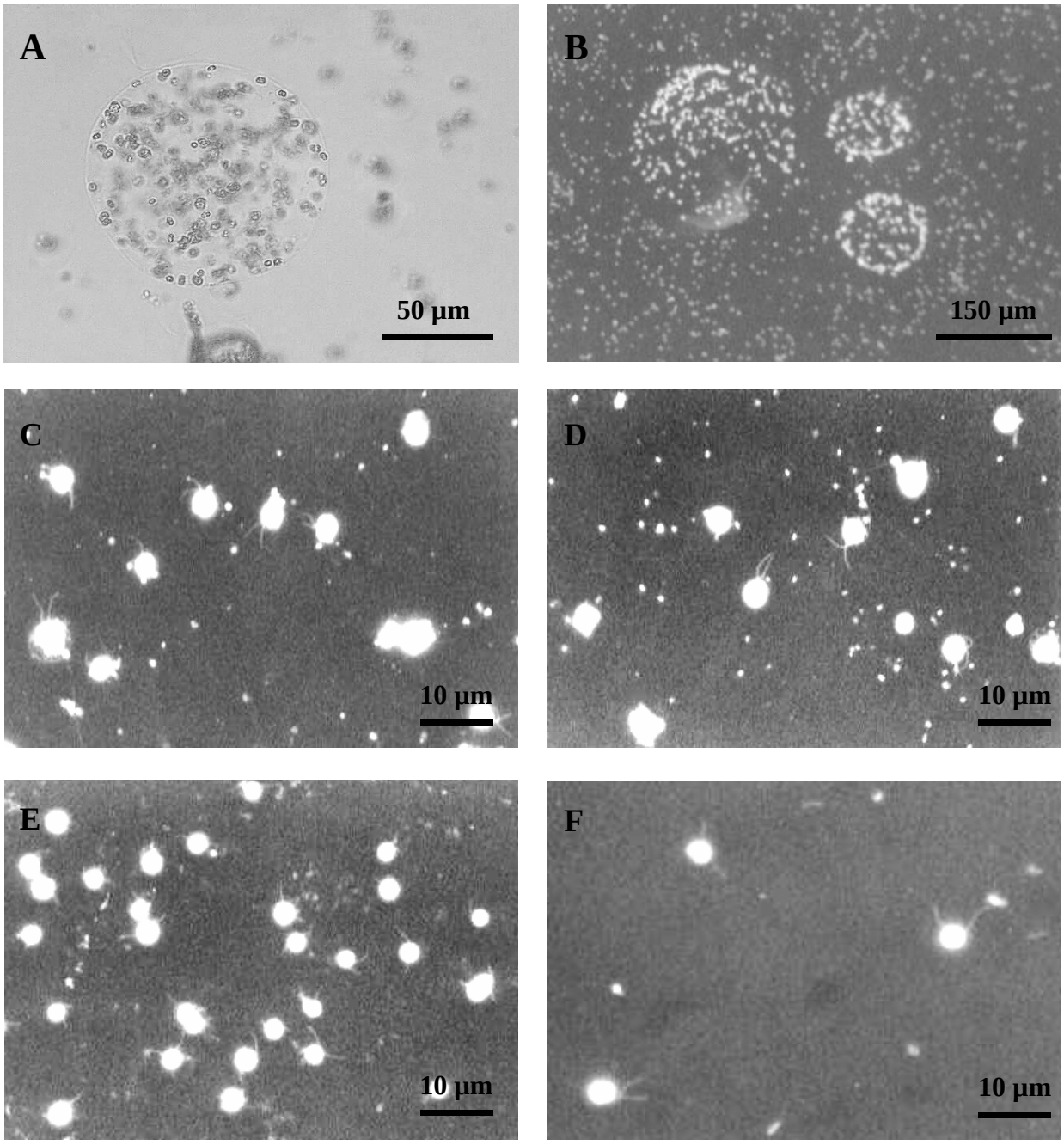

Figure II-2: A: Inverted microscope photograph of a field *Phaeocystis globosa* colony collected at station 330 of the Belgian coastal zone. B-F: epifluorescence microscopy photographs of *P. globosa* colonies (B), free-living flagellated and non-flagellated cells (C and D) from *Phaeocystis* colonial culture, and free-living flagellated cells (E and F) from the microflagellate culture.

strains kept in the Plymouth collection and may have lost its distal swelling. The cell body is covered by small rounded scales varying from 0.13 to 0.18 µm in diameter (Fig. II-3C,E) which, according to Zingone et al. (1999) correspond to the species *P. globosa* Scherffel. Parke et al. (1971) considered the strains they examined and described as belonging to *P. pouchetii* but a first analysis of the genome size and pigments placed them in a cluster of North European strains representing the true *P. globosa* (Vaulot et al. 1994). Molecular analyses of different *Phaeocystis* strains have shown that *P. pouchetii* and *P. globosa* are two distinct species (Medlin et al. 1994; Edvardsen et al. 2000) and that the description of the motile stages by Parke et al. (1971) is probably that of *P. globosa* (Edvardsen et al. 2000). It is now admitted that true colonial forms belong to three different species: *P. pouchetii*, *P. antarctica* and *P. globosa* (Lange et al. 2002). Because of the shape of the colony, the distribution of the cells in the colony and the geographical occurrence of the strain isolated, for which the motile form definitely belongs to the genus *Phaeocystis*, we identify it as *P. globosa*.

The cell-type identified in the microflagellate culture is very similar in shape and size (2-2.5 µm), but the haptonema is missing (Fig. II-3D). Moreover scales, when present, are completely different (Fig. II-3F,G) and correspond to those described for *Imantonia rotunda* Reynolds (Reynolds 1974, Green and Pienaar 1977). The two flagella are smooth, distally tapered, slightly unequal, with the bases widely divergent (Fig. II-3D) as in the revised diagnosis by Green and Pienaar (1977). The body scales are 0.52 to 0.8 µm in diameter and show the typical pattern of radiating ridges (Fig. II-3F,G). This scale pattern is particularly informative and leaves no doubt about the identity of this species, considered as *Imantonia rotunda*.

3.3. Pigment signature

Absorbance chromatograms of the two cultures are shown in Fig. II-4. In both cases fucoxanthin (fuco) was the dominant carotenoid pigment. Both cultures contained chlorophyll *a* (chl *a*), chlorophyll c_3 (chl c_3), chlorophyll c_1+c_2, diadinoxanthin, diatoxanthin, phytylated chlorophyll *c*-like and β,β-carotene. The main difference between the two haptophyte species is the presence in *I. rotunda* of two fucoxanthin derivatives, 19'-butanoyl and 19'-hexanoyloxyfucoxanthins (but-fuco and hex-fuco), whereas in *P. globosa* they were both undetectable. Table II-1 compares for each species values of carotenoids to chlorophyll a ratios which are commonly used as pigment signature for haptophytes.

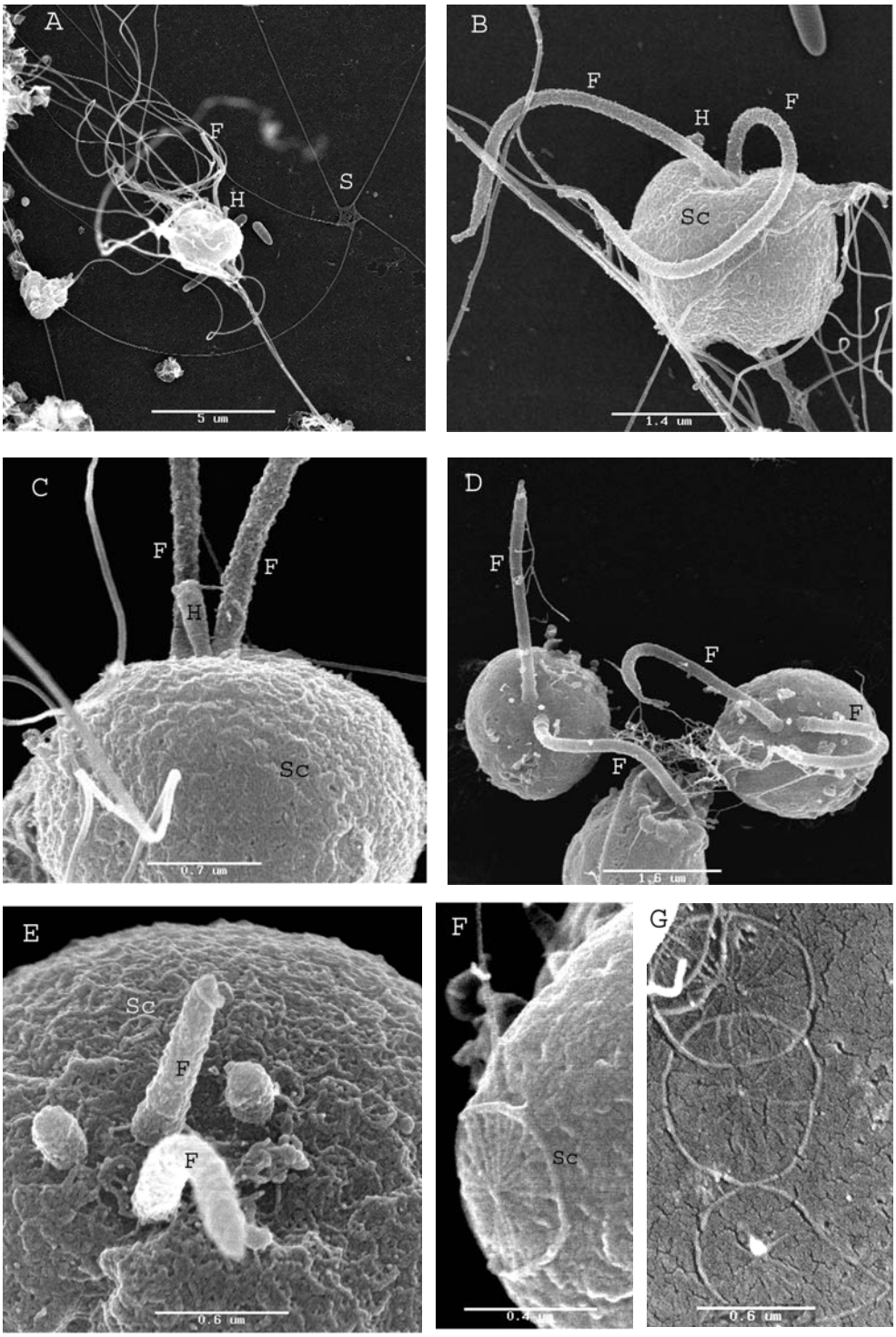

Figure II-3: Scanning electron micrographs of motile cells of *Phaeocystis globosa* (A-C and E) and *Imantonia rotunda* (D and F-G). *P. globosa* - A: a cell surrounded by many filaments and a star-like structure (S); B: a cell with two flagella (F) a haptonema (H) and its scale covering (Sc); C: detail of another cell showing the flagella departure, the haptonema and scales; E: detail of scales (Sc) on a cell with 4 growing flagella (F), this cell is probably undergoing a division. *I. rotunda* - D: two cells with their two sub-equal flagella widely divergent and no haptonema; F: a scale still on the body cell; G: three loose scales. All the scales show the typical pattern of radiating ridges.

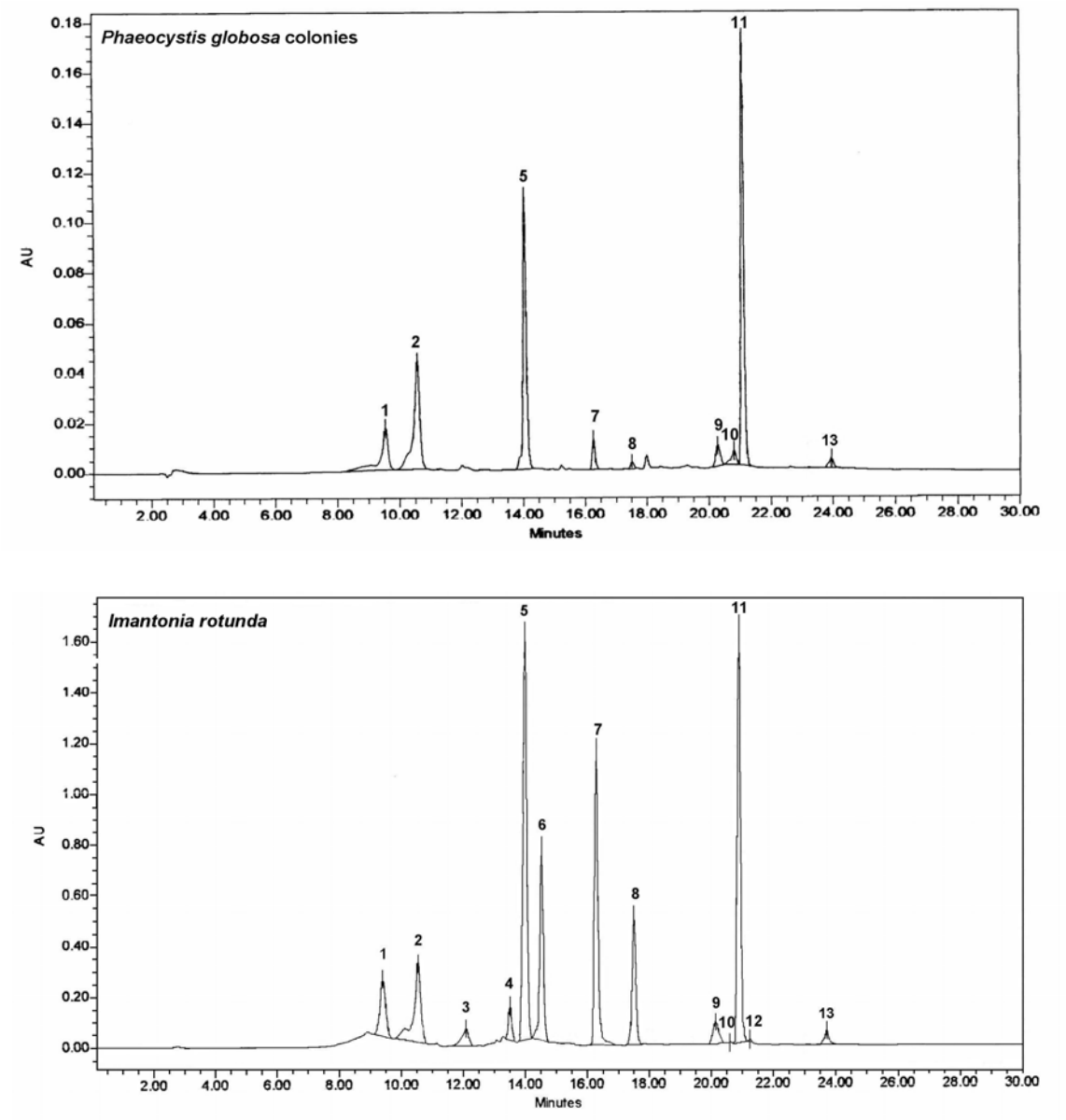

Figure II-4: HPLC absorbance chromatograms of cultures of *Phaeocystis globosa* colonies and *Imantonia rotunda* made at 436 nm. Retention time is given in minutes. AU = Absorption Units. Pigments: 1, chlorophyll c_3; 2, chlorophyll c_1+c_2; 3, cis-fucoxanthin; 4, 19'-butanoxyloxyfucoxanthin; 5, fucoxanthin; 6, 19'-hexanoxyloxyfucoxanthin; 7, diadinoxanthin; 8, diatoxanthin; 9, phytylated chlorophyll *c*-like; 10, chlorophyll *a* allomer; 11, chlorophyll *a* ; 12, chlorophyll *a* epimer; 13, β,β-carotene.

Table II-1: Haptophyte biomarker pigments of the two strains isolated from Belgian coastal waters expressed as ratios to chlorophyll *a*.

Strain	Ratio to Chl *a*			
	Chl c_3	But-fuco	Fuco	Hex-fuco
Phaeocystis globosa	0.208	0.000	0.350	0.000
Imantonia rotunda	0.269	0.060	0.746	0.296

3.4. In situ Phaeocystis globosa *biomass and biomarker pigments*

Microscopic observations of field samples revealed the presence of *P. globosa* at both locations whereas *I. rotunda* was not identified. The occurrence of *P. globosa* was particularly impressive in spring (Fig. II-5). *Phaeocystis* cells appeared in March and their biomass increased exponentially until reaching a peak in mid-April which was more important at station 330 compared to station 435 (382 and 249 µgC l^{-1} respectively). At the end of June, *Phaeocystis* disappeared from the water column until September when a low biomass of *Phaeocystis* was observed (16 and 25 µgC l^{-1} at stations 330 and 435 respectively).

As expected from the pigment analysis of *P. globosa* strain (Fig. II-4; Table II-1), the field biomass of *P. globosa* was well correlated with concomitant measurements of chlorophyll c_3 and fucoxanthin concentrations but not with hex-fuco (Table II-2). Concentration of but-fuco was under the detection limit in all field samples.

Table II-2: Correlations (Spearman rank) between *Phaeocystis globosa* carbon biomass and haptophyte specific pigments.

		Chl c_3	Fuco	Hex-fuco
Phaeocystis globosa	r_s	0.862	0.724	0.074
	n	25	25	25
	p	< 0.001	< 0.001	> 0.05

Chlorophyll c_3 was always detected in our samples except in November (Fig. II-5A, B). The concentration of this pigment was relatively high during *Phaeocystis* blooming (over 0.20 µg l^{-1} and up to 1.90 µg l^{-1} at the maximum biomass; Fig. II-5A,B) as compared to periods when *P. globosa* was absent (below 0.07 µg l^{-1}; Fig. II-5A,B).

Fucoxanthin (Fig. II-5C,D) was measured during the entire year as it is the dominant pigment of brown algae (e.g. diatoms, haptophytes and chrysophytes). Fucoxanthin concentration increased from mid-February onwards, with the early spring diatom bloom, and reached its maximum concentration (5.70 µg l^{-1}) in April at the time of maximum *Phaeocystis* biomass (Fig. II-5C,D). In mid-June we observed a second peak of fucoxanthin at station 330 (4.22 µg l^{-1}; Fig. II-5C) which corresponded with the occurrence of the diatoms *Guinardia delicatula* and *Rhizosolenia imbricata* (not shown).

Hex-fuco (Fig. II-5E,F) was not detected at the time of maximum *Phaeocystis* bloom. Rather significant concentrations although relatively low (0.02 - 0.06 µg l^{-1}; Fig. II-5E,F) were recorded outside the *Phaeocystis* spring bloom period, in January, May (after *Phaeocystis* bloom) and in early September.

4. Discussion

Light microscopy and scanning electron micrographs of *Phaeocystis* cells isolated from the Belgian coastal waters during the spring bloom suggest that the main *Phaeocystis* species occurring in these waters is *P. globosa.* This gives support to Baumann et al. (1994)'s reappraisal of the species status of *Phaeocystis* in the North Sea (sometimes wrongly reported in older literature as *P. pouchetii*) based on colony morphology and temperature tolerance.
The pigment composition of the cultured strain of *P. globosa* examined in the present work is characterized by the presence of chlorophyll c_3 and fucoxanthin, the latter pigment being surprisingly dominant. The fucoxanthin to chlorophyll a ratio (Table II-1) is comparable to values reported for diatoms (Mackey et al. 1996, Llewellyn and Gibb 2000). As the phytoplankton spring bloom community of Belgian coastal waters mainly consists of diatoms and colonies of *P. globosa* (Lancelot et al. 1998, Rousseau et al. 2002) we conclude that fucoxanthin has no value as biomarker of *P. globosa* occurrence in field samples. Chlorophyll c_3 on the other hand has been reported as an ubiquitous pigment of *Phaeocystis* species (Claustre et al. 1990, Vaulot et al. 1994, Breton et al. 2000, Cotonnec et al. 2001) but it is also found in many other haptophytes (Jeffrey and Wright 1994) some diatoms (Stauber and Jeffrey 1988, Llewellyn and Gibb 2000) and one chrysophyte (Vesk and Jeffrey, 1987). Microscopic observations of our samples revealed the co-occurence with *P. globosa* of three diatom species known to contain chlorophyll c_3. These are *Thalassionema nitzschioides*, *Rhizosolenia setigera* and *Asteroplanus karianus*. Their total biomass represented less than 3% of *P. globosa* biomass during the bloom. Hence most of the chlorophyll c_3 found in our

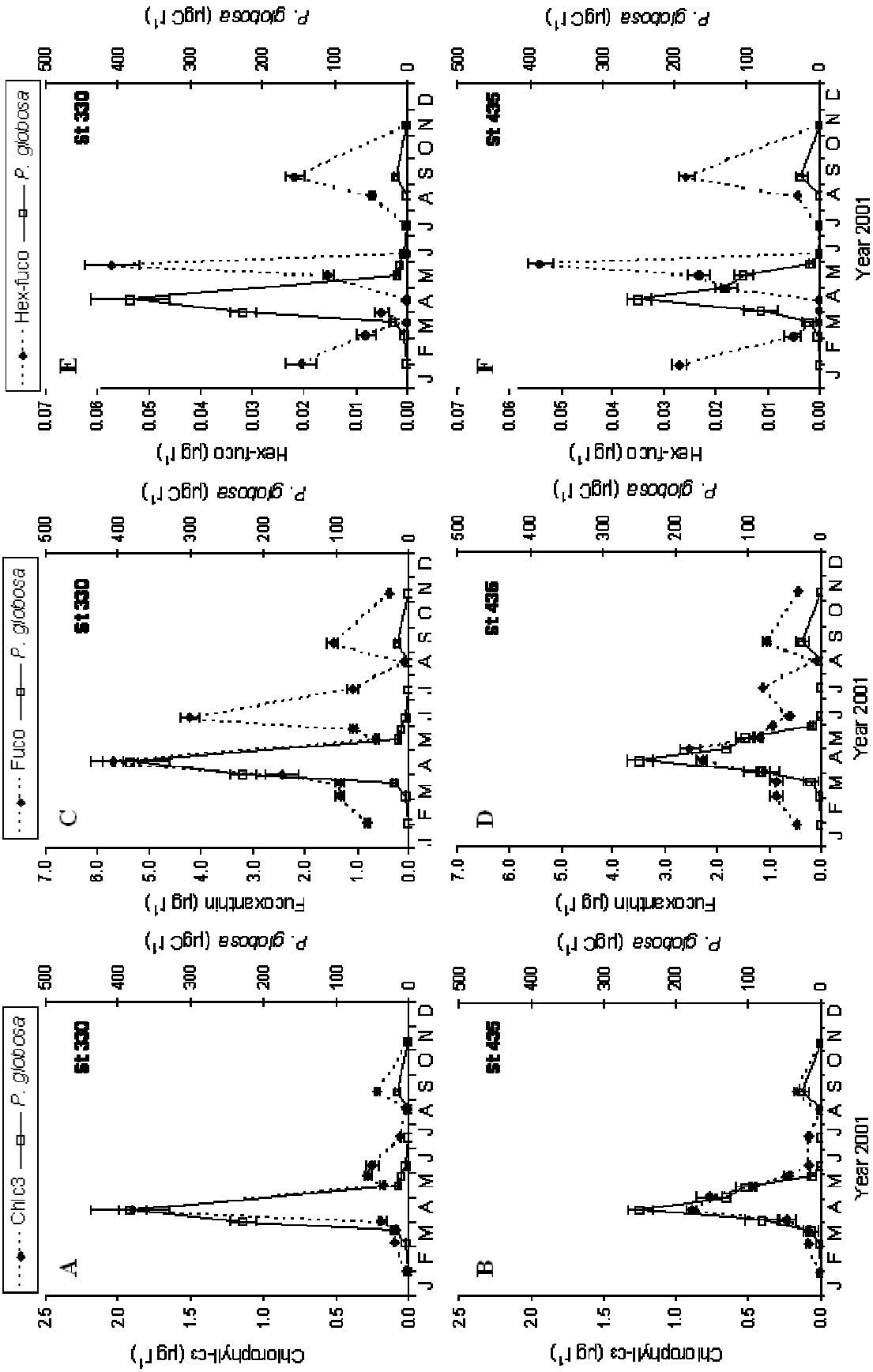

Figure II-5: Seasonal evolution of *P. globosa* cell carbon biomass (µgC l^{-1}) and chlorophyll-c_3 (A and B), fucoxanthin (C and D) and 19'-hexanoyloxyfucoxanthin (E and F) (µg l^{-1}) at stations 330 and 435 of the Belgian monitoring grid, in 2001.

samples could be attributed to *P. globosa*, as also suggested by the significant correlation between chlorophyll c_3 concentration and *P. globosa* biomass. We have however to admit that very few diatom species among those reported in our samples have been examined for their pigment composition. Further analysis of pigment composition and variability of main diatom species (e.g. *G. delicatula*, *R. imbricata* and *Pseudo-nitzschia delicatissima*) blooming during *Phaeocystis* bloom are needed prior the safely use of chlorophyll c_3 as indicator of *P. globosa* in field samples.

One important result of the pigment analysis of the *P. globosa* strain is the absence of the two fucoxanthin derivatives, hex-fuco and but-fuco. Existing literature on the pigment composition of cultured strains of *Phaeocystis* has shown a wide inter-specific as well as intra-specific variation of these two pigments. Hex-fuco is a dominant carotenoid in *P. antartica* with but-fuco being present at trace levels (Buma et al. 1991, Vaulot et al. 1994). Both pigments are also characteristic of *P. pouchetii* (Wright and Jeffrey 1987, Jeffrey and Wright 1994, Llewellyn and Gibb 2000) and were used as indicators of *P. pouchetii* in field samples (Wright and Jeffrey 1987). However Gieskes and Kraay (1986) reported that a strain of *P. pouchetii* isolated from the southern part of the North Sea did not contain hex-fuco (see comment in Bjørnland et al. 1988). Likewise Jeffrey and Wright (1994) reported that this pigment was absent in one of the six strains of *P. pouchetii* they have analysed. Vaulot et al. (1994) identified both pigments in North European strains including *P. globosa*, but at a much lower concentration than in tropical or Mediterranean strains. The fact that none of the acyl-fucoxanthin pigments were identified in our culture can thus be considered as a distinctive feature of *P. globosa* in Belgian coastal waters. This is confirmed by field observations showing no correlation between hex-fuco concentrations and *P. globosa* biomass. The relatively low concentrations of hex-fuco measured in May at the very end of *P. globosa* spring bloom (Fig. II-4), could result from nutrient limitation as suggested by Buma et al. (1991). These authors indeed observed a relative increase of hex-fuco during the stationary stage of a *Phaeocystis* culture isolated from the southern bight of the North Sea whereas this pigment was not detected during exponential growth. Recently, variations in light and iron conditions have also been proved to result in fluctuations of hex-fuco content in *Phaeocystis* sp. of several orders of magnitude (van Leeuwe and Stefels 1998, Schlüter et al. 2000). Records of hex-fuco in our field samples might also correspond to the presence of another hex-fuco containing-haptophyte species, such as *I. rotunda*. Indeed this species was accidentally isolated with *Phaeocystis* colonies from the April samples. The strain of *I. rotunda* that we isolated contained, in addition to its major carotenoids (fucoxanthin and hex-

fuco), chlorophyll c_3 and trace levels of but-fuco. The latter was not clearly identifiable in field samples, probably because of its low concentration. We assume then that *I. rotunda* abundance was negligible compared to that of *Phaeocystis.* As the two species cannot be easily distinguished with optical microscopy (inverted or epifluorescence), a confusion between *P. globosa* free-living flagellated cells and *I. rotunda* was possible (Fig. II-2C,D and Fig. II-2E,F). This species has been previously mentioned in Belgian coastal waters (M'harzi et al. 2000) in samples collected in February around sand banks of the Belgian coast. It was first isolated by Reynolds from surface waters near Bear Island (south-west of Spitsbergen), and has been found in many places: in the region of the Shetland Islands, the north-west of Ireland, the Plymouth area, the Mid-Channel (Reynolds 1974), in the Friday Harbor area, Washington (Green and Pienaar 1977), in the offshore waters of the East Australian current (Hallegraeff 1983), in the Kiel Bight and Kiel Fjord (Jochem 1990) and in the Inner Oslofjord (Backe-Hansen and Throndsen 2002). It is then recorded in places where *Phaeocystis* is also present and it is probable that both species co-occur but that *I. rotunda* is usually overlooked during *Phaeocystis* surveys.

5. Conclusion

This study confirms that there is a far greater range of pigment compositions across haptophyte species than originally reported. Recently the 4 haptophyte pigment types suggested by Jeffrey and Wright (1994) have been expended to eight (Zapata et al. 2004). Pigment distribution in *P. globosa* differs from others *Phaeocystis* species by the absence of hex-fuco and but-fuco. This result precludes the use of hex-fuco as indicator of *P. globosa* in phytoplankton samples (Breton et al. 2000; Meyer 2002) as well as biomarker of *Phaeocystis* ecological fate in the pelagic and the sediments. Especially it challenges conclusions of *Phaeocystis* resistance to copepod grazing in Belgian coastal waters and adjacent areas when based on the absence of hex-fuco in the copepod diet (Breton et al., 1999; Gasparini et al., 2000). Moreover the pigment suite of *P. globosa* is shared by some chlorophyll c_3 containing-diatoms of the Belgian coastal waters which make the interpretation of pigments in field samples difficult. *I. rotunda* which was identified in our samples thanks to electronic microscopy also contains chlorophyll c_3 as major pigment with fucoxanthin and the two acyl-fucoxanthins. These pigments are also characteristic of a marine chrysophyte and at least three bloom-forming dinoflagellates (Vesk and Jeffrey, 1987; Bjørnland and Liaaen-Jensen, 1989). We thus conclude that the pigment signature of *P. globosa* is not specific enough for

its safe identification in natural mixed assemblages. More generally we recommend that HPLC pigment analysis of field samples should always be coupled with microscopic studies of phytoplankton taxa from representative samples, and combined with pigment signature analysis of the major taxa present.

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Assessment of Cryptophyceae ingestion by copepods using alloxanthin pigment: a caution

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Abstract

The accessory pigment alloxanthin is a well-known taxonomic marker for Cryptophyceae in natural seawater. The use of alloxanthin to estimate *in situ* zooplankton grazing and selectivity on Cryptophyceae was studied using two dominant copepods of the southern North Sea, *Temora longicornis* and *Centropages hamatus*. High-performance liquid chromatography (HPLC) analyses of water samples and of freshly caught copepods, starvation experiments, and feeding experiments (shipboard bottle-incubations) were carried out. Although alloxanthin was always detected in copepod extracts, (1) no correlation was found with alloxanthin in seawater, and (2) no significant grazing on Cryptophyceae was observed. Results of our gut-evacuation experiments showed that at least 78 % of the initial alloxanthin content of *T. longicornis* and *C. hamatus* remained after 90 min, whereas fucoxanthin and chloropigments decreased rapidly with time. Alloxanthin and astaxanthin esters were the only pigments remaining in the body of *T. longicornis* after several hours' starvation. It is concluded that most of the detected alloxanthin did not originate from the gut but from the body tissues. Our results suggest that alloxanthin is not suitable as biomarkers for quantitative and qualitative estimates of copepod grazing on Cryptophyceae.

1. Introduction

In the North Sea, phytoplankton spring blooms consist mainly of Bacillariophyceae and Haptophyceae (Reid et al. 1990). Consequently, the ecological importance of these algae (Lancelot et al. 1998, Rousseau et al. 2002) as well as their contribution to zooplankton diet (Daro 1988, Fransz et al. 1991, Gasparini et al. 2000) have been extensively studied. In contrast, the presence of small flagellates (e.g. green algae and cryptomonads) has been less well documented. The main reasons are (1) lack of morphologic distinctive features of these algae and (2) damage caused during fixation that renders identification by classical microscopic methods difficult. Partly to overcome these problems, a chemotaxonomic approach based on detection of taxon-specific pigments by high-performance liquid chromatography (HPLC) has been used since the 1980s and is commonly employed to distinguish the main algae classes (see review in Jeffrey et al. 1999). Using this technique, the abundance and sometimes dominance of Cryptophyceae in natural seawater samples of the North Sea has been documented by the presence of alloxanthin pigment, a specific cryptomonad carotenoid (Gieskes and Kraay 1983, 1984, Brunet et al. 1996, Breton et al. 2000). In parallel, several studies on the natural diet of zooplankton have reported the presence of alloxanthin in copepod extracts, leading to the suggestion that Cryptophyceae contribute to the diet of these crustaceans (Pandolfini et al. 2000) and could even be strongly selected (Breton et al. 1999, Cotonnec et al. 2001). Meanwhile, the use of carotenoid pigments to study zooplankton grazing and selectivity has been challenged during the last decade because of their possible degradation into unidentified pigments and/or colourless products during gut passage. Descy et al. (1999) for instance, estimated that 91 % fucoxanthin, 88 % diadinoxanthin, 79 % lutein and 71 % alloxanthin were lost during gut passage in laboratory experiments conducted with the freshwater copepod *Diaptomus minutus*. Likewise, Kleppel (1998) showed that 60 to 100 % fucoxanthin was lost during gut passage in experiments conducted with the marine copepods *Acartia californiensis* and *Calanus pacificus* and concluded that it is necessary to exercise caution when attempting to use carotenoid pigments quantitatively.

In this paper, we focused on the *in situ* ingestion of Cryptophyceae by two dominant copepods of the southern North Sea, *Temora longicornis* and *Centropages hamatus*. HPLC analyses of water samples and of freshly caught copepods, starvation experiments, and feeding experiments (shipboard bottle-incubations) were performed. Our results challenge the

use of alloxanthin as quantitative and as qualitative indicator of Cryptophyceae ingestion by copepods.

2. Materials and methods

2.1. Sample collection

Sampling and experiments were carried out on board the RVs *Belgica* and *Zeeleeuw* in the relatively shallow (mean depth 24 m) Belgian coastal zone (Fig. III-1). Cruises were conducted at different time intervals, including all seasons, from 1998 to 2001 as part of the AMORE (Advanced MOdelling and Research on Eutrophication) project.

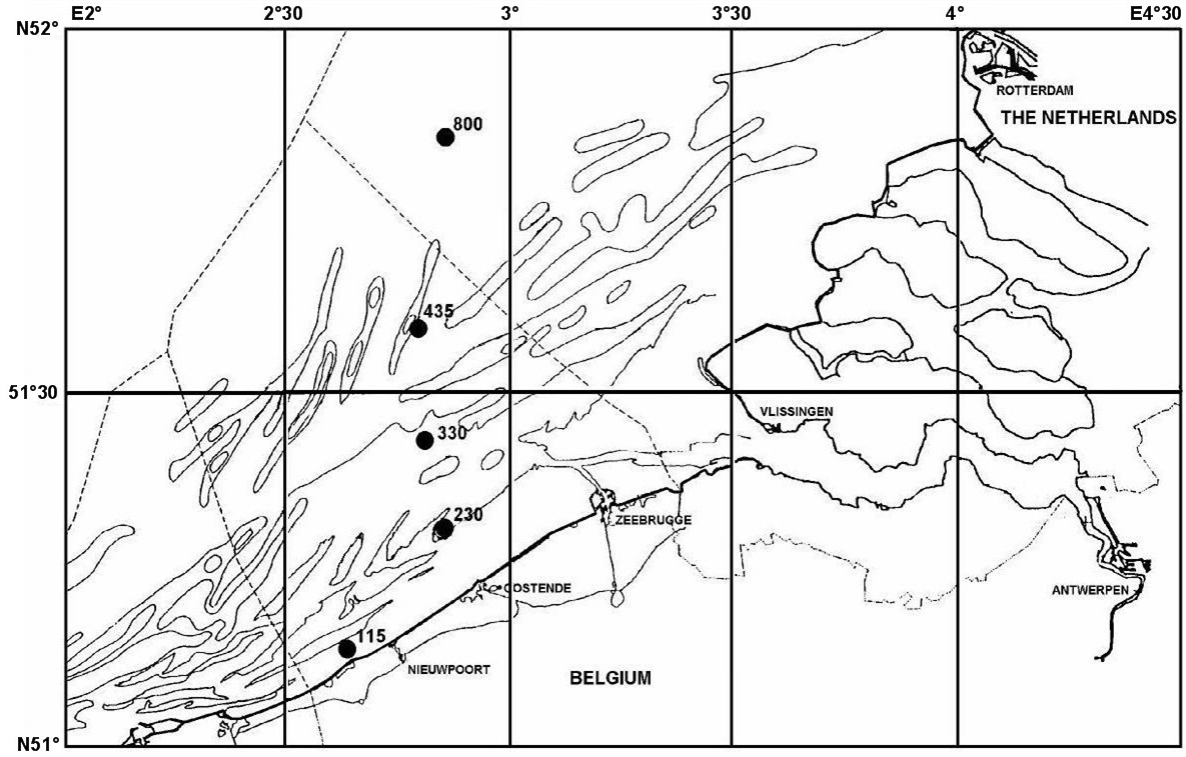

Figure III-1: Belgian coastal zone, showing location of the five stations sampled during the AMORE 1998-2001 cruises.

Water samples were taken with a 10 litres Niskin bottle from the subsurface, middle and near the bottom of the water column. Seawater subsamples (250-1500 ml) from the different depths were immediately filtered in triplicate using GF/F glass-fibre filters (47 mm diameter, 0.7 µm nominal pore size). All filters were folded in an aluminium foil and immediately stored in liquid nitrogen for HPLC pigment analysis. The rest of the seawater samples were then used for feeding experiments with copepods after mixing the water from the three depths (see later subsection).

Zooplankton samples were collected using a 200 µm mesh WP-2 plankton net which was towed obliquely between the surface and bottom (tow duration <5 min). From each zooplankton sample collected in 2000 and 2001, a subsample was taken with a 200 µm net, wrapped in aluminium foil, and frozen immediately in liquid nitrogen for analysis of the *in situ* pigment content of adult copepods by HPLC.

2.2. Evacuation experiment

In January 1998 and June 2001, evacuation experiments were carried out to determine the fate of pigments during gut passage. Immediately following capture, the copepods were sieved through a 1 mm mesh onto a 500 µm mesh, rinsed with filtered (0.7 µm) seawater, and transferred into a 25 litres aquarium of filtered seawater at ambient temperature. In January 1998 the starvation experiment was performed at 6.3°C, whereas in June 2001 it was performed at 15.7°C and corresponded to the period of alloxanthin maximum in water samples. Copepod subsamples were removed and frozen immediately in liquid nitrogen at 10 min intervals during a 100 min experiment in 1998 and at 5 to 15 min intervals during a 90 min experiment in 2001. An additional starvation experiment of 3 hours was performed in June 1998 for studying copepod pigments.

2.3. Feeding experiment

A total of 71 experiments to quantify grazing by *Temora longicornis* and *Centropages hamatus* and determined the fate of their prey in natural assemblages were performed from 1998 to 2001. For each experimental treatment, 30 adult copepods of the same species were selected, using a Pasteur pipette under a binocular microscope, and transferred into 500 ml of natural seawater. Incubation started within one hour of zooplankton collection and only individuals with normal swimming behaviour were selected. We immediately fixed three replicates of seawater (initial condition) in 1 % glutaraldehyde (final concentration), while three replicates without copepods (control treatments) and two to four replicates with copepods (experimental treatments) were incubated for 24 hours. All treatments were incubated in on-desk incubators with water circulation to maintain ambient temperature. At the end of the incubation, we controlled that the copepods were still alive and the replicates were fixed with 1 % glutaraldehyde.

2.4. Sample analysis

Upon return to the laboratory, all samples for HPLC analysis were stored in a freezer at -85°C for no longer than 6 months before analysis.

Samples for seawater pigment analysis were cut into small pieces (several mm x 1 cm) and sonicated in centrifuge tubes (on crushed ice), with 2 ml of 100 % cold acetone for 2 x 15 seconds at 50 W using a Labsonic sonicator equipped with a 4 mm diameter probe inserted directly into the solvent. After sonication, samples were kept in the dark for 2 hours at 4°C before centrifuged for three minutes at about 700 ×g. Supernatants were filtered onto 0.5 µm (Millex SR syringe equipped with 25 mm filter of 0.5 µm porosity), transferred to 1 ml vials and placed into the autosampler (kept at 4°C) prior to injection of 100 µl extract through the HPLC column.

Samples for copepod pigment analysis were rinsed with cold filtered seawater and placed under a binocular microscope under dim light. We sorted three replicates of 50 adult copepods per species and placed them into 500 µl of 90 % cold acetone. Replicates were then macerated with tissue grinders, while being held on crushed ice. After thorough grinding, they were refrigerated in the dark for two hours, filtered on a syringe filter (Acrodisc CR PTFE 0.45 µm) to remove suspended particles, and 200 µl of extract were injected into the HPLC system.

Reversed-phase HPLC analyses were conducted based on the slightly modified ternary gradient method of Wright et al. (1991) as described in Gasparini et al. (2000). Pigments were detected by absorption at 436 nm and identified based on comparison of their retention time and spectra with standards. High-purity HPLC standards for chlorophyll-*a*, pheophytin-*a*, chlorophyll-c_3, fucoxanthin, alloxanthin, peridinin and 19'-hexanoyloxyfucoxanthin were obtained from the International Agency for ^{14}C determination (Hørsholm, Denmark). Phaeophorbide-*a* was obtained from ICN Biomedicals. Other carotenoids were identified using retention time and spectra of well-characterised pigments (Jeffrey et al. 1997).

After pre-concentration following the method of Colijn et al. (1990) for phytoplankton and microzooplankton enumeration (see also Gasparini et al. 2000), samples from the feeding experiments were analysed by an inverted microscope provided with phase contrast (ZEISS Sedival) in 5 ml sedimentation chambers. Depending on cell density, random fields (60-100) or selected transects of the entire sedimentation chamber were counted for microplankton (×200) and small flagellates (×400). Clearance rates (volume swept clear of prey individual^{-1} unit time^{-1}) were quantified from the difference in prey concentration determined between

control and experimental treatments, using Frost's equations (1972). Ingestion rates were calculated per copepod species for each potential prey by multiplying clearance rates with the corresponding prey biomass in the water. Prey enumeration and biomass were estimated from the seawater samples fixed at initial conditions. The carbon content of diatoms was calculated on the basis of cell density and biometry determined for each species. A specific average biovolume of a cell population was measured throughout the period of its development (Rousseau et al. 2002, and pers. comm.). After application of the correction equation for fixation-induced volume change (Menden-Deuer et al. 2001), the biovolumes were converted into carbon biomass using the size-dependent density relationship recommended for diatoms by Menden-Deuer and Lessard (2000). Dinoflagellate, nanoflagellates and ciliates biomass were calculated by using carbon to volume relationships recommended by Menden-Deuer and Lessard (2000). Colonial *Phaeocystis* cells were free in our samples due to the dissolution of the colony matrix by the preserving agent. A conversion factor of 14.2 pgC $cell^{-1}$ for colonial cells and of 10.8 pgC $cell^{-1}$ for flagellated cells (microzoospores) was used to estimate *Phaeocystis* carbon biomass as recommended by Rousseau et al. (1990).

3. Results

Fig. III-2 shows seasonal variations in alloxanthin concentration integrated over the water column at different stations in the Belgian coastal zone in 2000 and 2001. Alloxanthin concentration varied between 0 and 0.44 μg l^{-1}, with maximum values observed from end-May to June 2001. The concentration of alloxanthin was significantly correlated with Cryptophyceae abundance (Fig. III-3). A bloom of Cryptophyceae was observed at the end of May 2001, when their abundance increased from a background level of less than 20 cells ml^{-1} to a maximum of 1060 cells ml^{-1}.

In copepods, the alloxanthin content was generally higher in *T. longicornis* than in *C. hamatus* (Mann-Whitney U-test, $U = 45$, $n_1 = 47$, $n_2 = 22$, $p < 0.001$) (Fig. III-4). The mean alloxanthin content of *T. longicornis* varied between 0.059 and 0.753 ng, with maximum values measured at the beginning of spring and in summer (>0.45 ng ind^{-1}). In *C. hamatus* alloxanthin values were between 0.011 and 0.219 ng ind^{-1}. Alloxanthin content was not significantly correlated with the alloxanthin concentration in the seawater (Fig. III-5) for either *T. longicornis* (Spearman's rank $r_s = 0.251$, $n = 47$, $p > 0.05$) or *C. hamatus* ($r_s = -0.075$, $n = 22$, $p > 0.05$).

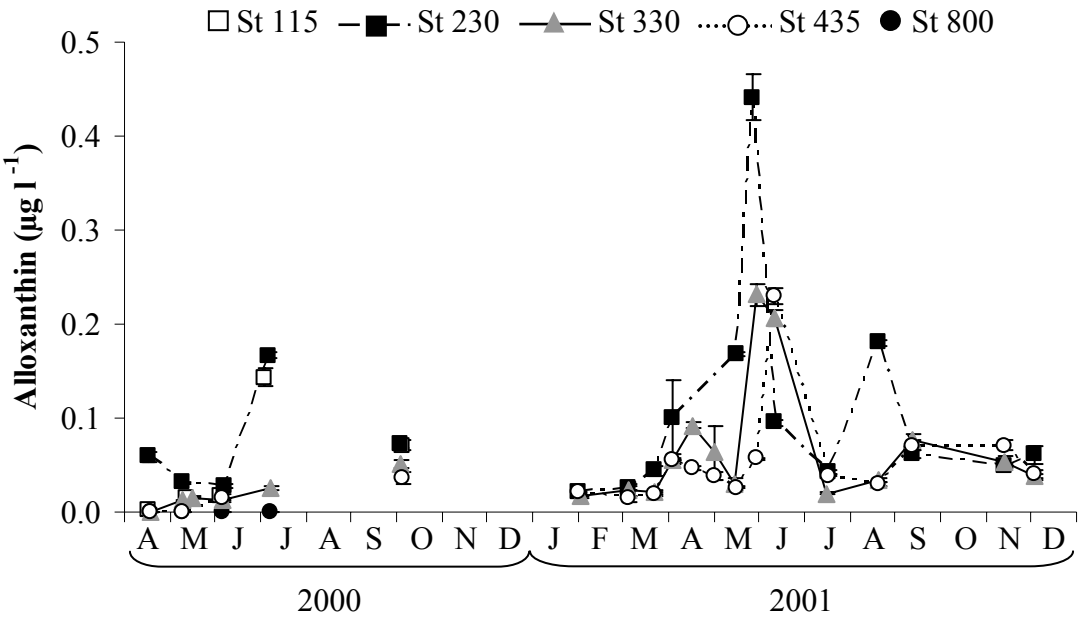

Figure III-2: Seasonal variations of alloxanthin concentration (mean ± SE) at five stations in Belgian coastal zone during AMORE 2000-2001 cruises.

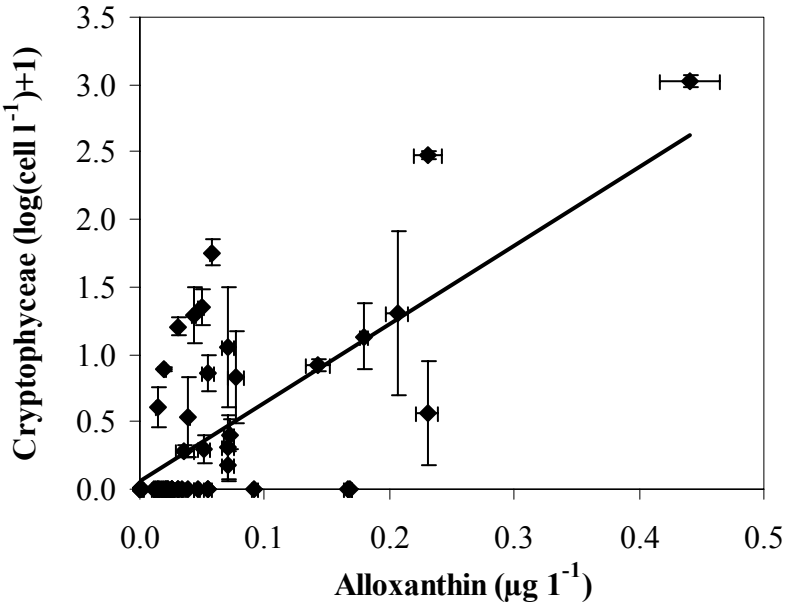

Figure III-3: Relation between concentration of alloxanthin (mean ± SE) and Cryptophyceae abundance (mean ± SE) in Belgian coastal waters during AMORE 2000-2001 cruises. Straight line: regression obtained by least square method (Spearman's rank $r_s = 0.592$, $n = 51$, $p < 0.001$).

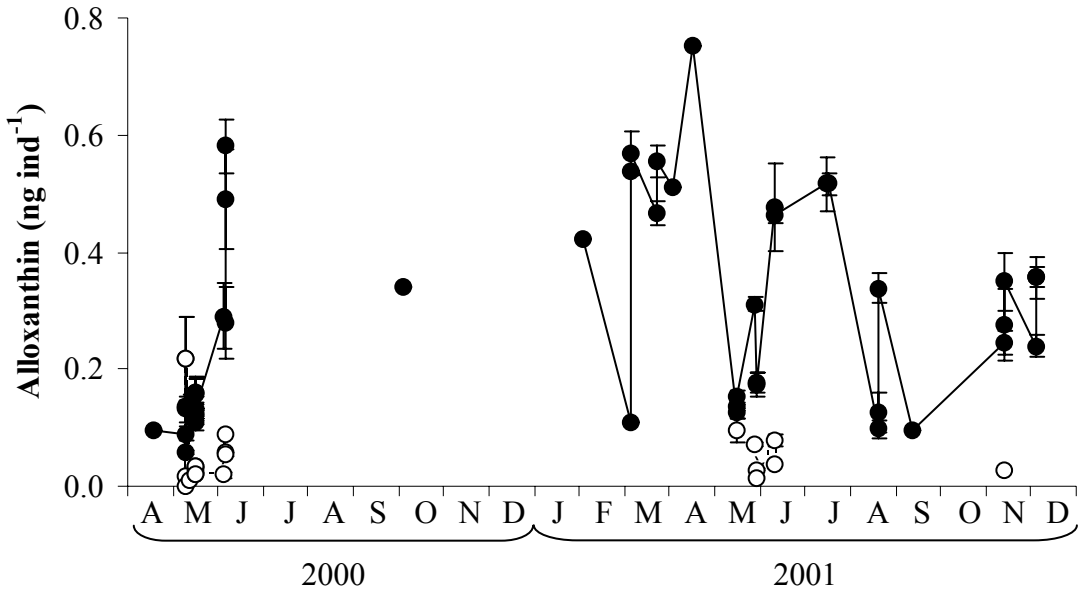

Figure III-4: *Temora longicornis* (●) and *Centropages hamatus* (○). Alloxanthin content (mean ± SE) during the AMORE 2000-2001 cruises.

As shown by the typical absorbance chromatograms obtained from HPLC analysis of copepod extracts in June 2001, fucoxanthin, diadinoxanthin, alloxanthin, diatoxanthin, chlorophyll-*a* and astaxanthin esters were detected in copepods at the beginning of starvation experiments (Fig. III-6A,C). Phaeophytin-*a* was not always apparent in absorbance chromatograms analysed at 436 nm because its absorption is low at this wavelength. However, it was clearly visible on fluorescence chromatograms (not shown). After 90 min of starvation chloropigments and fucoxanthin were no longer detected in *T. longicornis* (Fig. III-6B), nor diadinoxanthin in *C. hamatus* (Fig. III-6D). In June 1998, alloxanthin and astaxanthin esters were the only pigments remaining in the body tissues of *T. longicornis* after three hours starvation (not shown). Among the identified pigments, fucoxanthin, alloxanthin and chloropigments are commonly used as phytoplanktonic biomarkers. Diadinoxanthin and diatoxanthin, which are not class-specific, will not be considered here. The results of the two starvation experiments are shown in Figs. III-7 and III-8 for winter and summer, respectively. The loss of the phytoplanktonic biomarker pigments during the starvation experiments showed different pattern among pigments. Fucoxanthin (a marker of diatoms and golden-brown flagellates) disappeared completely from the copepod's body after 10 min starvation. Chlorophyll-*a* and pheophytin-*a* contents of *T. longicornis* and *C. hamatus* also decreased, but faster in summer than in winter. Less than 30 % of the initial value in chlorophyll-*a* remained in copepods after 10 min starvation in June 2001, whereas in January

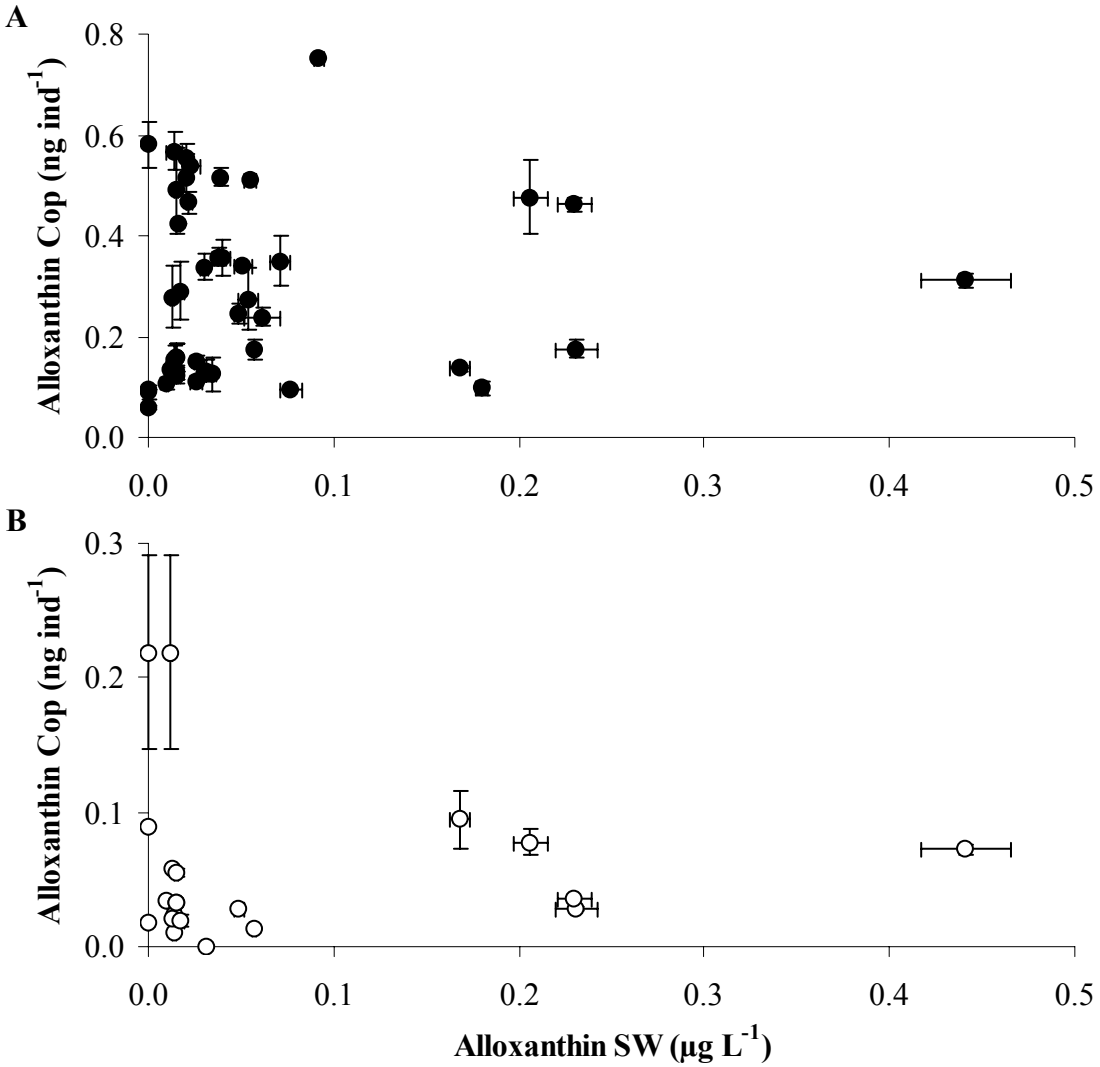

Figure III-5: (A) *Temora longicornis* and (B) *Centropages hamatus*. Relation between concentration of alloxanthin (mean ± SE) in seawater (SW) and alloxanthin content (mean ± SE) of copepods (Cop) during AMORE 2000-2001 cruises.

1998 this occurred after one hour incubation. In contrast to the other pigments, alloxanthin decreased little or not at all over time. In January 1998, the alloxanthin content of *T. longicornis* fell to 46 % of the initial value after 20 min before increasing and remaining above 70 % until the end of the starvation experiment (Fig. III-7). In June 2001, the alloxanthin content decreased to 45 and 43 % of the initial value after 60 min in *T. longicornis* and *C. hamatus*, respectively. In all experiments, the alloxanthin content of the copepods body tissues represented at least 78 % of the initial value in alloxanthin after 90 min of evacuation.

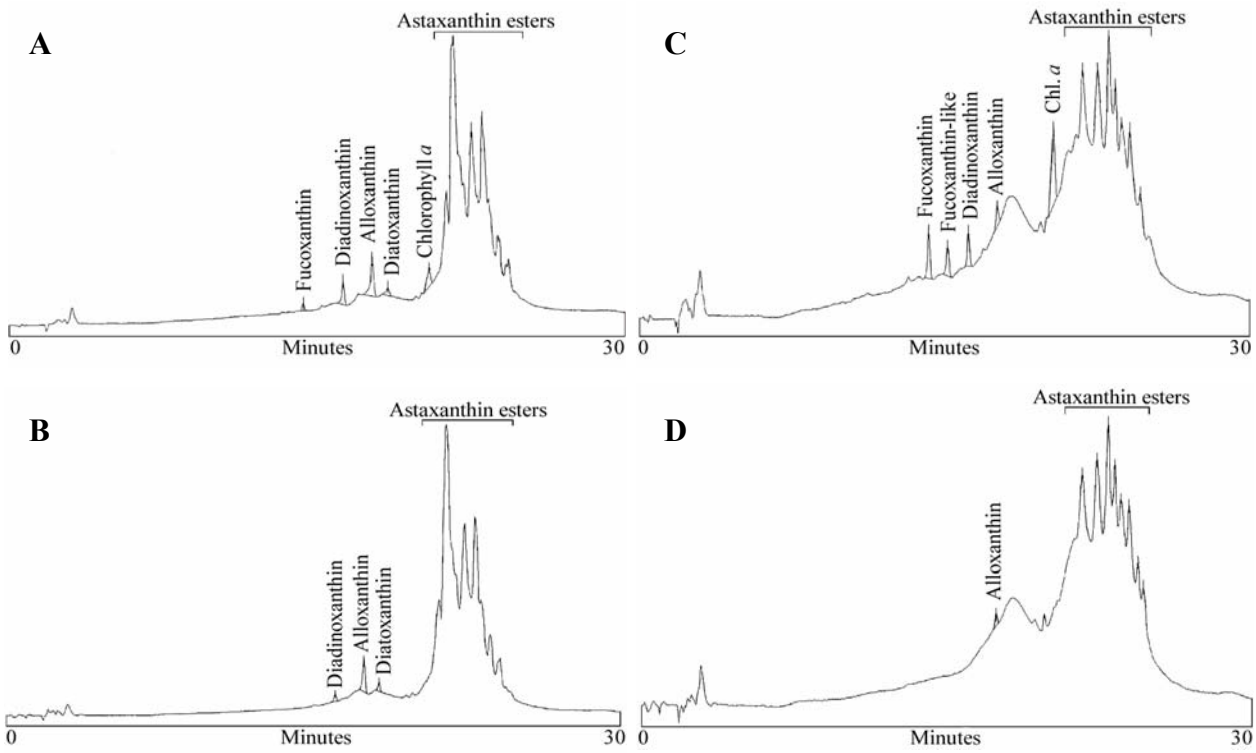

Figure III-6: (A)(B) *Temora longicornis*, (C)(D) *Centropages hamatus*. Absorbance chromatograms at 436 nm obtained from HPLC analysis of adult copepod extracts (50 individuals) at the beginning (A,C) and after 90 minutes (B,D) of starvation experiment in June 2001.

Among the 71 feeding experiments (Table III-1: 48 with *T. longicornis* and 23 with *C. hamatus*), significant ingestion rates were recorded for diatoms, dinoflagellates and ciliates. In contrast, we found no significant ingestion of Cryptophyceae or other nanoflagellates (Haptophyceae and Prasinophyceae). Detailed analysis of these grazing experiments has been included here, since we are focusing on the Cryptophyceae.

Table III-1: *Temora longicornis* (T) and *Centropages hamatus* (C). Number of experiments in which significant grazing rates (t-test, $p < 0.05$) were recorded for different prey items and copepod species.

	Bacillariophyceae		Haptophyceae		Cryptophyceae		Prasinophyceae		Dinophyceae		Ciliophora	
	T	C	T	C	T	C	T	C	T	C	T	C
number of experiments	48	23	48	23	48	23	48	23	48	23	48	23
significant ingestion	37	14	0	0	0	0	0	0	15	6	9	7
non-significant ingestion	11	9	48	23	48	23	48	23	33	17	39	16

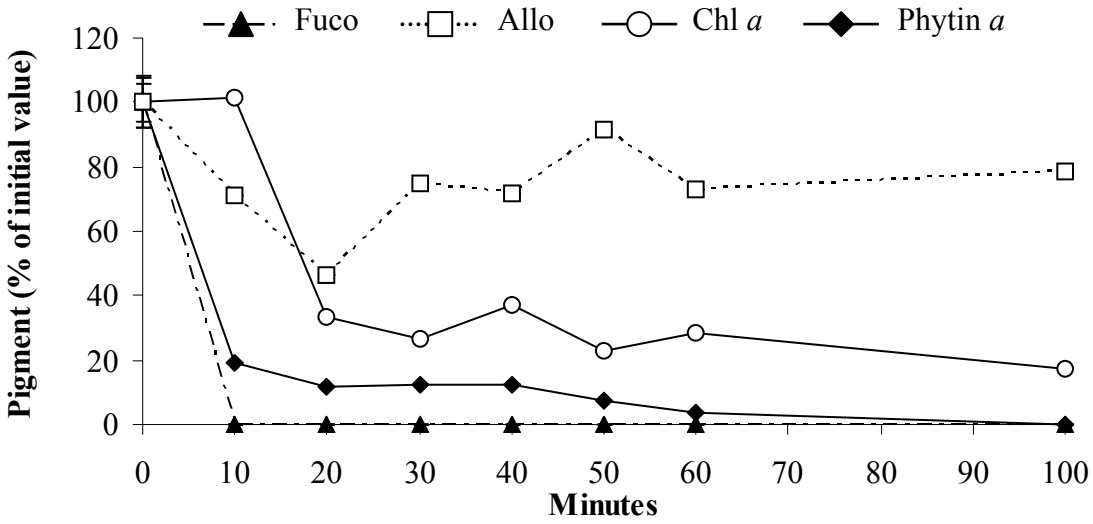

Figure III-7: *Temora longicornis*. Pigment content during 100 min starvation in January 1998.

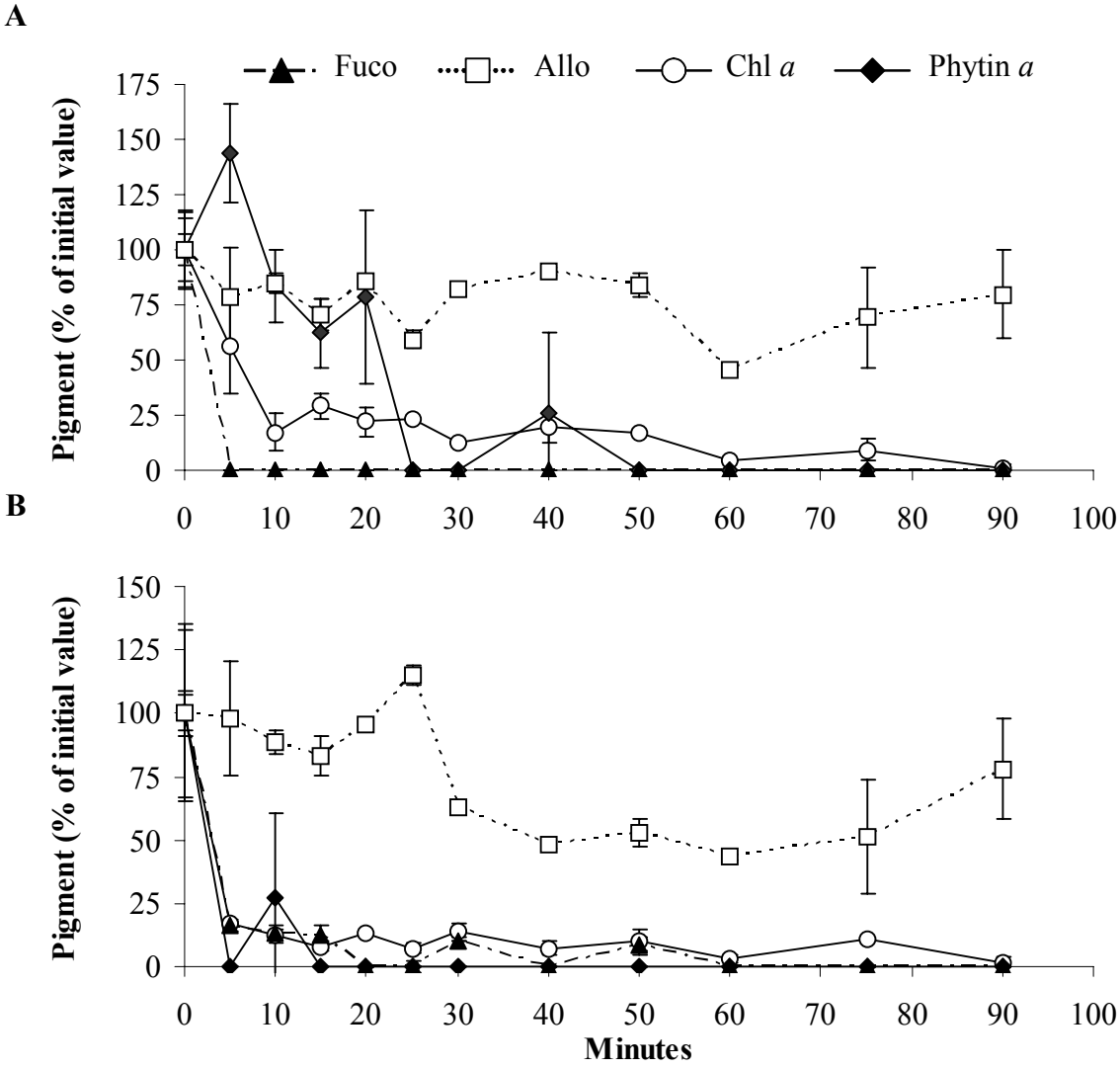

Figure III-8: (A) *Temora longicornis* and (B) *Centropages hamatus*. Pigments content (mean ± SE) during 90 min starvation in June 2001.

4. Discussion

The significant correlation ($p < 0.001$) between alloxanthin concentration and Cryptophyceae abundance confirms the chemotaxonomic marker quality of the pigment in natural seawater samples, as reported in the literature (Gieskes and Kraay 1983, Jeffrey and Vesk 1997, Breton et al. 2000). However, the origin of alloxanthin found in the copepods is problematic since (1) no significant correlation was found with alloxanthin in the seawater and (2) no significant grazing on Cryptophyceae was observed in feeding experiments whereas other potential prey were consumed. In fact, alloxanthin was present in the copepods even when this pigment was not detected in the water (Fig. IV-5). Such a discrepancy has also been reported by Breton et al. (1999) and Cotonnec et al. (2001), who found that (after fucoxanthin) alloxanthin was the main carotenoid in the body tissues of *T. longicornis*, whereas it was not detectable in the seawater samples, and that Cryptophyceae abundance observed by microscopy was low (10^2 - 10^3 cells l^{-1}). These observations led the authors to conclude that copepods fed selectively on Cryptophyceae. However, the results of our gut-evacuation experiments clearly show that alloxanthin was still present in copepod extracts after several hours of starvation whereas fucoxanthin and chloropigments decreased rapidly with time. If we assume that a chloropigment decrease indicates gut-emptying, it can be concluded that most of the detected alloxanthin did not originate from the gut but from body tissues. This conclusion could explain some intriguing laboratory observations reported in the literature. Breton (2000), for instance, reported the presence of alloxanthin in *T. longicornis* fed on monospecific cultures of diatoms, chlorophytes and *Artemia* sp. nauplii (which do not contain alloxanthin) and after three hours starvation. Likewise, Descy et al. (1999) reported the presence of alloxanthin in a freshwater copepod, *Diaptomus minutus*, starved for 8 hours in filtered water, and in copepods that were not fed Cryptophyceae.

It could be argued that copepod guts were not empty at the end of our starvation experiments and that the disappearance of pigments was due to degradation into colourless compounds. Indeed, fucoxanthin decreased faster than chloropigments, suggesting that a strong degradation of this pigment occurred during gut passage. Such degradation has already been suggested by Kleppel (1998) and is known to affect chloropigments as well (Conover et al. 1986, Head and Harris 1992, Tirelli and Mayzaud 1998, Pandolfini et al. 2000). However, this phenomenon does not challenge our assumption of an empty gut after more than one hour of starvation, since direct observation of copepods did not reveal any trace of food in the gut. Additionally, if we derive gut passage time from ambient temperature (Dam and Peterson

1988, Irigoien 1998), we obtain 24 and 39 min for experiments conducted at 15.7 and 6.3°C, respectively. Experiment durations (90 and 100 min) were consequently long enough to guarantee a complete evacuation of the gut.

Since copepod alloxanthin cannot be localised in gut contents, the question of its origin remains open. At least three hypotheses can be proposed: (1) the existence of endosymbiotic Cryptophyceae living in copepod tissues; (2) an accumulation in copepod body tissues of alloxanthin from ingested Cryptophyceae; (3) a metabolic transformation of other ingested pigments into alloxanthin by the copepods themselves. Concerning the first hypothesis, endosymbiotic Cryptophyceae are found in planktonic ciliates such as *Mesodinium rubrum* (Parsons and Blackbourn 1968, Hibberd 1977, Gieskes and Kraay 1983), in dinoflagellates such as *Gymnodinium acidotum* (Wilcox and Wedemeyer 1984) and in some *Dinophysis* spp. (Meyer-Harms and Pollehne 1998, Hackett et al. 2003), but not in copepods. The fact that other pigments usually found in Cryptophyceae were not detected in starved copepods make this hypothesis very speculative, but it cannot be completely excluded since the pigment dynamics of endosymbiotic Cryptophyceae are poorly known. The second hypothesis (accumulation of alloxanthin from ingested Cryptophyceae in copepod tissues) remains possible, since even if Cryptophyceae ingestion is very low (i.e. not detectable using grazing experiments), a whole life-time accumulation could lead to detectable amounts in copepods. Alternatively, copepods could transform other ingested algal carotenoids into alloxanthin. Like all animals, copepods cannot synthesise carotenoids *de novo* (Goodwin 1960). Thus, if carotenoids which are not present in the diet are found in the copepod tissues, they must have been produced from dietary carotenoids by oxidation. Copepods are capable of transforming the β-carotene and its derivatives present in ingested plant food into their own principal carotenoid, astaxanthin, by oxidative metabolic pathways via the intermediary pigments echinenone and canthaxanthin (Goodwin 1960, 1971, Simpson and Chichester 1981). Alloxanthin present in copepod body tissues could be an intermediary pigment leading to further metabolic transformation into astaxanthin. Indeed, alloxanthin has already been reported as a possible metabolic pathway to astaxanthin in goldfish *Carassius auratus* teguments (Ohkubo et al. 1999).

Our results do not permit a choice among the hypotheses considered or a combination of them, and further experiments will be necessary to elucidate the origin of alloxanthin in copepod bodies. In any case, our results suggest that alloxanthin is not suitable for quantitative and qualitative estimates of copepod grazing on Cryptophyceae. As a consequence, reports of alloxanthin in copepod extracts as an evidence of Cryptophyceae

ingestion (i.e. Breton et al. 1999, Cotonnec et al. 2001) need to be reconsidered. Furthermore we argue that copepod biomass may, at times, represent a substantial source of alloxanthin in the water column. A single copepod may contain sufficient alloxanthin in its gut to overshadow the Cryptophyceae alloxanthin in water samples. Thus, precautions must be taken to remove copepods from water samples before filtering. Similar problems could be encountered with other taxon-specific pigments such as echinenone and lutein which are sometime recorded in small amounts in copepod tissues (Lotocka and Styczynska-Jurewicz 2001).

Acknowledgements

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Contribution of herbivory to the diet of *Temora longicornis* (Müller) in Belgian coastal waters

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Abstract

The contribution of herbivory to the diet of *Temora longicornis* (Müller), an omnivorous calanoid copepod, and the degree of food limitation to its production were investigated in relation to microplankton availability during 2001 in Belgian coastal waters. The gut fluorescence method was combined with egg production measurements to estimate herbivorous and total feeding, respectively. Diatoms were the main phytoplankton component during the sampling period and constituted, with the colonial haptophyte *Phaeocystis globosa,* the bulk of phytoplankton biomass during the spring bloom. HPLC gut pigment analysis showed that diatoms were the main phytoplankton group ingested, whereas no evidence for ingestion of *P. globosa* and nanoflagellates was found. Further, our results showed higher phytoplankton ingestion by *T. longicornis* in spring, when small, chain-forming diatom species such as *Thalassiosira* spp. and *Chaetoceros* spp. were abundant, than in summer, when larger species such as *Guinardia* spp. and *Rhizosolenia* spp. dominated the diatom community. We showed that *T. longicornis* could be regarded as mainly herbivorous during fall and winter, while during spring and summer they needed heterotrophic food to meet their energetic demands for egg production. The phytoplankton spring bloom, either during diatom dominance or during *P. globosa* dominance, did not enhance the contribution of herbivory to the diet. We argue that when *T. longicornis* carbon requirements for egg production increase the contribution of non-phytoplankton food sources (microzooplankton and/or detritus) to total carbon ingestion becomes more important. Except during the early spring diatom bloom of *Chaetoceros socialis*, the egg production rates never reached the production potential of this species, including during the *P. globosa* bloom. This suggests that *T. longicornis* egg production was limited not only by food quantity but also by food quality and that ingestion of non-phytoplankton food sources did not allow the females to compensate for food limitation.

1. Introduction

The coastal waters of the southern North Sea are a well-known eutrophicated ecosystem due to anthropogenic sources of nutrients brought by the discharge of major west-European rivers (Lancelot 1995, Lancelot et al. 1998). The unbalanced nutrient environment of these coastal waters, characterized by an excess of nitrate over silicate and phosphate, induces an increase of spring algal blooms, with a major and sudden change in phytoplankton dominance from diatoms to the colonial flagellate *Phaeocystis globosa* (Lancelot 1995, Peperzak et al. 1998). This is also accompanied by a change in the species composition of the remaining diatoms, characterized by a shift from small chain-forming species toward an assemblage of larger diatom species throughout the spring period (Philippart et al. 2000, Rousseau et al. 2002).

The seasonal changes in phytoplankton abundance, cell size and taxonomic composition discussed above may affect the feeding behaviour of *Temora longicornis* (Müller), an omnivorous calanoid copepod that is present all year round in the coastal waters of the North Sea, with maximum abundances in spring (Daro 1988, Fransz et al. 1992, Williams et al. 1993). Several studies have demonstrated that *P. globosa* is not an adequate food source for *T. longicornis* (Daro 1985, Hansen and van Boekel 1991, Bautista et al. 1992, Breton et al. 1999, Gasparini et al. 2000), and it has been hypothesized that copepods switched to heterotrophic food to compensate for low phytoplankton ingestion (Daro 1985, Hansen and van Boekel 1991). Although field studies confirmed a preferential predation on microzooplankton by copepods during the *Phaeocystis* bloom (Brussaard et al. 1995, Gasparini et al. 2000), this predation seemed insufficient in terms of carbon to cover the copepods' nutritional needs and avoid food shortage (Gasparini et al. 2000). This food shortage could impact negatively not only on the next copepod generation (Bautista et al. 1992), but also on the energy transfer to higher trophic levels (Rousseau et al. 2000).

The general objectives of this study were (1) to examine the possible linkages between seasonal changes in phytoplankton availability and the *in situ* herbivorous feeding of adult *T. longicornis*, (2) to estimate the fraction of *T. longicornis* carbon requirements sustained by herbivory, and (3) to determine whether omnivorous feeding was sufficient to avoid the food limitation of egg production by *T. longicornis*. One approach to estimate omnivory is to measure both total carbon ingestion and algal ingestion and to quantify the degree of omnivory as the difference between the two. As outlined by Dam et al. (1994) and Peterson and Dam (1996) we used the gut fluorescence method combined with egg production measurement to estimate herbivorous and total feeding, respectively. We further analysed gut

pigment content by high-performance liquid chromatography (HPLC), and compared the results to the pigments and microzooplankton present in the water column in order to provide taxonomic information about the composition of *T. longicornis* diet (Kleppel and Pieper 1984, Buffan-Dubau et al. 1996).

2. Materials and methods

Sampling and egg production experiments were carried out in 2001 during field campaigns aboard the RVs *Belgica* and *Zeeleeuw* in Belgian coastal waters (southern North Sea) as part of the AMORE (Advanced MOdelling and Research on Eutrophication) project. Three stations, 230 (depth ~ 13 m), 330 (~24 m) and 435 (~32 m), were monitored fortnightly during spring and monthly for the rest of the year, weather permitting (Fig. IV-1). Vertical profiles of temperature and salinity were obtained using a Seabird Conductivity-Temperature-Depth (CTD) profiler system.

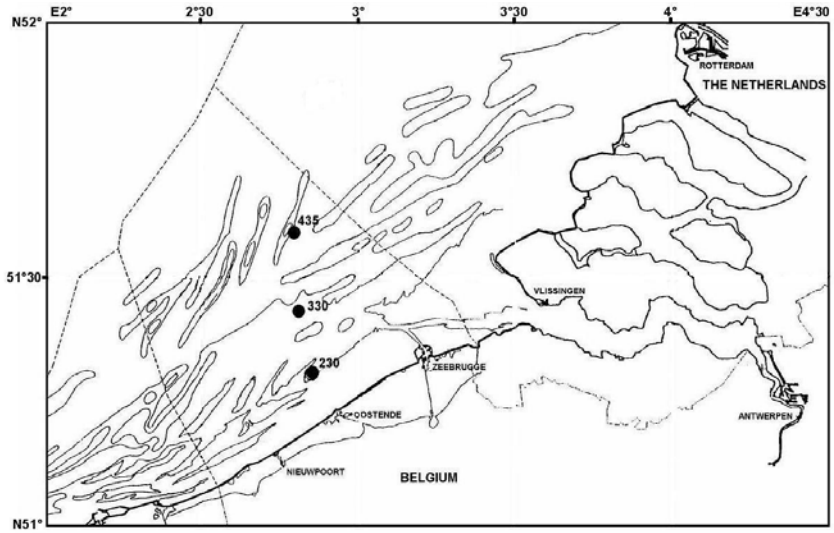

Figure IV-1: Belgian coastal zone, showing location of the three stations sampled during the AMORE 2001 cruises.

Water samples were taken with a 10 litre Niskin bottle from the subsurface, middle and near the bottom of the water column. Seawater subsamples (250-1500 ml) from the different depths were immediately filtered in triplicate using GF/F glass-fibre filters (47 mm diameter, 0.7 µm nominal pore size). All filters were folded in aluminium foil and immediately stored in liquid nitrogen for HPLC pigment analysis. Additional water subsamples (250 ml) were taken in triplicate, after mixing the water from the three depths, and were fixed with 1% (final concentration) glutaraldehyde for algal cell counting. HPLC pigment analysis and algal cell identification and counting followed the procedure described in Antajan and Gasparini (2004).

WP-2 plankton nets (200 µm) were towed obliquely between the surface and bottom (tow duration < 5 min) to collect animals for gut pigment content analysis and egg production experiments. From each zooplankton sample, a subsample was collected onto a piece of 200 µm gauze, wrapped in aluminium foil and frozen immediately in liquid nitrogen to prevent defecation losses. In the laboratory, three replicates of 50 *T. longicornis* adults were sorted under a binocular microscope, placed into 500 µl of 90 % cold acetone and macerated with a tissue grinder. After two hours of extraction in the dark at 4°C, the replicates were filtered on a syringe filter (Acrodisc CR PTFE 0.45 µm) to remove suspended particles, and pigments in the filtrates were analysed by HPLC (Gasparini et al. 2000, Antajan and Gasparini 2004). Chlorophyll-*a* (chl-*a*) and chl-*a* derivatives in gut contents were used as an index of phytoplankton ingestion, whereas carotenoid pigments were used as taxonomic indicators in order to characterize the ingested prey. The amounts of phaeopigments and chl-*a* were summed and the results were expressed as ng chl-*a* equivalents per copepod. No correction was made for possible pigment destruction during the gut passage. Phytoplankton ingestion rates were estimated by multiplying the gut chl-*a* equivalent by the gut clearance rate which was estimated from in situ temperature (Dam and Peterson 1988, Irigoien 1998). Algal carbon ingestion rates were calculated by applying a C:chl-*a* ratio of 50 (Banse 1977). *Temora longicornis* body carbon weight was calculated from the temperature-carbon weight relationships given by Halsband-Lenk (2001).

For egg production measurements, six to twelve replicates of five freshly caught *T. longicornis* females were transferred into 250 ml of filtered seawater (0.7 µm) inside a Plexiglas tube with 200 µm mesh false bottom to prevent egg cannibalism. It has been demonstrated that 24 h of incubation in filtered seawater does not affect egg production rates and reflects the feeding history of the females in the field prior to capture (Peterson and Bellantoni 1987, Tester and Turner 1990, Laabir et al. 1995, Hirche et al. 1997). All replicates were incubated in on-deck incubators and maintained at *in situ* temperature by immersion in a water bath with continuously circulating surface seawater for 24 hours. At the end of the incubation, female survival was visually checked, and eggs spawned during incubation were sieved on a 20 µm mesh and preserved with formalin (4 % final concentration). Into the laboratory eggs were counted and their diameters were measured to calculate their volume assuming a perfectly spherical shape. Then, the volume was converted into carbon content by applying a carbon/volume ratio of 0.14×10^{-6} µgC μm^{-3} (Kiørboe et al. 1985). Egg production rates were calculated as the total eggs divided by the number of females that survived through

the incubation. Females that died during incubation (< 1 %) were assumed to have been moribund at the beginning of the incubation and therefore to have laid no eggs. Egg-carbon production rates were then converted into total carbon ingestion rates by assuming a carbon-specific egg production efficiency of 33 % (Kiørboe et al. 1985, Peterson 1988, Båmstedt et al. 2000).

3. Results

Temperature and salinity profiles revealed that the water column was vertically well-mixed throughout the year. Figure IV-2 presents average temperature and salinity in the water column measured at the three stations in 2001. The seasonal variations of temperature show a winter minimum of about 5.5°C and a summer maximum of about 19.5°C. On average, the difference between stations 230 and 435 was about 1°C. Station 230 had an average salinity of 32 psu and seemed to be the more influenced by freshwater inputs, as shown by the amplitude of salinity variations. The average salinity average was 32.9 psu at station 330 and 33.6 psu at station 435.

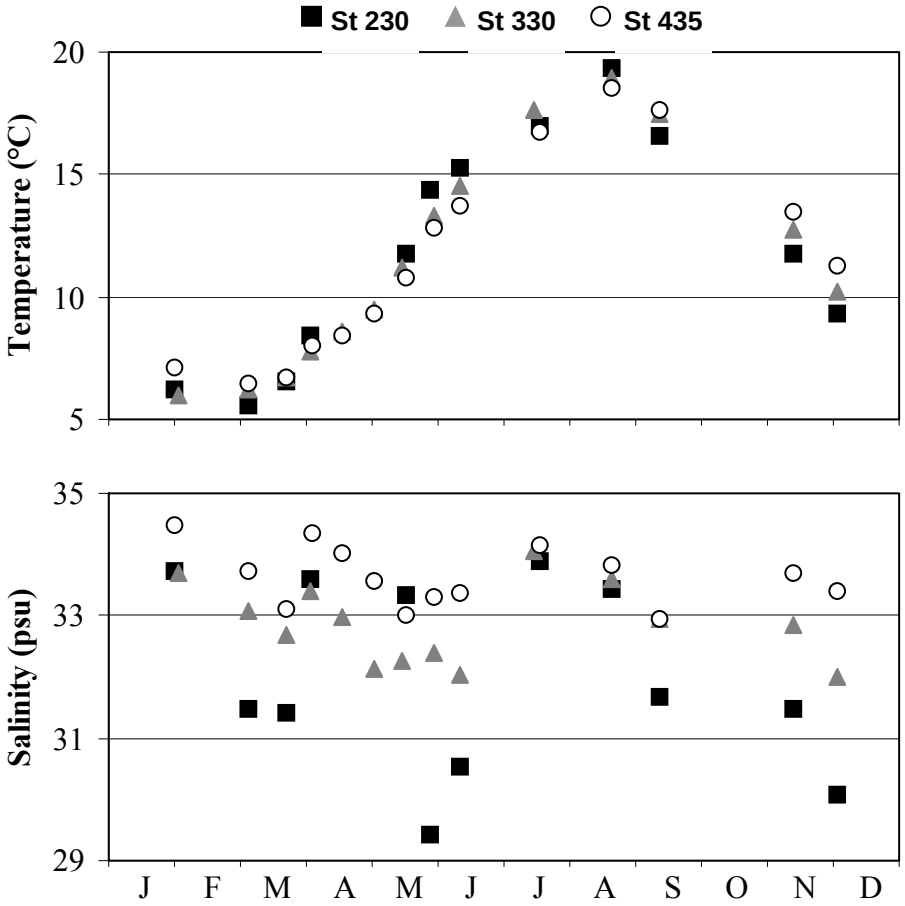

Figure IV-2: Temperature and salinity measured at the three sampling stations of the Belgian coastal zone during 2001.

3.1. Microplankton abundance and pigments biomarkers

Figure IV-3 shows the seasonal variation of diatom, *Phaeocystis globosa*, nanoflagellate (other than *P. globosa*), dinoflagellate and ciliate abundance at the three stations studied in 2001. Diatoms were present throughout the year, reaching maximum abundance in March (ca. 1700 cells ml^{-1}). At that time the genus *Chaetoceros*, largely composed of by *C. socialis*, became dominant, representing up to 90 % of the diatom community. In April and May *P. globosa* dominated diatoms and reached its maximum abundance around mid-April (more than 27,000 cells ml^{-1}). A second but rather modest *P. globosa* growth was observed in September (ca. 1,450 cells ml^{-1}). A bloom of nanoflagellates composed of Cryptophyceae, Prasinophyceae (*Pyramimonas* spp.) and other unidentified nanoplankton-sized flagellates occurred at the end of the *Phaeocystis* bloom in May and was particularly impressive at station 230 (ca. 2,300 cells ml^{-1}). Dinoflagellate and ciliate abundance increased at the wax and wane of the *P. globosa* bloom.

Representative fluorescence and absorbance chromatograms showing the elution pattern of chlorophyll and carotenoid pigments detected in water are presented in Fig. IV-4A,C, and the pigment concentration ranges are given in Table IV-1. Chlorophyll *a* concentrations integrated over the water column varied between 0.15 and 17.35 µg l^{-1} (Fig. IV-5). Increases in chl *a* concentrations were associated with increases in phytoplankton containing fucoxanthin (Spearman rank $r_s = 0.959$, $n = 40$, $p < 0.001$), and coincided with diatom (March and June) and *P. globosa* (April) blooms. Numerous carotenoid pigments were detected by HPLC. Our interest, however, was in those carotenoids that can be utilized as taxonomic markers of classes or groups of classes of phytoplankton. The major carotenoid, fucoxanthin, ranged in concentration from 0.08 to 6.57 µg l^{-1} and was significantly correlated with the abundance of the two dominant golden-brown algae ($r_s = 0.773$, $n = 32$, $p < 0.001$), diatoms and *P. globosa*. Chlorophyll c_3, which was highly correlated with *P. globosa* biomass, ($r_s = 0.751$, $n = 32$, $p < 0.001$) increased from concentrations < 0.10 µg l^{-1} in March up to 1.90 µg l^{-1} in April 2001, corresponding with the maximum abundance of *P. globosa*. Alloxanthin was commonly found in small amounts with highest values observed in May and June corresponding to an increase in cyptomonad abundance ($r_s = 0.597$, $n = 32$, $p < 0.001$). Both 19'-hexanoyloyfucoxanthin and peridinin were found as trace levels and were not correlated with any of the phytoplankton groups we examined. As no chlorophyll *b* standard was available we used chromatographic peak area to compare relative abundance of this pigment

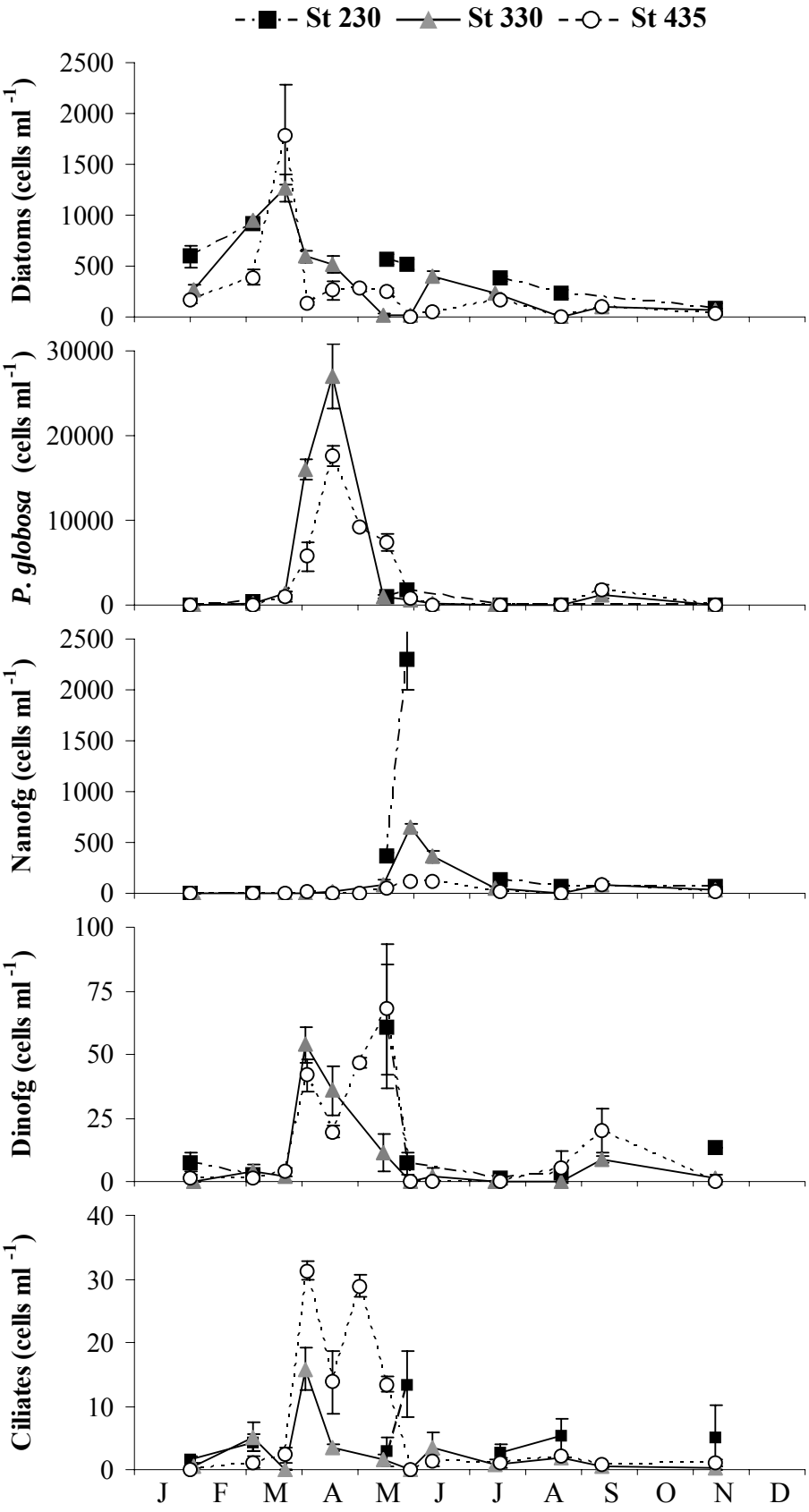

Figure IV-3: Abundance (mean ± SE) of diatoms, *Phaeocystis globosa*, nanoflagellates (other than *P. globosa*), dinoflagellates and ciliates at the three stations of the Belgian coastal zone during 2001. Note the vertical scales.

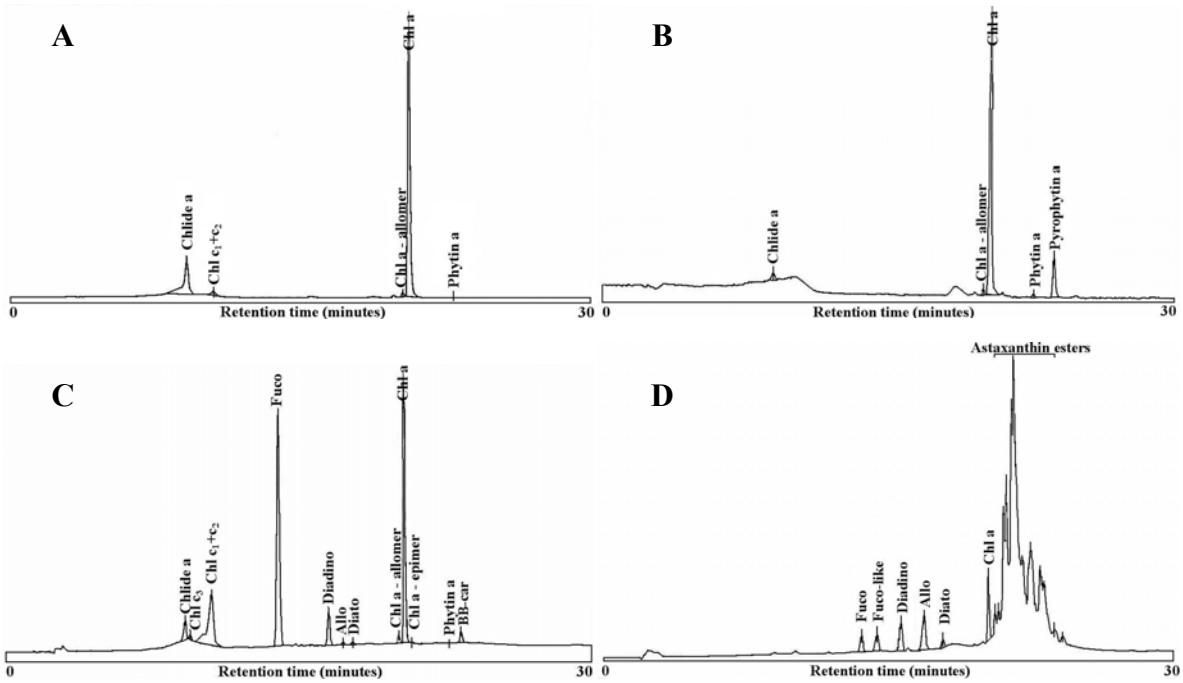

Figure IV-4: Typical fluorescence (A and B) and absorbance (C and D) chromatograms obtained from HPLC analysis of seawater samples (A and C) and of *Temora longicornis* (B and D; 50 adults) taken at station 330 of the Belgian coastal zone in March 2001. Abbreviations refer to the pigment name in Table IV-1.

Table IV-1: Concentration ranges (µg l^{-1}) and amount ranges (ng $ind.^{-1}$) of phytoplankton pigments detected in seawater samples and in extracts of *Temora longicornis* in Belgian coastal waters in 2001. (+) detected, (-) not detected.

Pigment name	Abbreviation	Seawater pigment (µg l^{-1})	Gut pigment content (ng $ind.^{-1}$)
Chlorophyllide *a*	Chlide *a*	0.01 – 1.11	0.00 – 0.09
Chlorophyll c_3	Chl c_3	0.00 – 1.90	-
Chlorophyll c_1+c_2	Chl c_1+c_2	+	+
Peridinin	Perid	0.00 – 0.12	-
Fucoxanthin	Fuco	0.08 – 6.57	0.00 – 1.83
Fucoxanthinlike absorption spectra	Fuco-like	-	
19'-Hexanoyloyfucoxanthin	Hex-fuco	0.00 – 0.11	-
Pheophyorbide *a*	Phide *a*	0.00 – 0.48	0.00 – 0.70
Diadinoxanthin	Diadino	+	+
Alloxanthin	Allo	0.01 – 0.44	0.07 – 0.75
Diatoxanthin	Diato	+	+
Zeaxanthin	Zea	+	-
Chlorophyll *b*	Chl *b*	+	-
Phytylated chlorophyll *c*	Phytyl-chl *c*	+	-
Chlorophyll *a* allomer	Chl *a* allomer	+	-
Chlorophyll *a*	Chl *a*	0.15 – 17.35	0.01 – 0.69
Chlorophyll *a* epimer	Chl *a* epimer	+	-
Pheophytin *a*	Phytin *a*	0.00 – 0.45	0.05 – 0.37
Pyropheohytin *a*	Pyrophytin *a*	-	
Astaxanthin esters	Asta	-	+
β-*β*cartene	*β*-*β*car	+	+

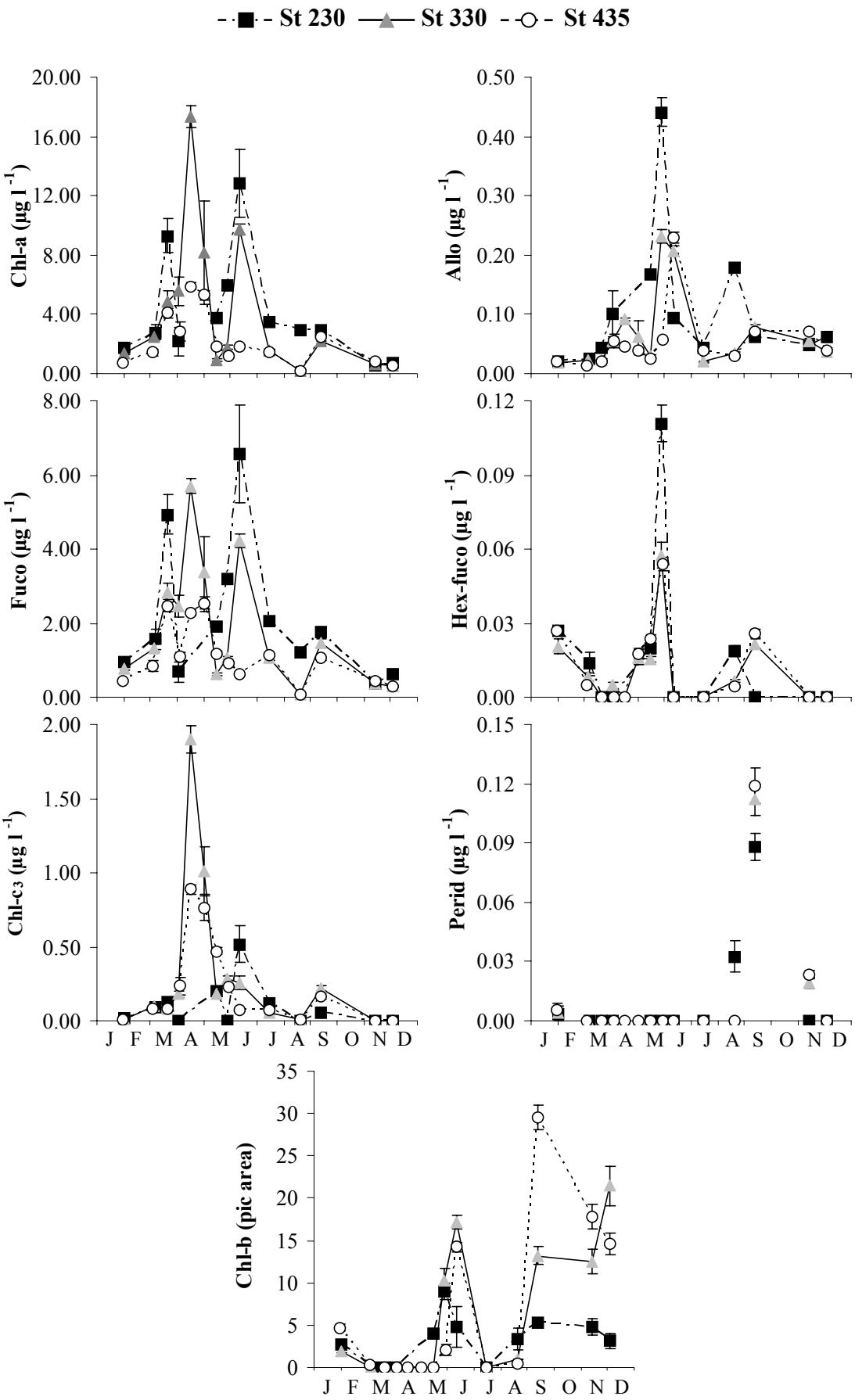

Figure IV-5: Pigment concentrations (mean ± SE) at three stations of the Belgian coastal zone during 2001. See Table IV-1 for pigment abbreviations. Note the vertical scales.

on a temporal scale. Chlorophyll *b* had most important chromatographic peak areas in June and in autumn and was significantly correlated with prasinophytes (r_s = 0.738, n = 32, p < 0.001).

3.2. Gut pigment content and phytoplankton ingestion rates

Representative fluorescence and absorbance chromatograms of the pigments detected in *Temora longicornis* are given in Fig. IV-4B,D and amount ranges of pigments in Table IV-1. Clearly, chl-*a* eq. gut content of *T. longicornis* (Fig. IV-6A) did not reflect the course of the chl *a* concentration in the water (Fig. IV-5). The highest values of chl-*a* eq. gut content were observed during the late winter-early spring period dominated by small chain-forming diatom species (such as *Thalassiosira* spp. and *Chaetoceros* spp.). Mean phytoplankton ingestion rate by *T. longicornis* estimated from gut fluorescence in February-March was 1.60 µgC ind.$^{-1}$ d^{-1} (± 0.18; SE), equivalent to 9.0 % (± 1.0; SE) of body carbon weight (Fig. IV-6B,C). During *P. globosa* bloom and the subsequent bloom of nanoflagellates (end-May), chl-*a* gut contents decreased drastically whereas the chl-*a* concentration in the water was at its maximum (Fig. 5). The corresponding phytoplankton ingestion rate fell down to 0.23 µgC ind.$^{-1}$ d^{-1} (± 0.09; SE), or 1.4 % of the body carbon at stations 330 and 435. In May, however, gut pigment contents at station 230 were significantly higher than the two other stations (Kruskal-Wallis test, n = 17, p < 0.01). This was also observed for chl-*a*, fucoxanthin and alloxanthin concentrations in seawater samples (Fig. 5), and was associated with the presence of a coastal bloom of small chain-forming diatoms, *Chaetoceros curvisetus* and *Asterionellopsis glacialis* (ca. 500 cells ml^{-1}). This difference in food composition and abundance has led to higher phytoplankton ingestion rates, 0.90 µgC ind.$^{-1}$ d^{-1} (± 0.07; SE) or 6.6 % (± 0.7; SE) of body carbon. After the spring bloom, chlorophyll-*a* gut contents remained very low the whole summer and until the end of the year, apart a slight increase in November. During this period, dominated by large diatoms (such as *Guinardia* spp. and *Rhizosolenia* spp.), mean phytoplankton ingestion rate was 0.335 µgC ind.$^{-1}$ d^{-1} (± 0.054; SE), equivalent to 2.6 % (± 0.4; SE) of the *T. longicornis* body carbon weight. Overall, phytoplankton ingestion was positively related to abundance of small diatoms (< 3,000 µm^3; r_s = 0.78, n = 26, p < 0.001).

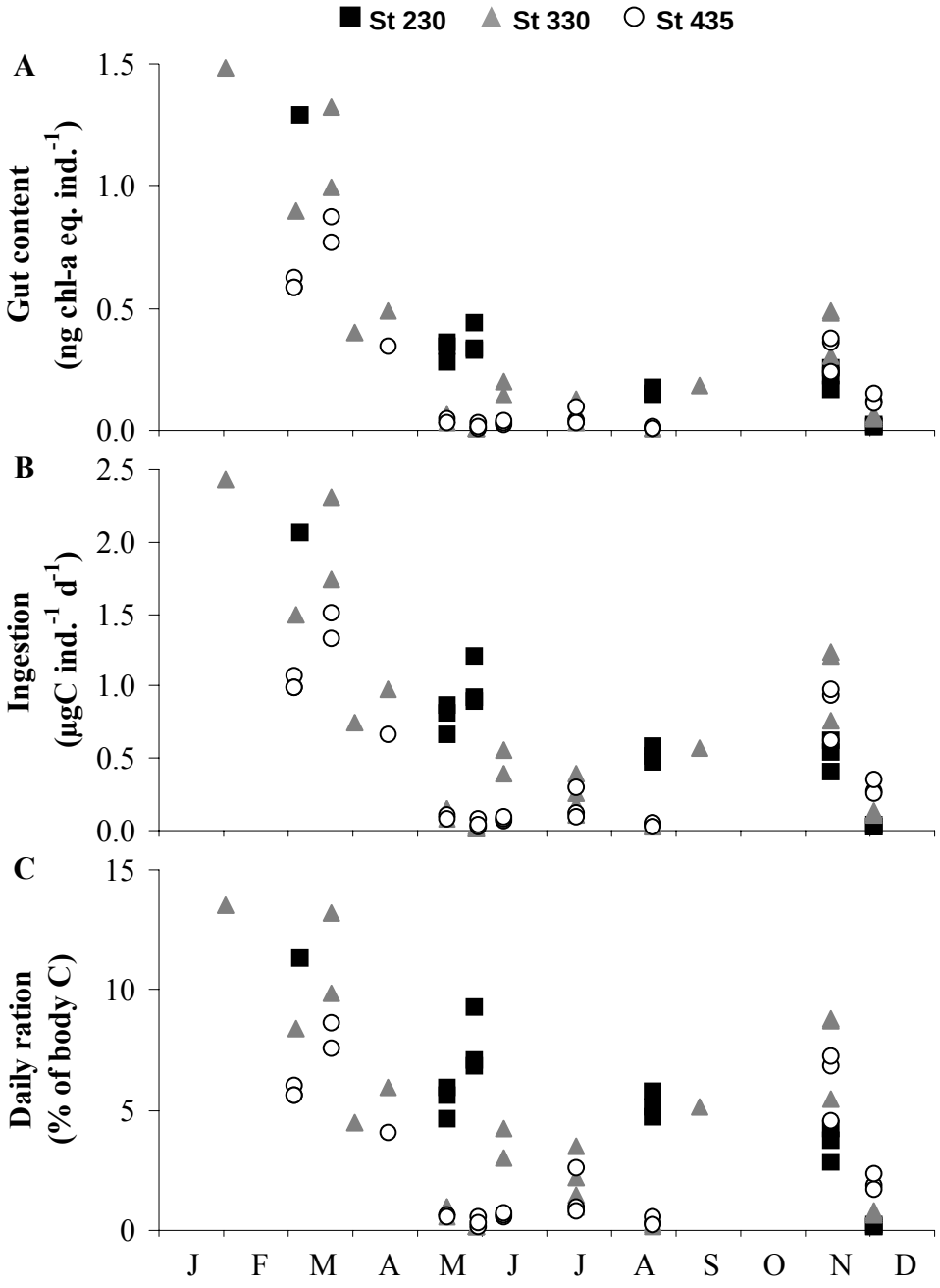

Figure IV-6: *Temora longicornis* adults. Chloropigment gut contents (A) and daily phytoplankton ingestion rates expressed in ngC fem.$^{-1}$ d^{-1} (B) and as percentage of the body carbon weight (C) at three stations of the Belgian coastal zone during 2001

Among biomarker pigments found in seawater samples only fucoxanthin and alloxanthin were detected in *T. longicornis*. Fucoxanthin gut contents presented the same temporal pattern as chl-*a* equivalent gut contents (Fig. IV-7A), with highest values in March (mean value: 1.05 ng ind.$^{-1}$). Alloxanthin gut contents varied between 0.07 and 0.75 ng ind.$^{-1}$ and did not show any clear pattern against time (Fig. IV-7B). The other pigments found in *T. longicornis* were chlorophyll c_1+c_2, diadinoxanthin, diatoxanthin and astaxanthin esters (Fig. IV-4B, Table IV-1).

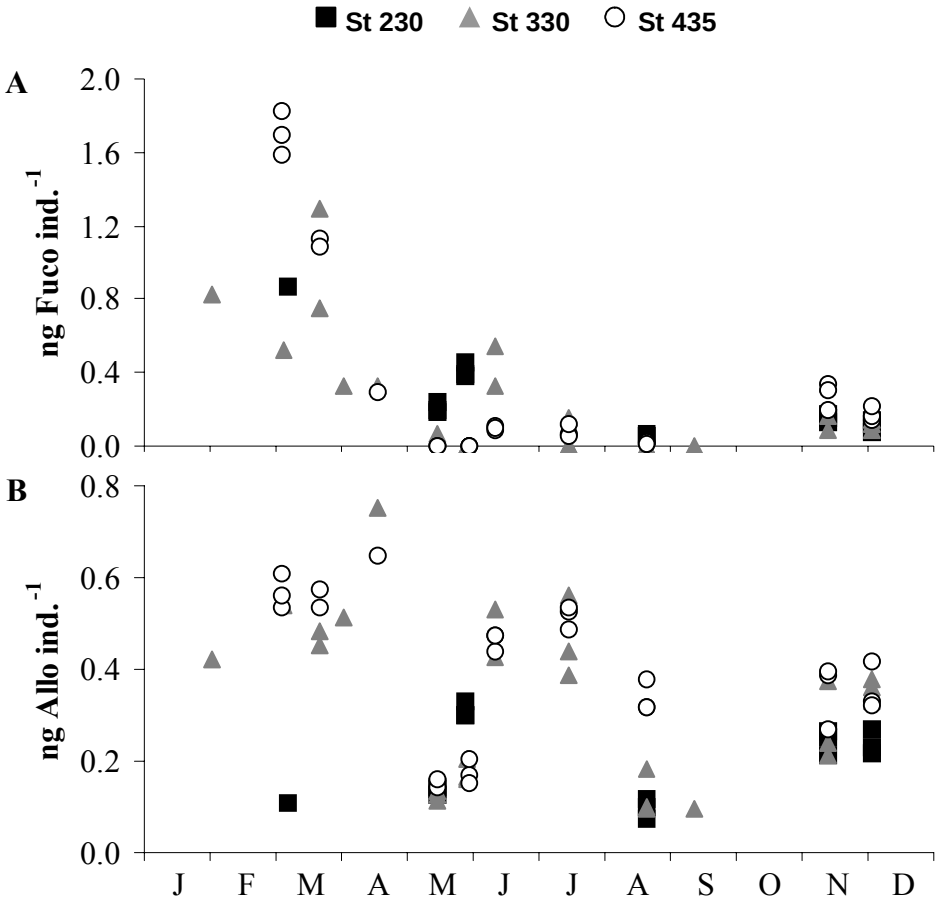

Figure IV-7: *Temora longicornis* adults. Fucoxanthin (A) and alloxanthin (B) gut contents at three stations of the Belgian coastal zone during 2001. Note the vertical scales.

3.3 Egg production rates and carbon requirements

Temora longicornis females produced eggs throughout the year (Fig. IV-8A). The highest daily mean rate, 42 eggs fem.$^{-1}$ d^{-1}, and the highest individual egg production, 61 eggs fem.$^{-1}$ d^{-1}, were measured in March during the bloom of *C. socialis*. During this period the total ingestion rates (i.e. the carbon ingestion required for covering egg production rates) ranged from 3.26 to 8.09 µgC fem.$^{-1}$ d^{-1} with a mean value of 5.01 µgC fem.$^{-1}$ d^{-1} (± 0.38; SE) equivalent to 29.0 % (± 2.2; SE) body carbon weight (Fig. IV-8B,C). In mid-April during the peak of *P. globosa* bloom the mean rate of egg production was reduced to 14 eggs fem.$^{-1}$ d^{-1} corresponding to an ingestion of 2.00 µgC fem.$^{-1}$ d^{-1} (± 0.29; SE) or 12.1 % (± 1.8; SE) body carbon weight. After that, mean *T. longicornis* egg production rates remained below 20 eggs fem.$^{-1}$ d^{-1} and total ingestion rates ranged from 1.98 µgC fem.$^{-1}$ d^{-1} in June to 0.87 µgC fem.$^{-1}$ d^{-1} in November, equivalent to 15 and 6 % of the body carbon weight, respectively.

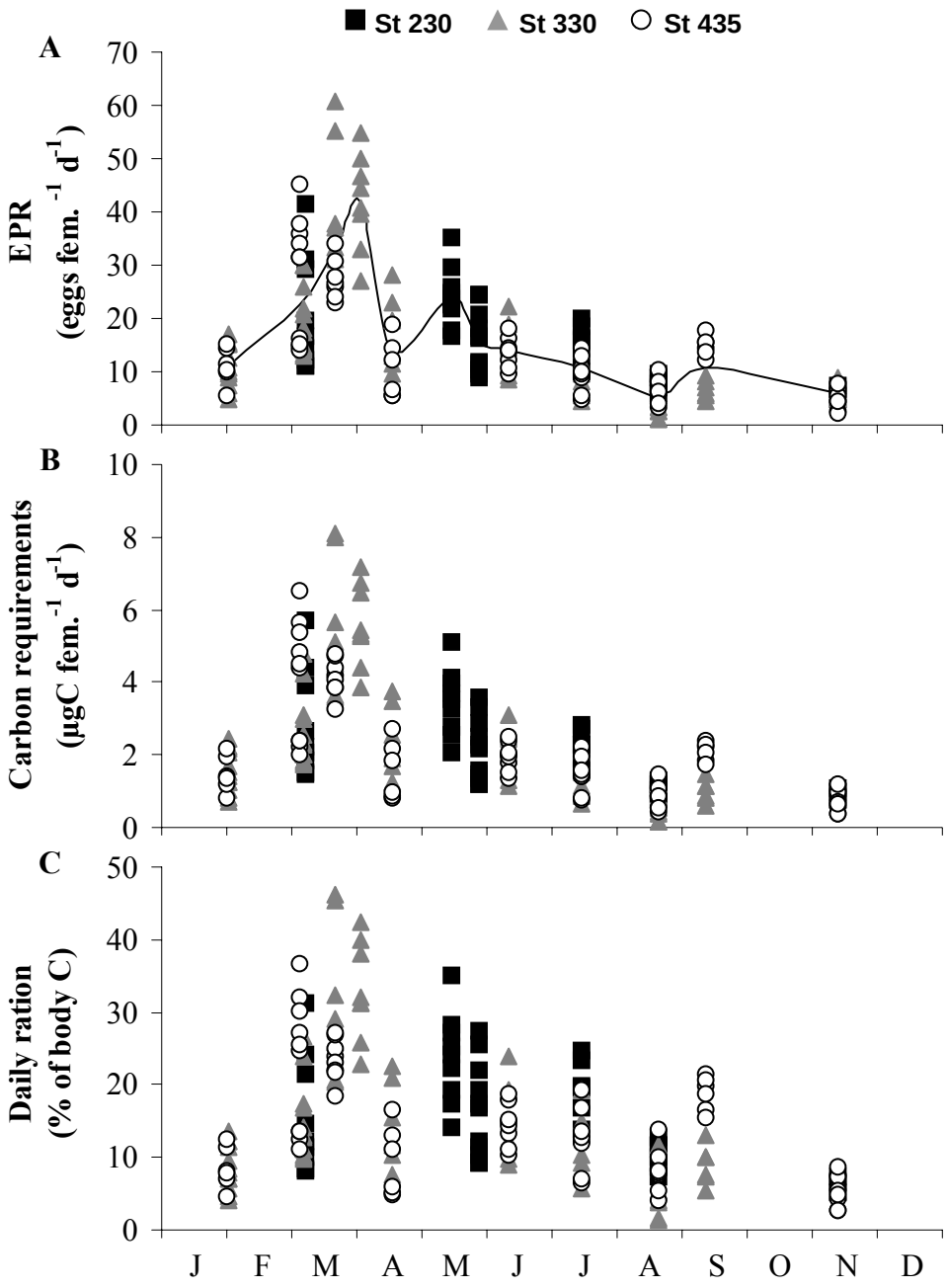

Figure IV-8: *Temora longicornis* females. Egg production rates (A), and carbon requirement for egg production expressed in µgC fem^{-1} d^{-1} (B), and as percentage of the body carbon weight (C) at three stations of the Belgian coastal zone during 2001. In (A) the line represents smoothed mean.

As for phytoplankton ingestion, egg production rates were positively related with small diatom abundance (r_s = 0.71, n = 25, $p < 0.001$). In order to estimate whether herbivorous feeding was sufficient to maintain the observed egg production we compared phytoplankton ingestion estimated from chl *a* gut contents to the estimated total ingestion needed for the egg production rates measured (see method). In winter herbivorous feeding contributed on average 65 % of the observed egg production, whereas, during the spring

bloom and summer the contribution was < 30 %. In fall, however, measured phytoplankton ingestion was sufficient to cover egg production carbon requirements (Table IV-2).

Table IV-2: *Temora longicornis*. Egg production rate (EPR) with standard error of the mean (SE), carbon requirements for egg production (see method for explanation), phytoplankton ingestion and percentage of the carbon requirements covered by phytoplankton ingestion in Belgian coastal waters in 2001.

	EPR (SE) (eggs fem.$^{-1}$ d^{-1})	C needs for EPR (µgC fem.$^{-1}$ d^{-1})	Herbivory (µgC ind.$^{-1}$ d^{-1})	% EPR covered by herbivory
Winter	18.3 (1.4)	2.71	1.77	65
Spring	23.1 (1.5)	3.22	0.92	29
Summer	8.8 (0.6)	1.02	0.24	23
Fall	5.8 (0.5)	0.82	0.83	100

4. Discussion

4.1 Microplankton availability and herbivorous diet of Temora longicornis

The observed phytoplankton seasonal pattern and species composition was typical of the non-stratified Belgian coastal waters as recorded at station 330 since 1988 (Lancelot et al. 1998, Rousseau et al. 2002). Diatoms were the main phytoplankton component during the sampling period and constituted, with the colonial haptophyte *Phaeocystis globosa,* the bulk of phytoplankton biomass during the spring bloom. The initiation of spring succession by a moderate bloom of small chain-forming diatoms, dominated in March by *Chaetoceros socialis*, was followed by a massive bloom of *P. globosa* colonies in April-May. Another notable event in spring was a short increase of nanoplankton-sized flagellate forms (mainly cryptomonads and prasinophytes) from the end of May to June. This change in the composition of the phytoplankton population was also accompanied by a change in cell size of the diatom population. The diatom assemblage co-occurring with *P. globosa* was indeed dominated by larger diatoms such as *Guinardia delicatula* and *Rhizosolenia imbricata*, which remained present throughout summer. Finally, dinoflagellates and ciliates appeared to be more abundant from April to May.

Temora longicornis gut contents and phytoplankton ingestion rates measured during this study reflect changes in phytoplankton abundance and taxonomic composition. As expected from other studies (Daro 1985, Hansen and van Boekel 1991, Bautista et al. 1992, Breton et al. 1999, Gasparini et al. 2000), gut fluorescence and ingestion rates were much lower during the *P. globosa* bloom than during the pre-bloom of diatoms in early spring. The

predominance of fucoxanthin in copepod guts suggests that *T. longicornis* was feeding principally on golden-brown algae. Further, fucoxanthin was the major accessory pigment found in seawater samples, in agreement with the dominance of golden-brown algae represented overall by diatoms and *P. globosa*. The pigment composition of a *P. globosa* strain isolated from Belgian coastal waters in April 2001 revealed that fucoxanthin was the main carotenoid pigment of that species, and that its fucoxanthin to chl-*a* ratio was comparable to values reported for diatoms (Antajan et al. 2004). Therefore, in samples in which both diatoms and *P. globosa* were abundant fucoxanthin did not enable distinguishingment between them. However, chlorophyll-c_3, the second major pigment of *P. globosa* in our area (Antajan et al. 2004), was highly correlated with *P. globosa* abundance. Although other algal species may contain this pigment (e.g. other haptophytes and some diatoms) it seems reasonable in our case to use chlorophyll-c_3 as biomarker of that alga, at least during *P. globosa* dominance (see discussion in Antajan et al. 2004). As chlorophyll-c_3 was never detected in copepod guts and chloropigment gut contents decreased during *P. globosa* bloom, most of the fucoxanthin found in copepod guts probably originated from diatom ingestion instead of *P. globosa* ingestion. This argument is also reinforced by the positive relationship found between phytoplankton ingestion rates and diatom abundance. Further, our results showed higher phytoplankton ingestion when small chain-forming diatom species such as *Thalassiosira* spp. and *Chaetoceros* spp. were abundant (February-March, and May at station 230), than when larger species such as *Guinardia* spp. and *Rhizosolenia* spp. dominate the diatom community in summer.

The use of HPLC in gut pigment analysis allowed the detection of significant amounts of alloxanthin, a specific Cryptophyceae carotenoid pigment (Gieskes and Kraay 1983, Breton et al. 2000), in *T. longicornis*. A gut evacuation experiment performed in June 2001 has demonstrated that alloxanthin was not present in the gut but in the body tissues of *T. longicornis* and remained detectable after several hours of starvation (Antajan and Gasparini 2004). Then, alloxanthin contents in *T. longicornis* can not be used as an index of recent feeding on Cryptophyceae.

Contrary to a preliminary study which had detected trace amount of peridinin in *T. longicornis* gut contents (Gasparini et al. 2000) we never found this pigment in any of our gut content analysis. Peridinin, which is an unequivocal marker for autotrophic Dinophyceae (Mackey et al. 1996, Jeffrey and Vesk 1997, Breton et al 2000), was only detected from August to November in seawater samples. Interestingly, maximum dinoflagellates abundance occurred in April and May, yet peridinin was not detected in any seawater samples from those

months. Recently, heterotrophic dinoflagellates were shown to account for 77 % of the total microzooplankton standing stock in the coastal waters of the southern North Sea in spring (Stelfox-Widdicombe et al. 2004). Then, the absence of peridinin, even in samples in which dinoflagellates were detected by microscopy, precluded the evaluation of the contribution of dinoflagellates in copepod diet based on the presence of this pigment in copepod gut.

The importance of the small green flagellates in oceanic phytoplankton is now well established from pigment analysis through the presence of chlorophyll-*b* (Jeffrey 1976, Rodriguez et al. 2002) In spite of the fact that chlorophyll-*b* could not be quantified in this study its relative abundance (chromatographic peak area) was significantly correlated with Prasinophyceae abundance (mainly *Pyramimonas* spp.), representing up to 40 % of the nanoflagellate abundance in June. 19'-hexanoyloxyfucoxanthin is an accessory pigment found in many Haptophyceae (Jeffrey and Wright 1994, Zapata et al. 2004). Apart from *P. globosa*, which does not contain this pigment in our study area (Antajan et al. 1994), light-microscopic counts did not allow the identification of any other haptophyte species. However, previous studies have shown than other haptophytes species are common in Belgian coastal waters (M'harzi et al. 1998, Antajan et al. 2004). Neither chlorophyll-*b* nor 19'-hexanoyloxyfucoxanthin have been detected in gut contents of *T. longicornis*, indicating that these algae did not contribute greatly to their diet.

4.2. The contribution of herbivory to the diet of Temora longicornis

Many authors have already pointed out that heterotrophic dinoflagellates and ciliates may be an important food source for *T. longicornis* (Turner 1984, Kleppel et al. 1988, Gasparini et al. 2000, Vincent and Hartmann 2001). Astaxanthin is an animal pigment that occurs in crustaceans (Castillo et al. 1982) and other microzooplankton (Kleppel and Lessard 1992). It could potentially reflect microzooplankton ingestion (Kleppel et al. 1988), but quantification of astaxanthin from *T. longicornis* chromatograms was difficult because the majority of the pigment was esterified and eluted over a broad range of retention times (Fig 4D). Furthermore, this pigment was also detected in starved *T. longicornis* (Antajan and Gasparini 2004) and the astaxanthin content in the body tissues has been shown to vary on a time scales of hours to a day (Juhl et al. 1996). These findings make it difficult to separate the tissue astaxanthin content of *T. longicornis* from the gut astaxanthin content and preclude the use of this pigment as biomarker for predation on heterotrophic protists (Juhl et al. 1996).

In this study, we compared phytoplankton carbon ingestion to egg production carbon requirements, in an attempt to evaluate the importance of autotrophic versus heterotrophic feeding to sustain *T. longicornis* egg production rates throughout the season. We showed that *T. longicornis* can be regarded as mainly herbivorous during fall and winter, whereas during spring and summer they needed non-phytoplankton food to meet their energetic demands for egg production. A weakness of our approach is a possible degradation of chloropigments during the gut passage leading to an underestimation of herbivory. Many authors have reported highly variable percentages of pigment destruction during copepod digestion, with values ranging from 0 to 95 % (see review in Pasternak 1994). Large losses of pigment have been attributed to experimental artefacts and pigment losses in the range of 10 to 30 % seem to be a more realistic process (Kiørboe and Tiselius 1987, Dam and Peterson 1988, Pasternak 1994, Båmstedt et al. 2000). If we admit pigment losses of 30 % in the guts of *T. longicornis*, the herbivory contribution to the diet would have been of 86 % in winter, 37 % in spring, 30% in summer and 130 % in fall. In any case, this correction does not affect the observed shift from a herbivorous to a more omnivorous feeding behaviour at the beginning of the growth season. Another possible source of underestimation of the degree of *T. longicornis* herbivory in this study is the fact that gut contents were examined for animals caught in daytime. Day-night differences could have been a source of error for phytoplankton ingestion calculation if diel feeding rhythms occurred. In the southern North Sea, diel feeding rhythms were not always observed for *T. longicornis* and seem to vary seasonally (Daro 1985, Hansen and van Boeckel 1991). A preliminary study conducted on the dominant copepod species in Belgian coastal waters has shown good agreement of the gut fluorescence method we used in the present study with phytoplankton ingestion rates obtained from the disappearance of cells in bottle incubations and from the radiotracer method (Gasparini et al. 2000). As gut fluorescence and phytoplankton ingestion rates measured in our study were within the range reported in the literature for *T. longicornis* (Dam and Peterson 1991, Bautista et al. 1992, Hansen and van Boeckel 1991, Breton et al. 1999, Gasparini et al. 2000), we considered that the gut fluorescence method was a valuable proxy of phytoplankton ingestion for studying seasonal differences.

The phytoplankton spring bloom, either during diatom dominance or during *P. globosa* dominance, did not enhance the contribution of herbivory to the diet. Additionally, the high egg production rates observed in early spring corresponded to a low herbivory contribution. One hypothesis that could explain these observations is that when *T. longicornis* carbon requirements for egg production increase, the contribution of non-phytoplankton food

sources to total ingestion becomes more important. In order to verify this hypothesis, we compared the contribution of herbivory to the diet with the carbon requirements for egg production (Fig. 9). We observed that above a certain level of carbon requirements (ca. 1.7 µgC fem.$^{-1}$ d^{-1}) phytoplankton ingestion was never sufficient to cover egg production needs, whereas below this limit herbivory may have been sufficient in some cases.

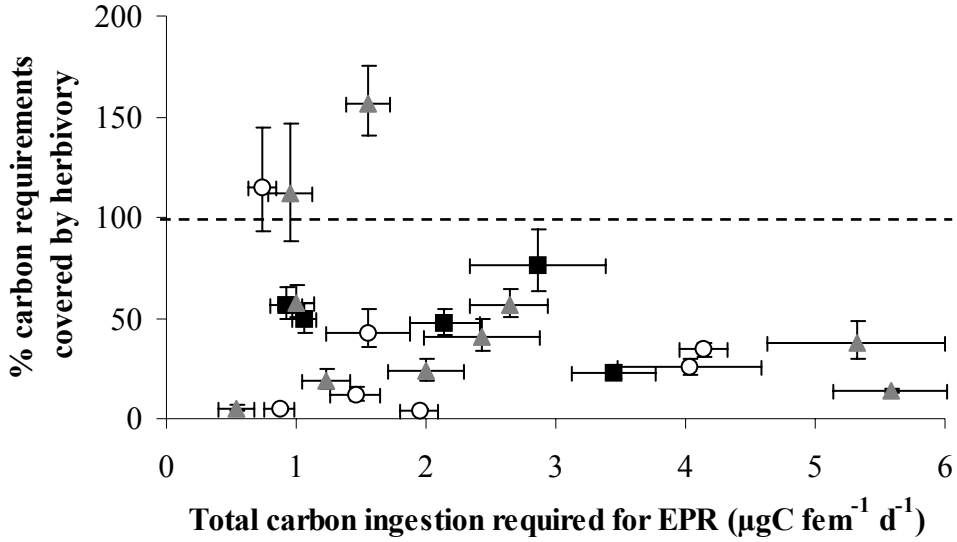

Figure 9: *Temora longicornis*. Relationship between the percentage of the carbon requirements covered by phytoplankton carbon ingestion and the total carbon ingestion required to sustain the egg production rates (EPR) measured (mean ± SE). The hatched line represents the level where 100 % of carbon needs were covered by herbivory.

If such relationship between carbon requirements for egg production and degree of herbivory in diet is confirmed for other copepod species in other areas this would help in understanding and predicting the complex link existing between copepods and their potential prey, phytoplankton and microzooplankton.

4.3. Is food availability limiting Temora longicornis *egg production?*

The final goal of our study was to examine the degree of food-limitation of *T. longicornis* in Belgian coastal waters. It has been demonstrated that egg production of *T. longicornis* reflects short-term feeding history (Peterson 1985, Peterson and Bellantoni 1987, Peterson et al. 1991). Therefore, lower egg production rates in comparison with maximum egg production observed under optimal feeding conditions, at similar temperature, would indicate food limitation. The highest egg production rates observed during this study in early spring were similar to maximum rates reported for *T. longicornis* during diatom spring blooms (40 to 65 eggs female^{-1} d^{-1}; Peterson and Kimmerer 1994, Kiørboe and Nielson 1994,

Peterson and Dam 1996, Halsband and Hirche 2001). During the rest of the year, including the peak of *P. globosa* bloom in April, the production potential of this species was generally not achieved. This suggests that *T. longicornis* egg production was limited not only by food quantity but also by food quality. Some studies have already shown that *P. globosa* has insufficient content of specific compounds which are essential for copepod egg production such as polyunsaturated fatty acids (Claustre et al. 1990, Cotonnec et al 2001, Tang et al. 2001), vitamin C (Claustre et al. 1990) and nitrogen (Bautista et al. 1994). The fact that egg production was below the potential maximum during the major part of the year suggests that ingestion of non-phytoplankton food sources did not allow the females to compensate for food limitation. This latter assumption is in agreement with recent studies showing that mixed-food diet and addition of ciliates to diatoms in the food offered to *T. longicornis* did not enhance egg production rate (Dam and Lopez 2003) and may even limit secondary production during periods of low algal abundance (Klein Breteler et al. 2004).

On the other hand, maximum egg production rates are usually observed from March to May even in very contrasting environmental feeding conditions (Peterson 1985, Kiørboe and Nielsen 1994, Peterson and Kimmerer 1994, Peterson and Dam 1996, Halsband and Hirche 2001, this study). Therefore, we can not exclude that the season itself is an important factor in *T. longicornis* egg production dynamics and this latter assumption would need further investigation.

Acknowledgements

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Diet composition and feeding selectivity of *Temora longicornis* and *Centropages hamatus* in Belgian coastal waters

1. Introduction

Traditionally, copepods have been considered to be the main path of transfer of organic matter from primary producers to higher trophic levels. However, it has become apparent that the linear food chain concept of diatoms – copepods – fish needs to be expanded to include the microbial community (see Kleppel 1993 and references therein). Copepod diet tends to be diverse, reflecting the variety of phytoplankton and microzooplankton taxa present in the environment. In the previous chapter we have shown non-autotrophic food sources to be important contributors to the diet of *Temora longicornis* allowing them to sustain their reproduction rate. A second aspect of interest in the copepod's response to a food environment composed of many kinds of particles deals with selective feeding behaviour. Calanoid copepods are known to be capable of selective feeding i.e. they can actively search for, capture and choose to ingest or reject potential food particles (e.g., Nejstgaard et al. 1997, Meyer-Harms et al. 1999, Tackx et al 2003, Rollwagen Bollens and Penry 2003, Broglio et al. 2004). A growing number of field investigations support the revised concept of copepods as omnivores, often actively selecting ciliates and dinoflagellates, both key agents in the functioning of microbial food web (Sherr et al. 1986, Sherr and Sherr 1988), faster than diatoms of equivalent size (Tiselius 1989, Fessenden and Cowles 1994, Atkinson 1996, Vincent and Hartmann 2001, Zeldis et al. 2002, Broglio et al. 2004). In spite of the relevance of considering feeding selectivity in predicting the impact of copepods on the different components of the natural communities, this factor in not taken into account in the actual version of the biogeochemical model MIRO (Lancelot et al. 2004), which synthesised current knowledge on the kinetics and the factors controlling the main auto- and heterotrophic processes in Belgian coastal waters. Few field data exist, however, on the actual process and degree of selective feeding by copepods in this coastal ecosystem (Daro 1985, Gasparini et al. 2000). Therefore, our major objective in this chapter was to examine the selective feeding behaviour of two major copepod species in Belgian coastal zone, *T. longicornis* and *Centropages hamatus*, over a wide range of prey conditions. We conducted incubation experiments designed to estimate diet composition, prey selectivity and feeding rates upon natural prey assemblage.

2. Materials and methods

Copepods and prey for the incubation experiments were collected from stations 230, 330 and 435, locations regularly visited by the Belgian monitoring network. The characteristics of these stations are given in chapter I (see Table I-1 and Fig. I-2).

2.1. Copepod feeding experiments

We conducted 56 sets of experiments, 40 with *T. longicornis* and 16 with *C. hamatus*, between 1999 and 2001, mainly during the growth season. Copepods for grazing and predation incubation experiments were collected using a 200 µm mesh WP-2 plankton net which was towed obliquely between surface and bottom (tow speed 0.5 m s^{-1}; duration < 5 min). For each experimental treatment, 30 adult copepods of the same species were sorted from the diluted cod end content, and transferred into 500 ml of unscreened natural seawater. This was collected from subsurface, middle and near the bottom of the water column with a 10 l-Niskin bottle. The water collected from the three depths was then gently mixed and used to measure clearance rates and prey preference upon natural prey assemblage. Vertical profiles obtained by the CTD indicated that the water column was well mixed and allow us to assume that the natural plankton assemblage was not disturbed by the mixing process. Incubations started within one hour of zooplankton collection and only undamaged individuals with normal swimming behaviour were selected. We immediately fixed three replicates of seawater (initial condition) in 1 % glutaraldehyde (final concentration), while three replicates without copepods (control treatments) and two to four replicates with copepods (experimental treatments) were incubated for 24 hours. All treatments were incubated in on-deck incubators with flowing sea water at sea-surface temperature which creates a gentle movement to prevent sedimentation in the bottles. At the end of the incubation the replicates were fixed with 1 % glutaraldehyde.

2.2. Cell counts

After pre-concentration following the method of Colijn et al. (1990), samples from the feeding experiments were analysed using an inverted microscope, provided with phase contrast (ZEISS Sedival), in 5 ml sedimentation chambers for phytoplankton and microzooplankton enumeration (Gasparini et al. 2000, Antajan and Gasparini 2004). Depending on cell density, random fields (60-100) or selected transects of the entire

sedimentation chamber were counted for microplankton (×200) and small flagellates (×400). The identified cells were grouped into one of the following major prey categories: small and large diatoms, *P. globosa*, nanoflagellates, small and large dinoflagellates, small and large ciliates. The 18 µm threshold was used to distinguish between small and large prey categories, rather than the typical size boundary of 20 µm between nanoplankton and microplankton, based on the size distribution of diatoms in the natural assemblage (Philippart et al. 2000). Only diatoms were identified to species level. As abundance of each species was measured as individual cell counts, diatom species with individual cell size < 18 µm were included in the small diatom category even though some diatom genera (such as *Chaetoceros* and *Asterionellopsis*) often form chains that could be long enough to be perceived as microplankton by grazer. The carbon content of diatoms was calculated on the basis of cell biometry and density determined for each species. A specific average biovolume of a cell population was measured throughout the period of its development (Rousseau et al. 2002, and pers. comm.). After application of the correction equation for fixation-induced volume change (Menden-Deuer et al. 2001), the biovolumes were converted into carbon biomass using the size-dependent density relationship recommended for diatoms by Menden-Deuer and Lessard (2000). Dinoflagellate, nanoflagellate and ciliate biomass were calculated using carbon to volume relationships recommended by Menden-Deuer and Lessard (2000). Colonial *Phaeocystis* cells were free in our samples due to the dissolution of the colony matrix by the preserving agent. A conversion factor of 14.2 pgC $cell^{-1}$ for colonial cells and of 10.8 pgC $cell^{-1}$ for flagellated cells (microzoospores) was used to estimate *Phaeocystis* carbon biomass as recommended by Rousseau et al. (1990).

2.3. Feeding rates and selectivity

Clearance rates (ml $copepod^{-1}$ h^{-1}) of *T. longicornis* and *C. hamatus* were calculated using the equations of Frost (1972), for those species for which a significant difference in concentration between control and experimental bottles was found at the end of the incubation (t-test, $p < 0.05$). However, due to high variability within replicates, on several occasions, differences between control and grazing bottles were not significant, although computed clearance rates were in general positive and high. Conservatively, we opted to include these clearance values in the work, although pointing out when the estimate had a significant statistical value. When computing clearance rates, negative values were levelled off to zero. For bottles with copepods added, the grazing rate, g (h^{-1}) was calculated as:

$$g = \frac{\ln C_1 - \ln C_1^*}{t}$$

where C_1 and C_1^* are the prey concentration in the control and in the experimental treatments, respectively, at the end of the incubation, and *t* is the duration of the incubation. Results from three replicate bottles in each set of experiment were averaged. The clearance rate, *F* (ml copepod^{-1} h^{-1}), defined by Frost (1972) as 'the volume swept clear' or 'the volume of ambient medium from which cells are completely removed by copepods' per unit time, was calculated by the following equation:

$$F = \frac{V \cdot g}{N}$$

where *V* (ml) is the volume of the bottle and *N* is the number of copepods in the bottle. Ingestion rates (*I*) during the incubations were calculated according to Marin et al (1986) as the product of clearance rate (*F*) of a prey category and its initial concentration (C_0) in the incubation water:

$$I = FC_0$$

Selective feeding behaviour of *T. longicornis* and *C. hamatus* during the investigation period was characterised by comparing the distribution of prey types in the copepod diet with their distribution in the available medium in each experiment using the chi-square (χ^2) goodness-of-fit (Kleppel et al. 1996):

$$\chi^2 = \sum_{i=1}^{k} \frac{(f_i - \hat{f}_i)^2}{\hat{f}_i}$$

Here, f_i is the distribution of prey i observed in copepod diet, $\hat{f}_i$ is the distribution of the prey i expected if the null hypothesis is true (i.e. the frequency of the prey in available medium), and the summation is performed over all k categories of prey available. Significant ($p < 0.05$) differences between these prey distributions were interpreted as selective feeding behaviour by the copepod predators (i.e. that certain prey are ingested in a higher or lower proportion than expected from their relative abundance in the environmental medium).

Prey preferences among the different prey categories were evaluated in two different ways. First, a simple visual way to approach selection is the one by plotting the prey contribution to the copepod diet (r_i) against its availability (n_i) (Broglio et al. 2004):

$$r_i = \frac{R_i}{\sum_{i=1}^{m} R_i} \quad \text{and} \quad n_i = \frac{N_0}{\sum_{i=1}^{m} N_0}$$

where m is the number of prey categories, and R_i is the number of individuals of each prey category consumed by the copepods in each experiment and determined as follows:

$$R_i = \frac{(N_0 - N_1)}{2} - N_1^*$$

where N_0 and N_1 where the mean number of the prey i in the control treatments at the beginning and at the end of the incubation, respectively, and N_1^* was the mean number of the prey i in the experimental bottles (with added copepods) at the end of the incubation. In this kind of plot, values that fall above the line 1:1 are indicative of prey preference, whereas values falling below the line represent deterrence for the specific prey. Secondly, we determined prey preference among the different prey categories by using the Vanderploeg and Scavia's (1979 a,b) electivity index (E^*):

$$E_i^* = \frac{W_i - (\frac{1}{m})}{W_i + (\frac{1}{m})}$$

where W_i is defined by the following equation:

$$W_i = \frac{\frac{r_i}{n_i}}{\sum_{i=1}^{m} \frac{r_i}{n_i}}$$

where m, r_i and n_i are as described above. Neutral preference was indicated by an E^* of 0, with positive values up to +1 representing increasing preference and negative values down to -1 representing increasing avoidance. Of the several electivity indices described in the literature, in particular Chesson's α (Chesson 1983) and Ivlev's E (Ivlev 1961), E^* is the most appropriate for this sort of feeding experiment (Rollwagen Bollens and Penry 2003). As reviewed by Lechowicz (1982) and Confer and Moore (1987), E^* is the only index sufficiently stable where both the number of prey types and proportion varies between experiments.

3. Results

Patterns of change in abundance and taxonomic composition of the protist community in Belgian coastal waters have already been presented and discussed in detail in chapter one, and we will here focused on copepod diet composition and selection among the available prey categories.

3.1. Diet composition

Figure V-1 shows the ingestion rates of *Temora longicornis* and *Centropages hamatus* for the different prey categories during the course of the year. Total carbon ingestion rate range from 0.3 to 190 ngC ind.$^{-1}$ h^{-1} for *T. longicornis* and from 1 to 104 ngC ind.$^{-1}$ h^{-1} for *C. hamatus*. The contribution of the different prey categories to the total carbon ingestion of copepods varied through the seasons (Table V-I). In winter diatoms represented 74 % of the total carbon ingestion of *T. longicornis*, whereas dinoflagellates and ciliates represented 7 % and 19 %, respectively. In spring dinoflagellates and ciliates contributed to 20 and 34 %, respectively, of the *T. longicornis*' diet and, 24 % and 18 %, respectively, of the *C. hamatus*' diet. In summer diatoms represented more than 80 % of *T. longicornis*' diet, whereas *C. hamatus* ingested mainly diatoms (58 %) and dinoflagellates (36 %). In fall ingestions rates of *T. longicornis* were very low (< 2 ngC ind.$^{-1}$ h^{-1}) with dinoflagellates and ciliates representing 40 % and 30 % of the diet, respectively, whereas both diatoms and nanoflagellates represented ca. 16 % of their diet. *Phaeocystis globosa* never represented more than 3 % of the total carbon ingestion of both copepod species.

Table V-1: Relative contribution of each prey category to the total carbon ingestion of *Temora longicornis* and *Centropages hamatus* in Belgian coastal waters through the seasons.

	Winter	Spring		Summer		Fall
	Temora	*Temora*	*Centropages*	*Temora*	*Centropages*	*Temora*
Diatoms < 18 µm	21 %	15 %	10 %	4 %	0 %	14 %
Diatoms > 18 µm	53 %	27 %	42 %	78 %	58 %	2 %
Phaeocystis globosa	0 %	3 %	2 %	0 %	0 %	0 %
Nanoflagellates	0 %	0 %	4 %	3 %	3 %	15 %
Dinoflagellates < 18 µm	1 %	7 %	10 %	6 %	34 %	10 %
Dinoflagellates > 18 µm	6 %	13 %	14 %	5 %	2 %	30 %
Ciliates < 18 µm	0 %	5 %	12 %	0 %	3 %	2 %
Ciliates > 18 µm	19 %	29 %	6 %	4 %	0 %	28 %

When comparing ingestion rates of both copepod species for experiments performed the same day at a same station, these were not significantly different (*t*-test, $p > 0.05$). In order to better understand the variability in the relative importance of the different ingestion rates for each prey category from one period to another, the ingestion rate values on a specific prey category were plotted against the available biomass of that prey category in the water (Fig. V-2). In such a graph, an increase of ingestion rate versus prey biomass is expected up to a satiety threshold at which maximum ingestion rate is reached. During our experiments, we did not observed satiety threshold for any prey of the two copepod species studied.

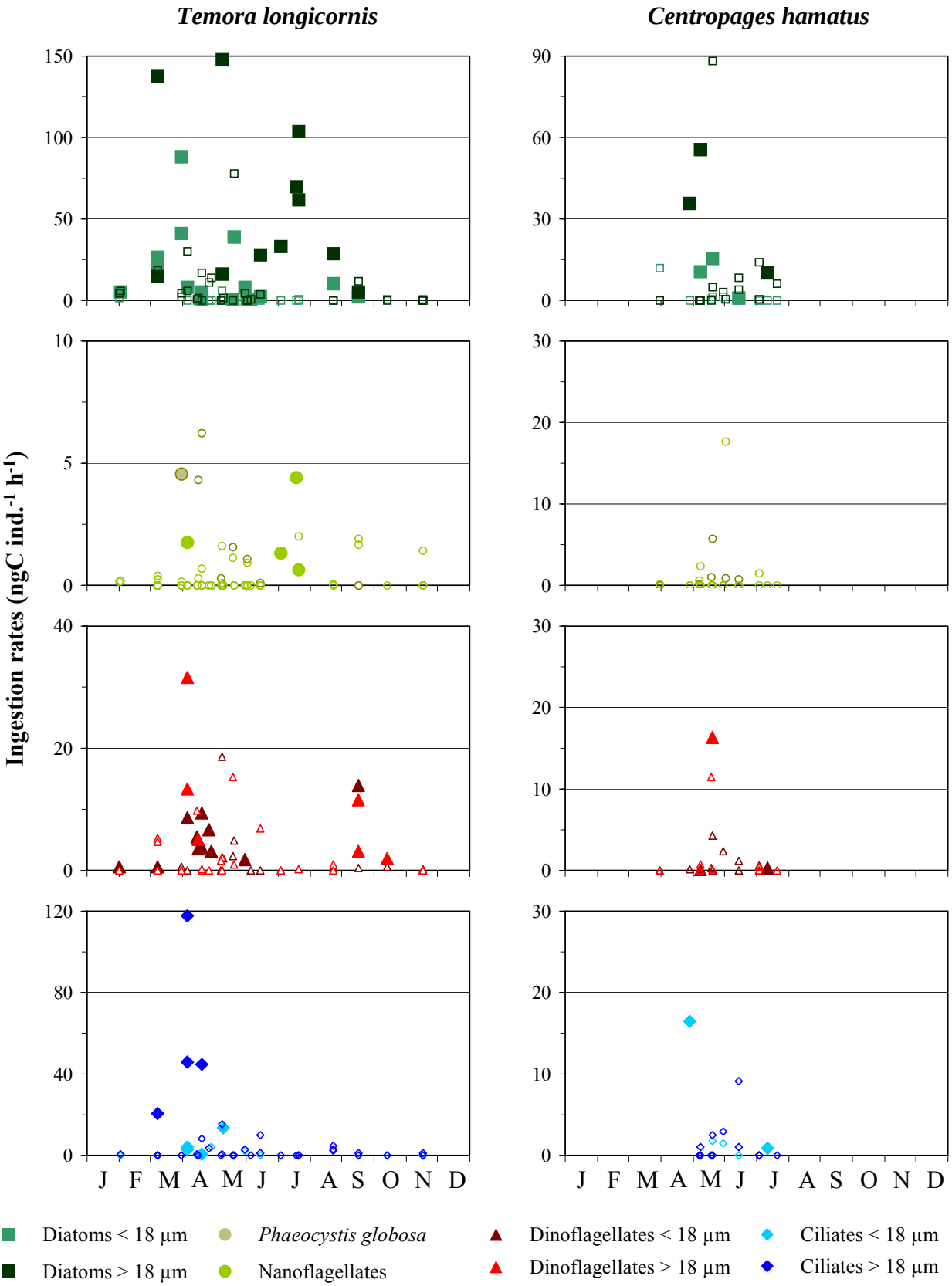

Figure V-1: *Temora longicornis* and *Centropages hamatus*. Ingestion rates in incubation experiments with unfiltered seawater from Belgian coastal waters between 1999 and 2001. Full symbols represent cases when concentration in experimental bottles was significantly lower ($p < 0.05$, *t*-test) than concentration in the control bottles at the end of the incubation.

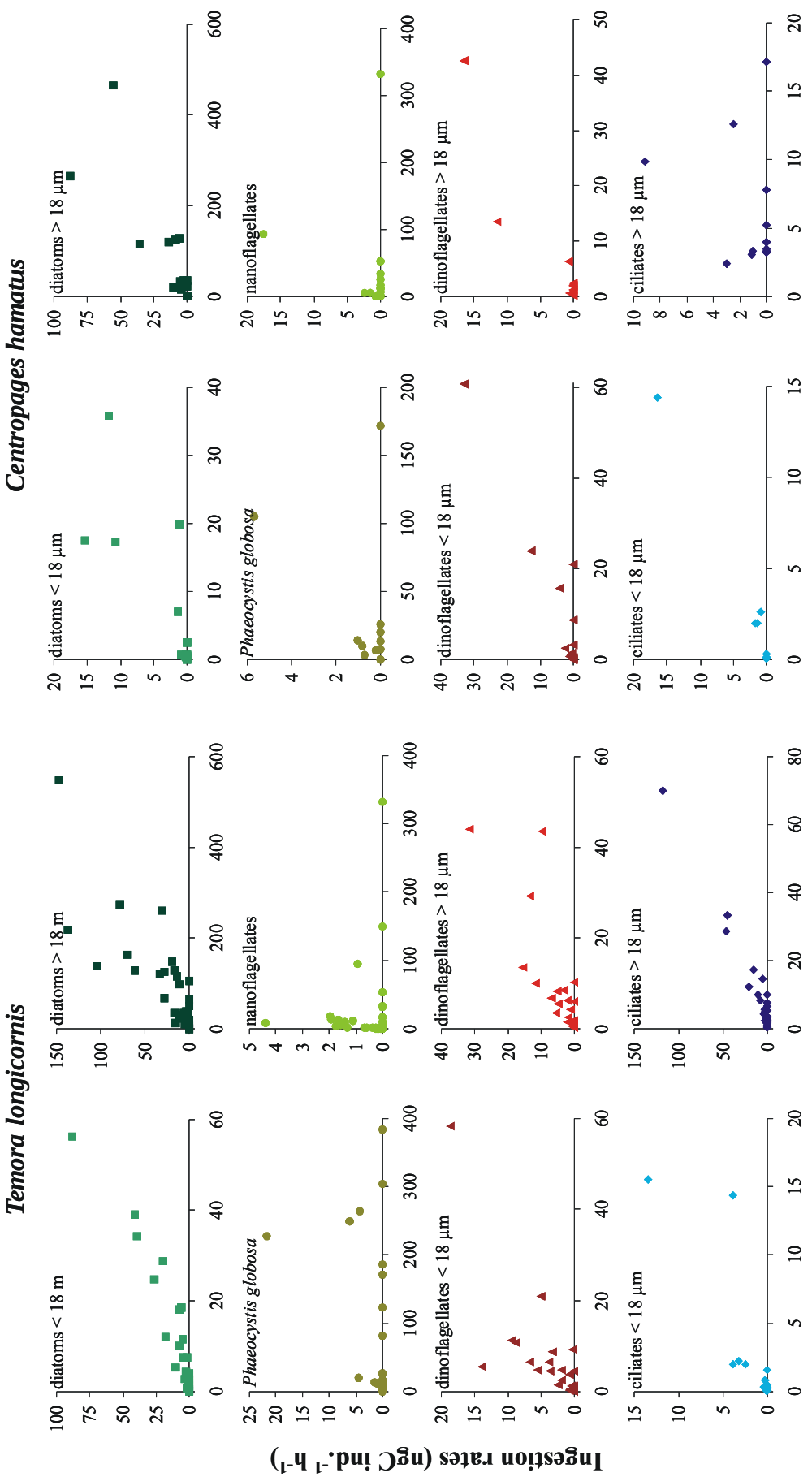

Figure V-2: Relations between the carbon ingestion rate of *Temora longicornis* and *Centropages hamatus* on a certain prey category and the carbon biomass of that prey category from 1999 to 2001 in Belgian coastal waters. (see Table V-2 for Spearman's rank correlation)

The total carbon ingestion according to the food biomass available *in situ* does not show any satiety threshold either (Fig. V-3). This indicates that maximum ingestion rate was not reached during our experiments and that *T. longicornis* and *C. hamatus* were both food-limited. Further, ingestion rates on some prey categories were not correlated with their availability in the water (Table V-2). No correlation was observed between ingestion rates and *P. globosa* or nanoflagellate biomass both for *T. longicornis* and *C. hamatus*, neither for large dinoflagellates and ciliates for *C. hamatus*.

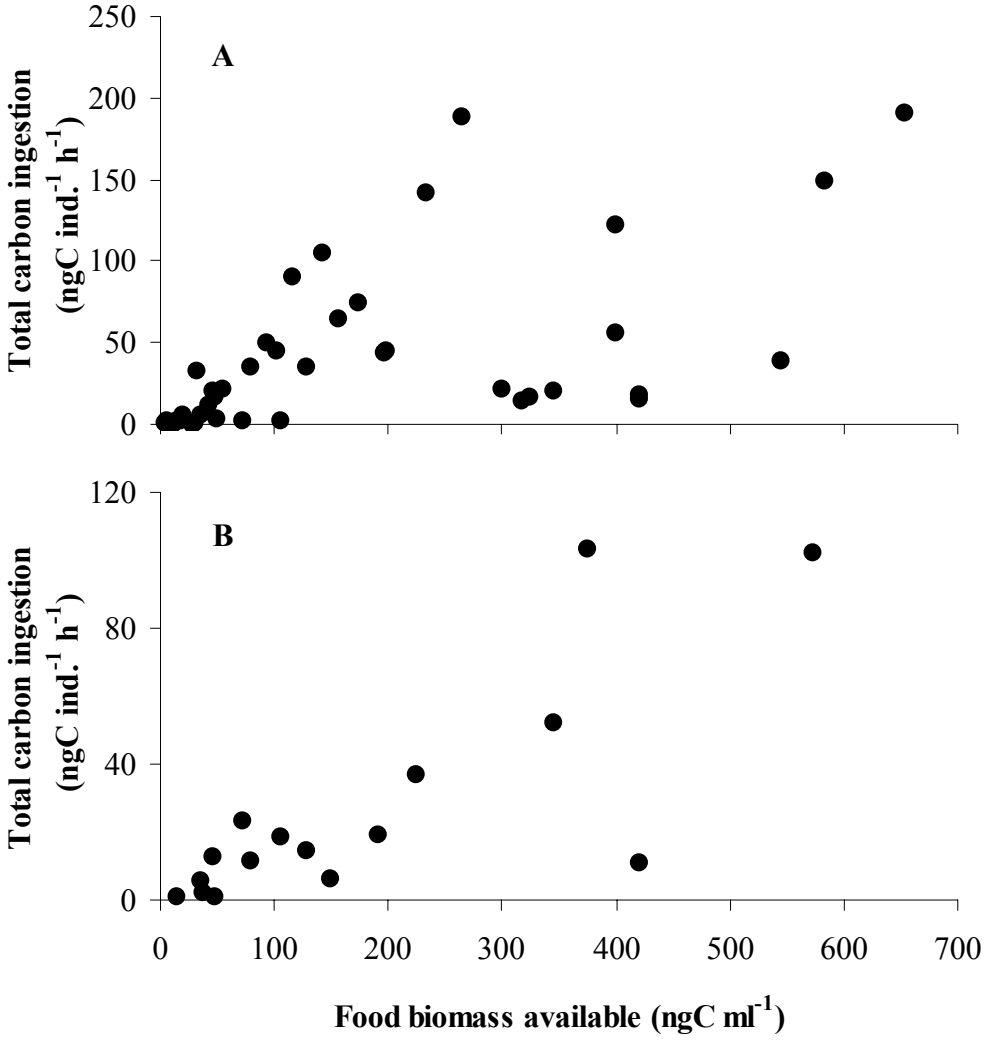

Figure V-3: (A) *Temora* longicornis and (B) *Centropages* hamatus. Relation between total carbon ingestion and food biomass available from 1999 to 2001 in Belgian coastal waters. (Spearman rank $r_s = 0.68$, $n = 40$, $p < 0.001$ and $r_s = 0.73$, $n = 16$, $p < 0.001$ for *T. longicornis* and *C. hamatus*, respectively).

Table V-2: Spearman correlation coefficient (R_s) between carbon ingestion rates of *Temora longicornis* and *Centropages hamatus*, and carbon biomass of available prey categories.

		Diatoms		*Phaeocystis globosa*	Nanoflagellates	Dinoflagellates		Ciliates	
		< 18 µm	> 18 µm			< 18 µm	> 18 µm	< 18 µm	> 18 µm
Temora longicornis	R_s	**0.73**	**0.77**	0.19	0.04	**0.75**	**0.79**	**0.68**	**0.72**
	n	40	39	26	39	29	23	16	23
	p	< 0.001	< 0.001	> 0.05	> 0.05	< 0.001	< 0.001	< 0.01	< 0.001
Centropages hamatus	R_s	**0.69**	**0.7**	0.02	-0.37	**0.58**	0.67	0.75	-0.19
	n	15	16	11	16	13	7	6	9
	p	< 0.01	< 0.01	> 0.05	> 0.05	< 0.05	> 0.05	> 0.05	> 0.05

3.2. Selection for major prey categories

Throughout the year, *Temora longicornis* and *Centropages hamatus* demonstrated a strongly selective feeding behaviour as measured by highly significant (χ^2, $p < 0.001$) differences in the distributions of prey items in their diet compared to the distributions of prey available in the feeding medium in all experiments (Fig. V-4). The selective feeding behaviour of *T. longicornis* showed clearly a seasonal pattern with highest selective feeding (highest χ^2) in spring and fall, whereas in winter and summer the selectivity was lower. The chi-square was positively related to the concentrations of *Phaeocystis globosa* (Spearman's rank $R_s = 0.69$, $n = 26$, $p < 0.001$) and dinoflagellates < 18 µm ($R_s = 0.53$, $n = 33$, $p < 0.01$) in the water (Fig. V-5).

It is difficult to conclude about a possible seasonal selective feeding behaviour of *C. hamatus* since this species was present in sufficient abundance to performe incubation experiments only from April to July. Further, selectivity of *C. hamatus* was not related with any of the prey categories we looked at.

Figures V-6 and V-7 show the prey contribution to *T. longicornis* and *C. hamatus* diet, respectively, as function of its relative availability in Belgian coastal waters. Overall, both copepod species showed positive feeding selection for dinoflagellates and ciliates (i.e. most values are above the 1:1 line). For diatoms the responses were very scattered. The selection for diatoms < 18 µm was either positive, negative or neutral whereas, when diatoms > 18 µm represented more than 10 % of the total prey abundance in the water, they were consumed in proportion of their availability in the environmental medium. Significant decrease in cell concentration between control and experimental (with copepods) bottles were rarely observed for *P. globosa* (only once in 38 experiments) and nanoflagellates (4 times

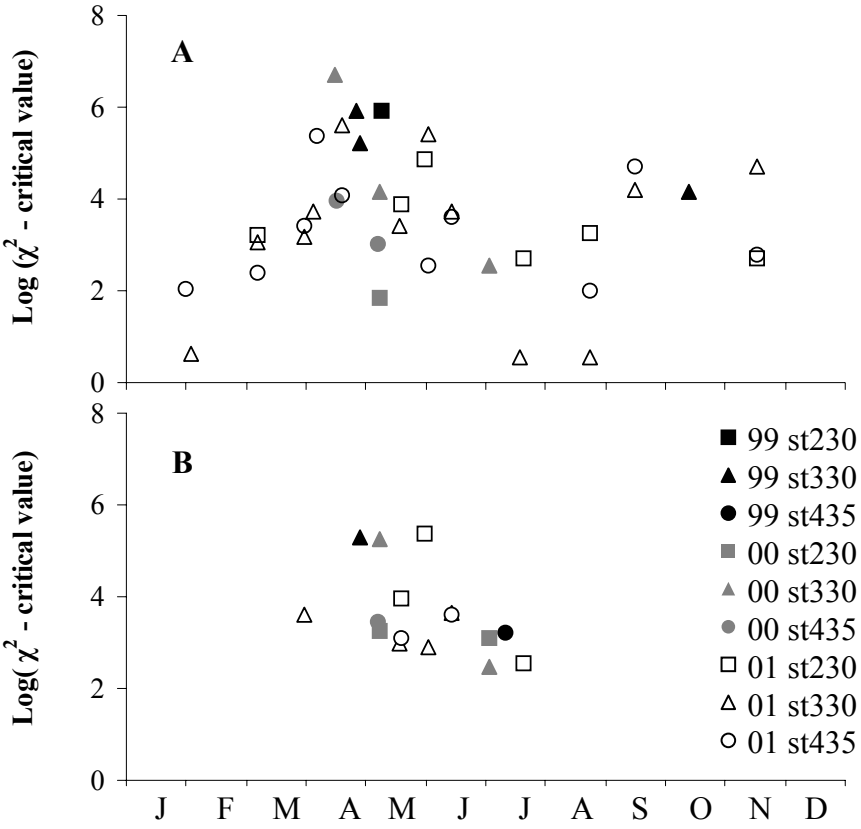

Figure V-4: (A) *Temora longicornis* and (B) *Centropages hamatus*. The critical values ($\chi^2_{0.001,(k-1)}$) for *k* number of prey categories was subtracted to the calculated chi-square (χ^2) goodness-of-fit between the distribution of prey categories in the diet and in the Belgian coastal waters from 1999 to 2001. The larger the chi-square the smaller the probability that H_0 (the distribution of prey categories in the diet is similar to their distribution in the water) is true.

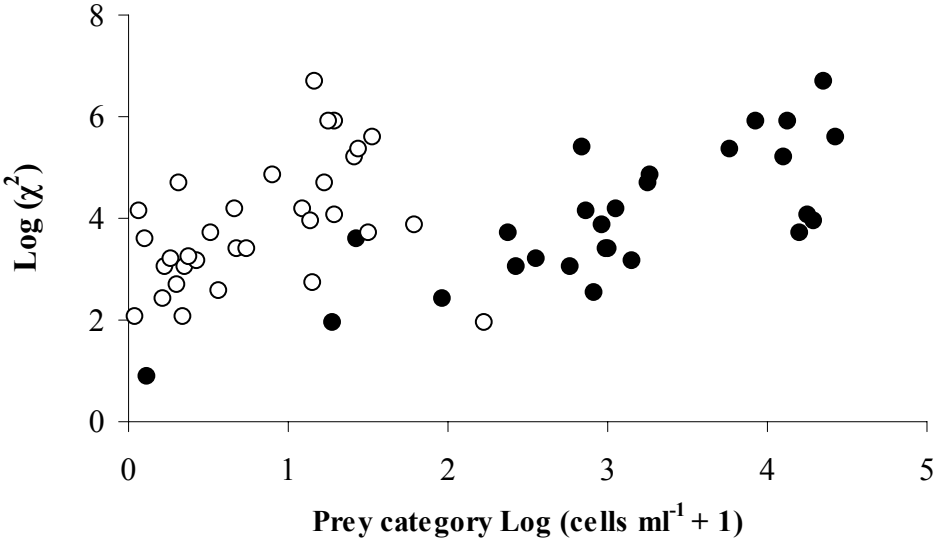

Figure V-5: *Temora longicornis*. Relation between chi-square (χ^2) value and (●)*Phaeocystis globosa* and (○) dinoflagellates <18 µm abundance in Belgian coastal waters from 1999 to 2001.

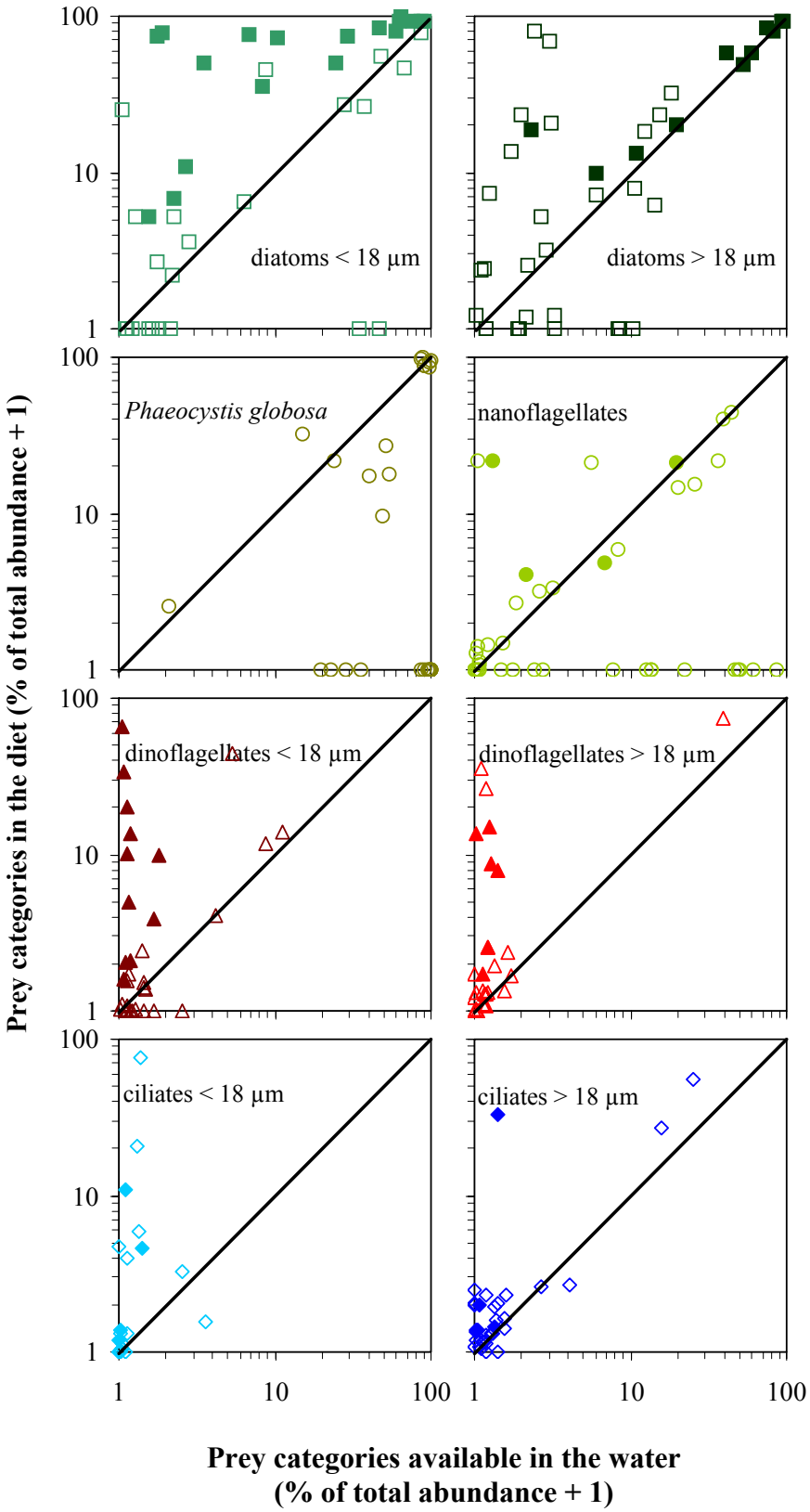

Figure V-6: *Temora longicornis*. Relative presence of a prey category in the diet as function of its relative abundance in Belgian coastal waters from 1999 to 2001. Data above the 1:1 line indicate positive feeding selection for that prey category. Full symbols represent cases when concentration in experimental bottles was significantly lower ($p < 0.05$, *t*-test) than concentration in the control bottles at the end of the incubation.

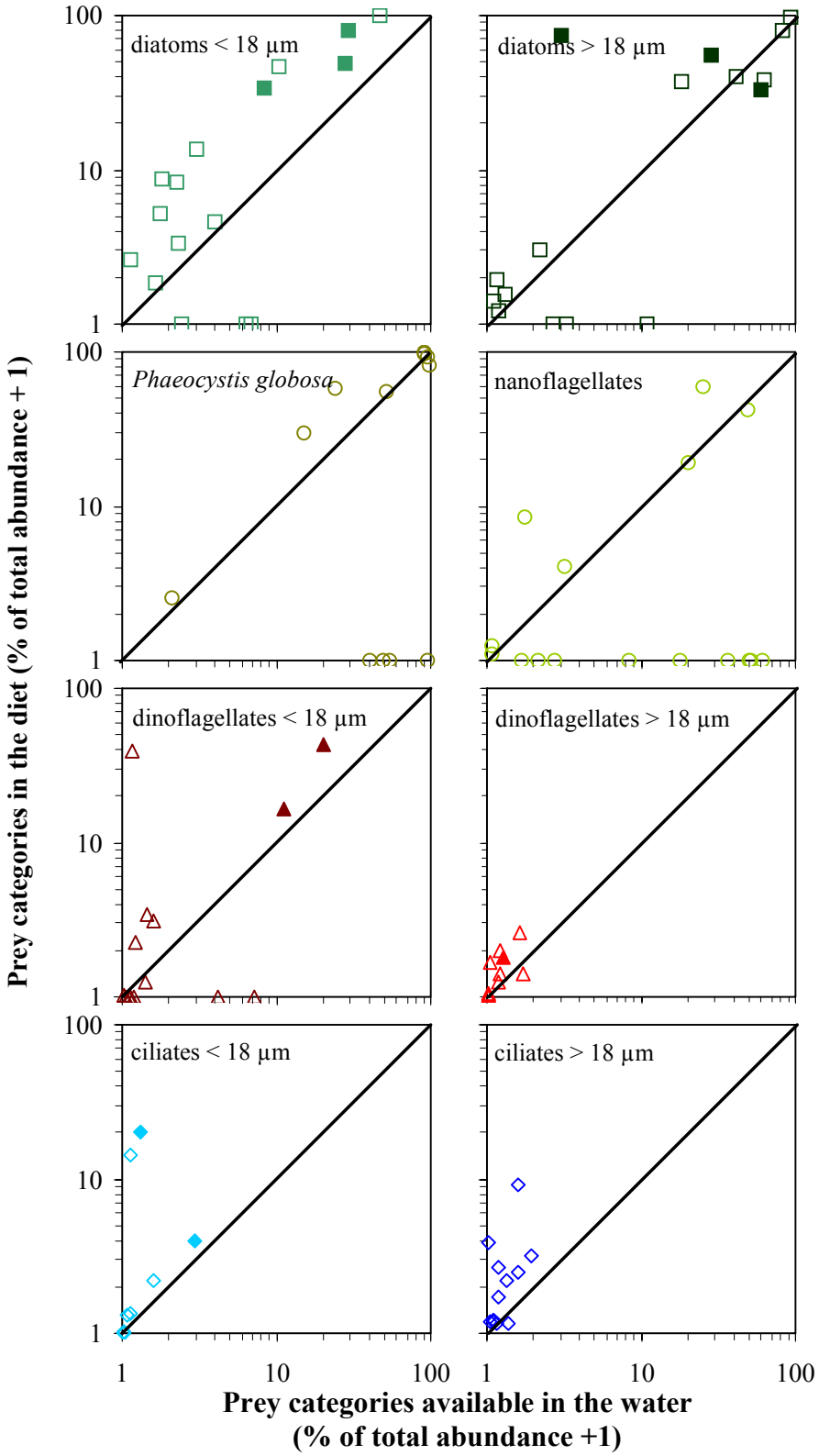

Figure V-7: *Centropages hamatus*. Relative presence of a prey category in the diet as function of its relative abundance in Belgian coastal waters from 1999 to 2001. Data above the 1:1 line indicate positive feeding selection for that prey category. Full symbols represent cases when concentration in experimental bottles was significantly lower ($p < 0.05$, *t*-test) than concentration in the control bottles at the end of the incubation.

in 55 experiments), whereas no disappearance of prey (i.e. avoidance) was observed in more than half of the experiments where these prey categories were present. When a significant decrease of abundance was found in the experimental bottles, *P. globosa* was consumed at a lower rate than expected from its relative abundance (i.e. negative selection) and nanoflagellates were consumed in proportion of their availability. Although the ciliate concentrations were lower in the copepod bottles in most cases (80 % for the small and 90 % for the large ciliates), only a few cases were statistically significant from the control bottles (Fig. V-8). Ciliates often accounted for < 1% of the available prey and variability in the obtained cell counts was large between replicates, which could explain our difficulties to observe significant differences between control and experimental bottles.

3.3. Selectivity patterns

One must be aware, however, that selection, either negative or positive for a prey category may depend not only on its proportion in the water but also on its absolute availability, which could partially explain the variability in response within and between prey species. Copepods may shift from a preferred prey item to another if this last one becomes much more abundant. To have an idea of changes in prey selection patterns of *T. longicornis* and *C. hamatus* as function of prey availability changes through the seasons, figures V-8 and V-9 present the prey categories' availability in combination with electivity index (E^*; Vanderploeg and Scavia 1979a,b) and clearance rates.

Diatoms were the main prey available through the seasons, with large diatoms biomass being 10 fold higher than that of diatoms < 18 µm. Both diatom-size categories were significantly ingested, with clearance rates on small diatoms being generally higher than those on large diatoms (median: 0.44 and 0.20 ml ind.$^{-1}$ h^{-1}, respectively, for *T. longicornis* and 0.33 and 0.12 ml ind.$^{-1}$ h^{-1}, respectively, for *C. hamatus*). However electivity values were very variable through the seasons and independent of diatom availability. Clearance rates on *P. globosa* were always very low and all but one not significantly different from zero (*t*-test, $p > 0.05$). However, in 6 sets of experiments with *T. longicornis*, the concentration of *P. globosa* in control bottles was significantly lower than in the experimental ones, suggesting trophic cascade effects masking possible grazing by copepods on *P. globosa*. Nanoflagellates were usually selected against by the two copepod species and no changes in selectivity pattern (from a negative to a positive selection) were observed during high abundance of this prey category occurring after the *Phaeocystis* bloom. As explained in the first chapter, the huge

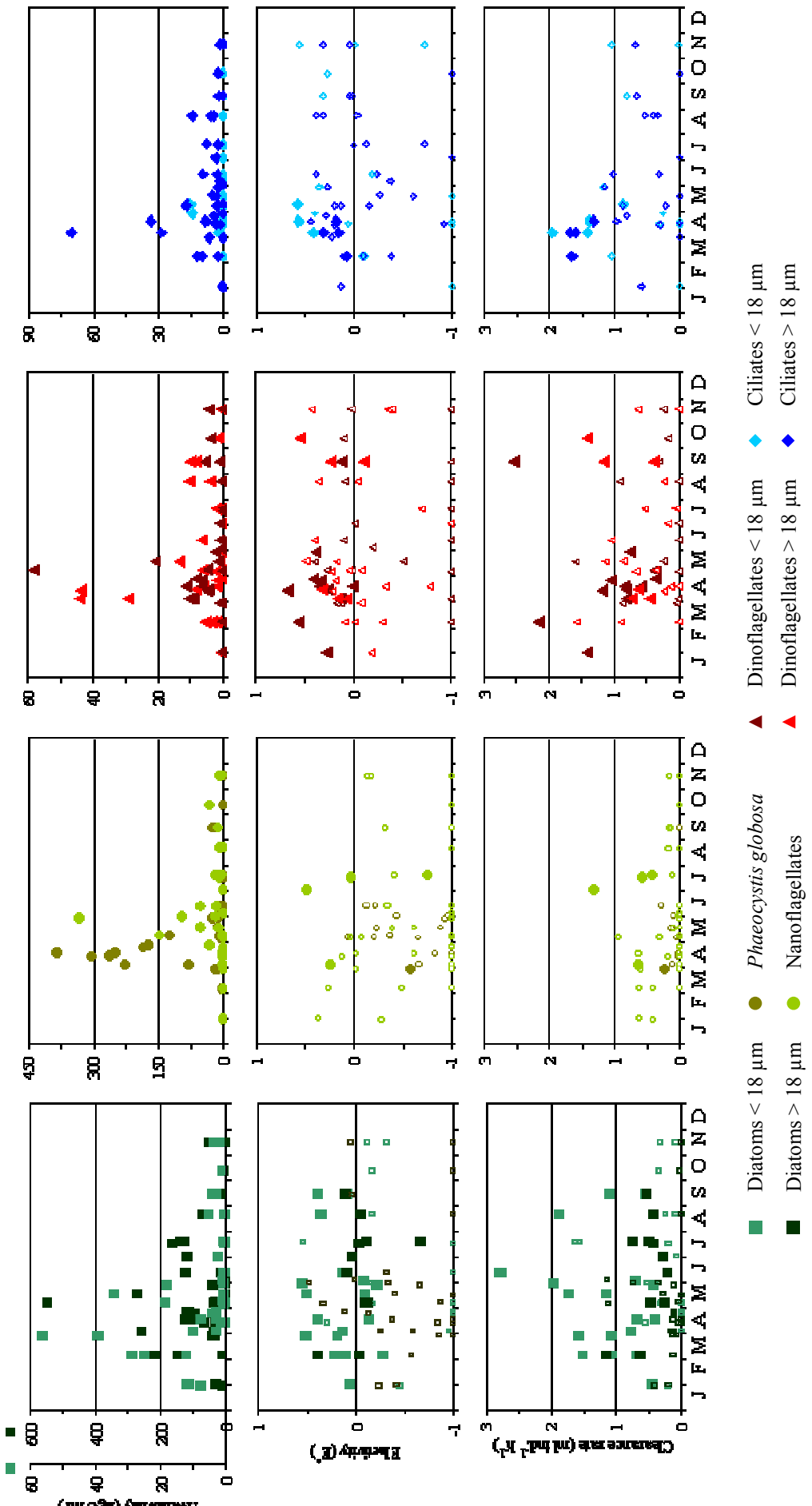

Figure V-8: *Temora longicornis*. Prey categories availability, electivity and clearance rates in incubation experiments with unfiltered seawater from Belgian coastal waters between 1999 and 2001. Full symbols represent cases when concentration in experimental bottles was significantly lower ($p < 0.05$, *t*-test) than concentration in the control bottles at the end of the incubation.

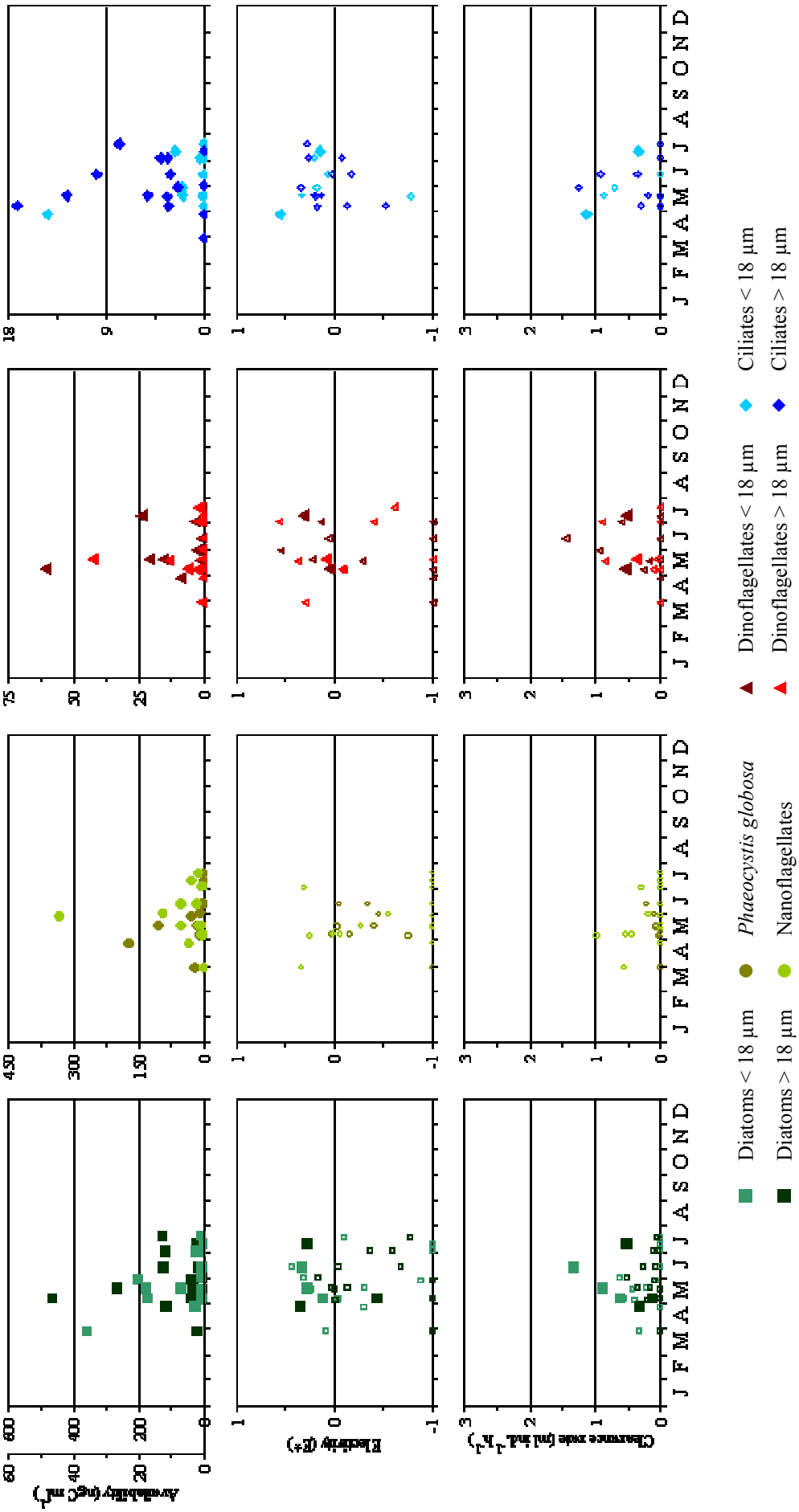

Figure V-9: *Centropages hamatus*. Prey categories availability, electivity and clearance in incubation experiments with unfiltered seawater from Belgian coastal waters between 1999 and 2001. Full symbols represent cases when concentration in experimental bottles was significantly lower ($p < 0.05$, *t*-test) than concentration in the control bottles at the end of the incubation.

increase in *P. globosa* biomass in spring was accompanied by an increase in dinoflagellate and ciliate biomass, whereas diatoms, especially the small ones, decreased. Although dinoflagellates and ciliates were in low relative biomass during the *P. globosa* blooms, *T. longicornis* demonstrated a clear preference for these prey categories (whatever their size), as measured by high electivity indices as well as high clearance rates (Fig. V-10). *Centropages hamatus,* on the contrary, did not show any shift or preference in prey selection during the spring period dominated by *P. globosa*. Apart from the one during the *Phaeocystis* bloom, we did not observe any other shift in prey selection related to the presence of a particular prey category.

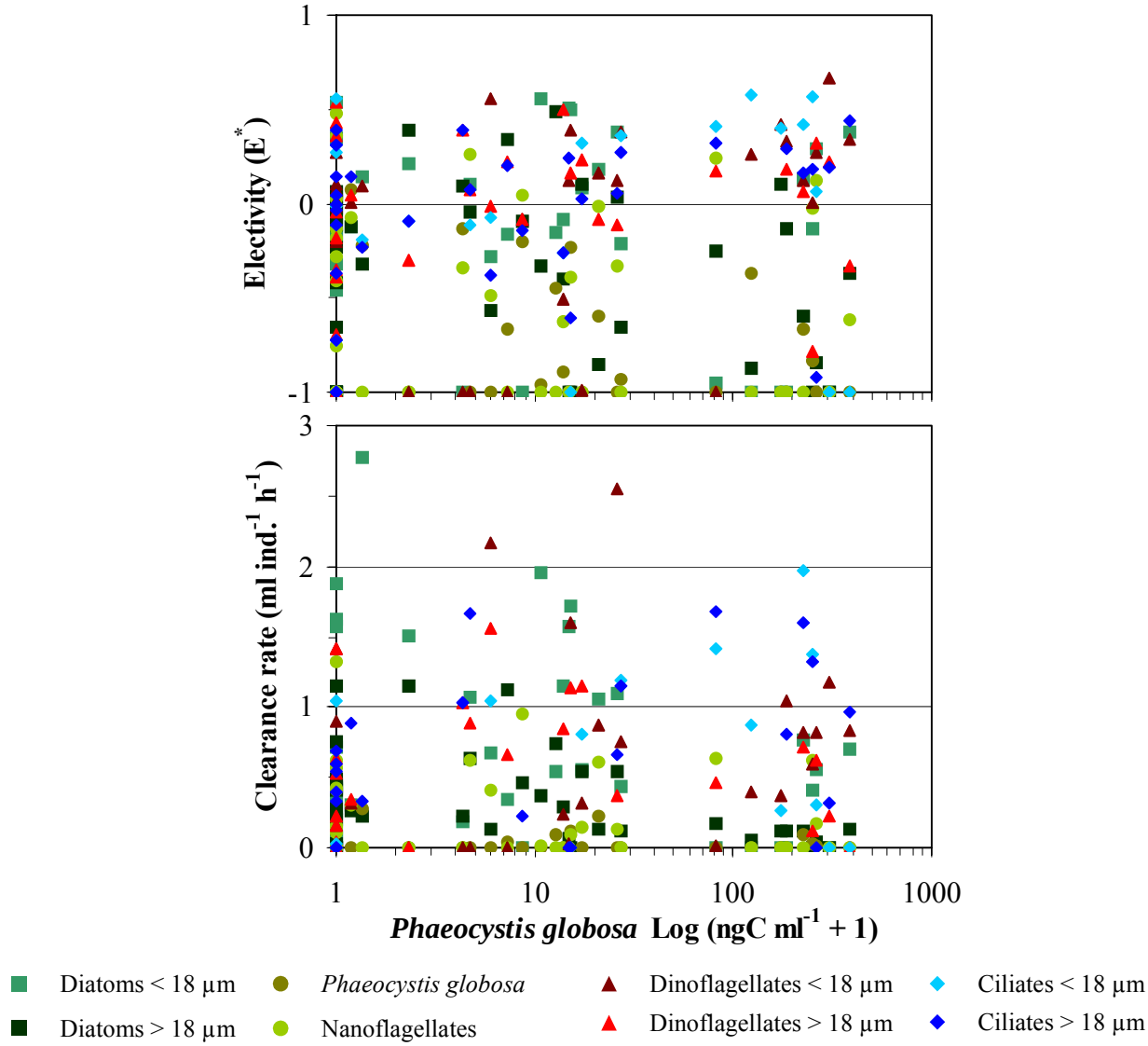

Figure V-10: *Temora longicornis*. Electivity and clearance rates for the different prey categories versus *Phaeocystis globosa* biomass in Belgian coastal waters from 1999 to 2001.

3.4 Preference among prey categories

Electivity indices and clearance rates of both copepod species were very variable through the seasons and no clear pattern of prey preference could be seen on a seasonal base. In order to determine, however, what were the preferred food sources over all the feeding experiments performed, the frequency distributions of the electivity index of *T. longicornis* (Fig. V-11) and *C. hamatus* (Fig. V-12) for each prey category were compared. These figures confirm the generally low preference for *P. globosa* and nanoflagellates as food source for both copepod species studied. Frequency distribution of electivity index indicates avoidance of *P. globosa* in 96 % and 92 % of experiments where this prey was available for *T. longicornis* and *C hamatus*, respectively. Nanoflagellates were selected against in 75 % and 82 % of cases for *T. longicornis* and *C. hamatus*, respectively. On the contrary, some prey categories were regularly preferred, such as the small dinoflagellates and large ciliates by *T. longicornis* (positive selection in 60 % and 57 % of cases, respectively), and both size-categories of ciliates by *C. hamatus* (89 % and 67 % for small and large ciliates, respectively). Electivity index (either negative or positive) on small and large diatoms was very variable and most values were comprised between -0.25 and +0.25, which seems to indicate a neutral preference for these prey categories.

To resume the diverse distributions observed and to determine an order of preference among the available prey, we calculated the mode and the median for each prey category based on the electivity values grouped in classes. As expected from our observations, *P. globosa* and nanoflagellates were the less preferred food over all the experiment performed both for *T. longicornis* and *C. hamatus*, whereas ciliates < 18 μm were their preferred food. Both copepod species presented the same order of preference among the available prey category, except for the small dinoflagellates which were more often avoided by *C. hamatus* than by *T. longicornis*.

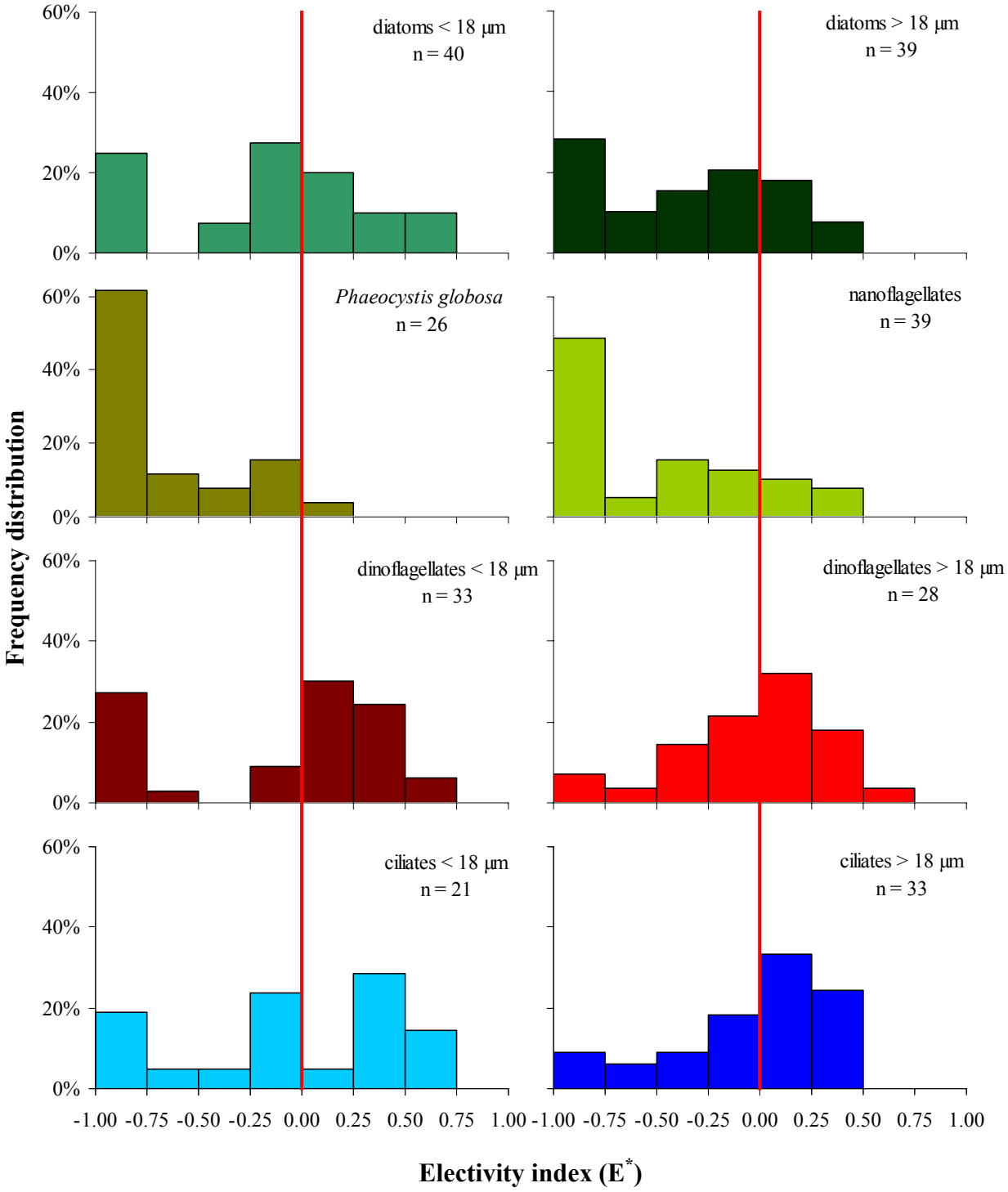

Figure V-11: *Temora longicornis*. Frequency distribution of electivity index (E^*) for each prey category. The number, *n*, of experiment where the prey category was available is indicated.

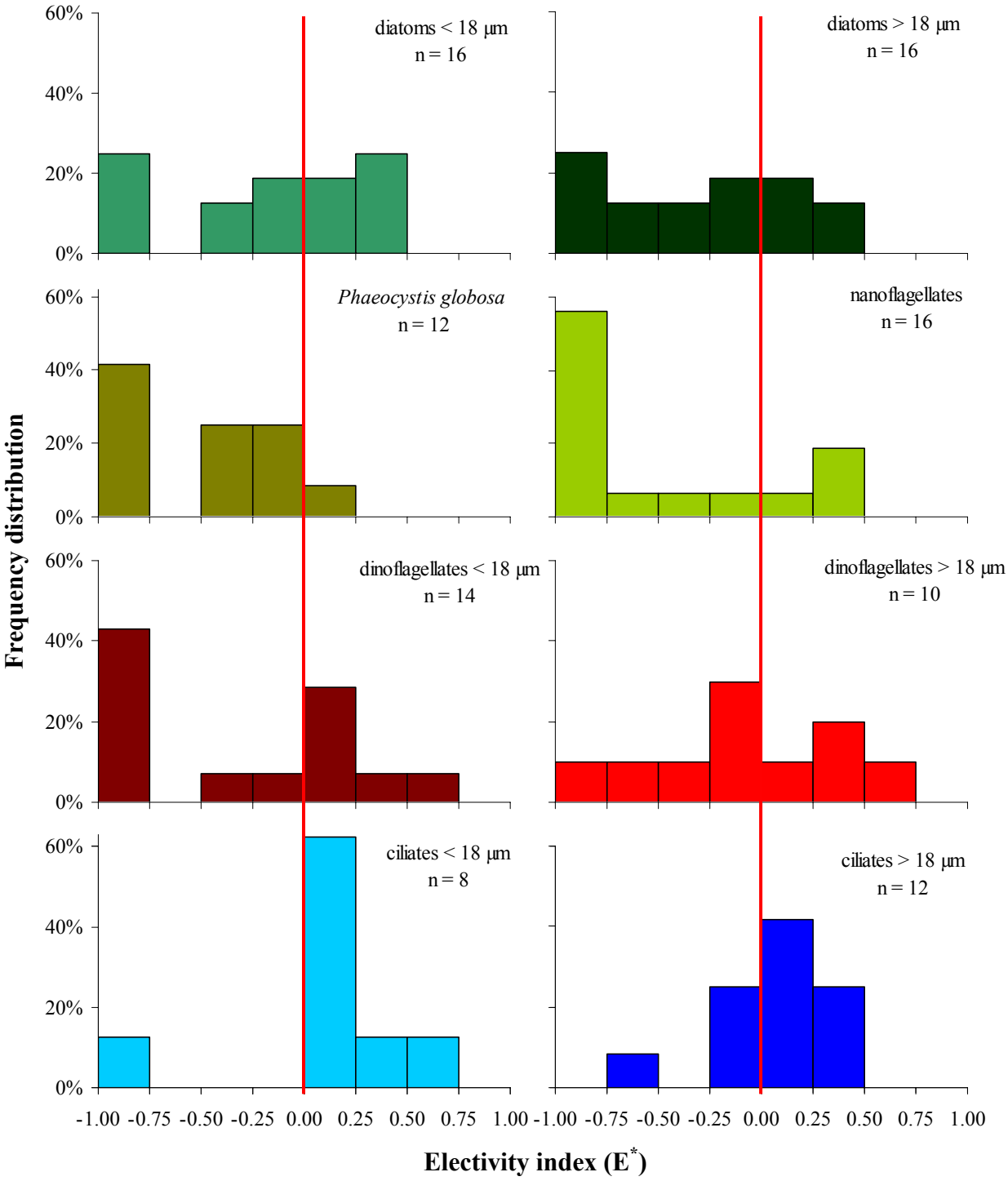

Figure V-12: *Centropages hamatus*. Frequency distribution of electivity index (E^*) for each prey category. The number, *n*, of experiment where the prey category was available is indicated.

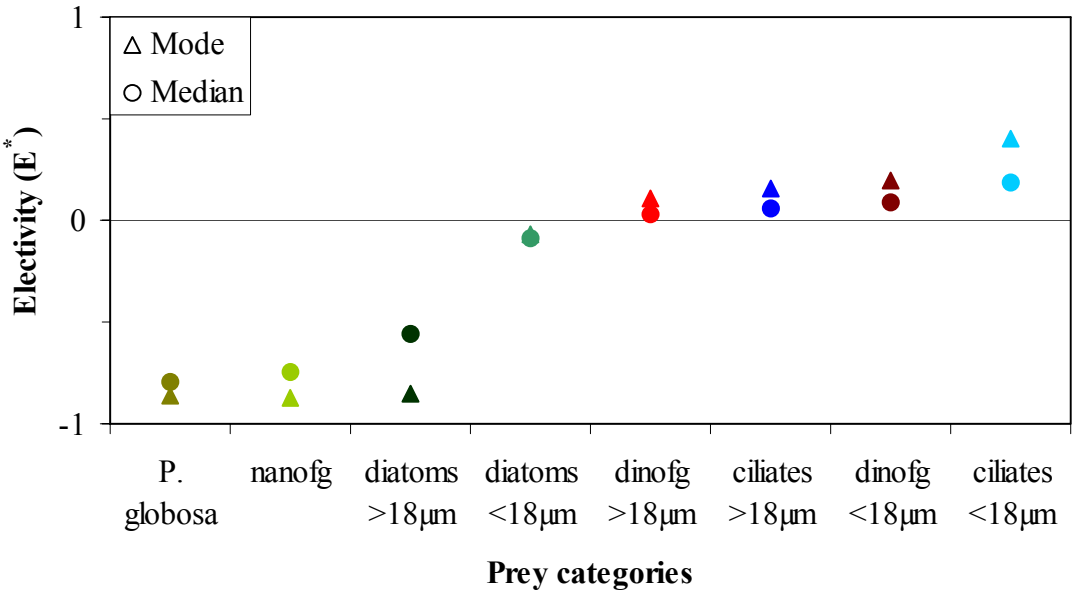

Figure V-13: *Temora longicornis*. Mode and median of electivity index for each category of prey over all the incubations performed in Belgian coastal waters from 1999 to 2001.

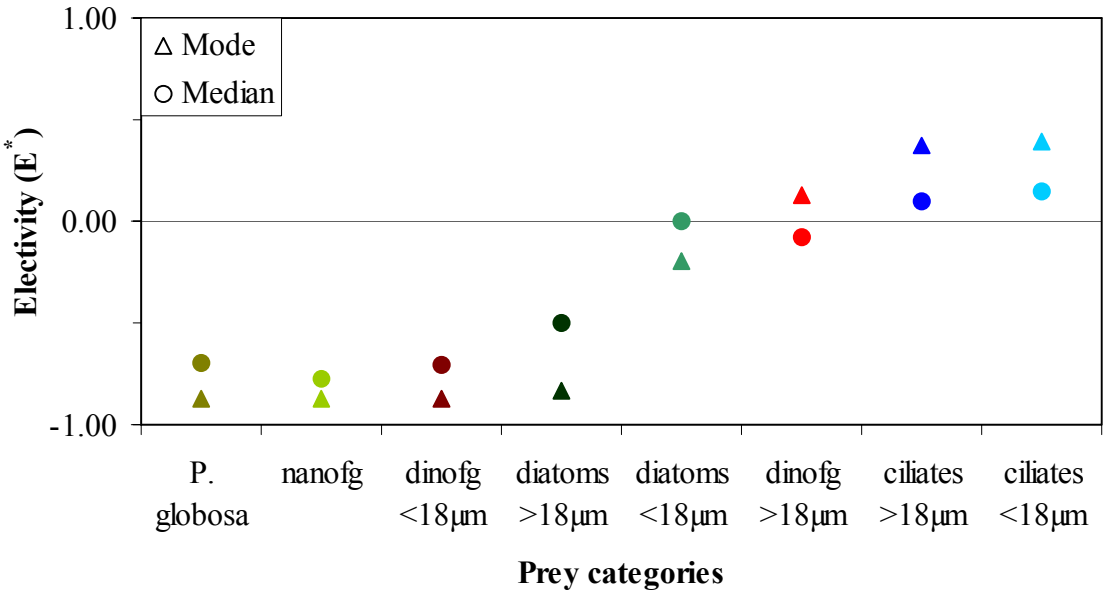

Figure V-14: *Centropages hamatus*. Mode and median of electivity index for each category of prey over all the incubations performed in Belgian coastal waters from 1999 to 2001.

4. Discussion

4.1. Ingestion rates and dietary diversity

The estimates of total carbon ingestion were very variable in this study and ranged from 0.01 to 4.54 µgC ind.$^{-1}$ d^{-1} for *T. longicornis* and < 0.01 to 2.50 µgC ind.$^{-1}$ d^{-1} for *C. hamatus*. Ingestion rates of *T. longicornis* were similar to previous data in Belgian coastal zone ranging from 1.70 to 4.24 µgC ind.$^{-1}$ d^{-1} and based on the ^{14}C tracer method (Daro 1985). Our values were also consistent with *in situ* values ranging from 0.15 to 4.81µgC ind.$^{-1}$ d^{-1} reported for *T. longicornis* feeding on natural population in the Bay of Biscay from April to May (Bonnet 2001). A few *in situ* measurements of ingestion rates in *C. hamatus* have been reported in the literature. Nicolajsen et al. (1983) measured ingestion rates of *C. hamatus* feeding on natural phytoplanktonic patch to be ranged from 0 to 15.6 ng chl *a* ind.$^{-1}$ d^{-1} and Kiørboe et al. (1985) reported maximum ingestion rates of 18 ± 9 ng chl *a* ind.$^{-1}$ d^{-1}. Assuming a C/chl *a* ratio of 50 (Banse 1977), these values, estimated from gut pigment content, are equivalent to 0 - 0.78 and 0.9 ± 0.45µgC ind.$^{-1}$ d^{-1}, respectively, and lower than our results. On the other hand, the daily food rations calculated from our data (see chap IV for method explanation) ranged from 0.1 to 25 % and from < 0.01 and 22 % of the body carbon weight for *T. longicornis* and *C. hamatus*, respectively. These values are quite below satiation determined in the laboratory for these species, 170 % body C d^{-1} for *T. longicornis* (Klein Breteler et al. 1990) and 85 % body C d^{-1} for *C. hamatus* (Kiørboe et al. 1982). This would indicate that both species were food limited in our area. The fact that carbon ingestion rate do not reach satiety threshold against prey biomass availability also supports the view that copepods could be food limited on most occasions. We cannot exclude, however, the possibility that other possible food sources not considered in the present study (e.g. detritus) may have contributed to increase our daily food rations estimates. To verify this hypothesis we compared total carbon ingestion estimated for *T. longicornis* from our incubation experiments with the estimated carbon requirements for egg production rates measured at the same time (Fig. V-14; see chap IV for explanation). In most of the cases the total carbon ingestion (phytoplankton + microzooplankton) measured on the natural plankton assemblage was not sufficient to cover egg production carbon requirements. As *T. longicornis* is known not to accumulate lipid reserve in the form of wax esters, which could be used to sustain egg production in response to a period of food shortage (Fraser et al 1989), our results favour the above hypothesis that another possible source of carbon was not taken into account in our incubation experiments. Detritus is often suspected as an important source of nutrition for

copepod (Roman 1984) but this has rarely been quantified (Gottfried and Roman 1983, Tackx et al.1989) and will need to be subject to further investigation in the Belgian coastal zone.

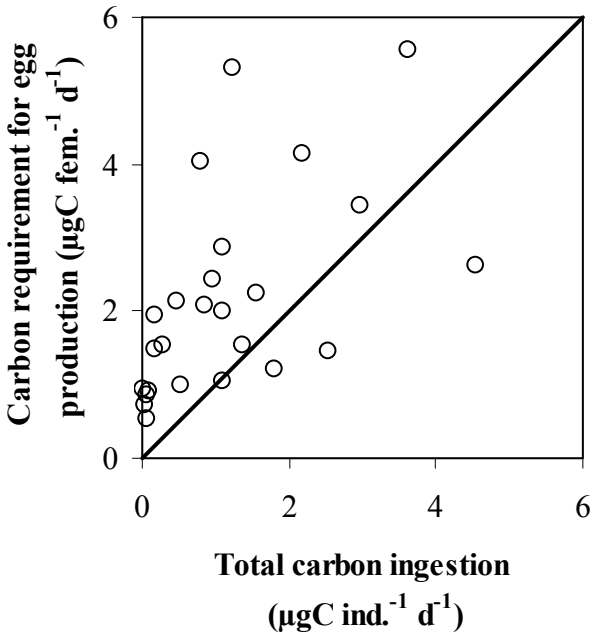

Figure V-14: *Temora longicornis*. Comparison between estimated total food carbon ingestion (from cell counts) and minimal dietary carbon needs based on egg production experiments in Belgian coastal waters in 2001.

The omnivorous character of *Temora longicornis* and *Centropages hamatus* was evident as diatoms, nanoflagellates, dinoflagellates and ciliates occurred in their diet. Diet composition of *T. longicornis* was very variable from one season to another, with diatoms representing the major part of the ingested carbon in winter (74 %) and summer (82 %), whereas dinoflagellates plus ciliates dominated in spring (54 %) and fall (70 %). Diatoms generally contributed to more than half of the total carbon ingestion in *C. hamatus* and dinoflagellates plus ciliates to ca. 40 %. Other field studies of *T. longicornis* and *C. hamatus* diet have shown similar diversity (Tiselius 1989, Gasparini et al. 2000, Vincent and Hartmann 2001, Bonnet 2001). This ability to eat a variety of food seems to be an adaptative advantage for copepods, allowing them (1) to adjust their diet under different conditions of food availability, and (2) to increase the probability for obtaining a nutritionally complete ration (Kleppel 1993).

4.2. Selectivity pattern and prey preference

Our results showed that copepods were not only omnivorous but highly selective as well, consuming the different prey categories in a different proportion than expected from

their availability *in situ*. The pattern of selectivity was different for the diverse prey categories defined in this study. There was an overall positive selection for ciliates by both copepod species, with ciliates < 18 µm being the preferred prey category, whereas *P. globosa* and nanoflagellates were selected against. The copepods showed a variable food preference for the different dinoflagellate categories. *Temora longicornis* showed a clear preference for small dinoflagellates and a more neutral selection for dinoflagellates > 18 µm. Although diatoms were an important contributor to the copepod's diet in this study, they were only ingested according to their abundance or even slightly rejected.

Selective feeding behaviour is the result of complex interactions. It is influenced not only by the availability of a prey in the water column and its size, but also by the specific composition of the potential prey population and their nutritional value (Kleppel 1993). Size-selective feeding has often been shown to be important for the two copepod species considered in this study (Tiselius 1989, Tackx et al. 1990, Vincent and Hartmann 2001) or for congeners of the same genera in feeding incubations using natural plankton assemblages, including *Temora stylifera* (Kleppel et al. 1996), *Centropages brachiatus* (Cowles 1979), and *C. chierchiae* (Vincent and Hartmann 2001). Contrary to studies mentioned above, the data presented here suggest that it was not the size of the prey which was the main reason for food selection. For instance, for a same size category, *T. longicornis* positively selected ciliates and dinoflagellates < 18 µm, rejected nanoflagellates and had a more or less neutral preference for diatoms < 18 µm. This suggests that other factors than size were also driving copepod prey selectivity in our experiments.

In coastal area were food is available in sufficient quantity, it has been suggested that food quality, may be the deciding factor for food selection. The nutritional value of a prey item is generally defined by biochemical compounds. For instance, ciliates and dinoflagellates have been suggested to be qualitatively important as food source for copepods because of their low carbon to nitrogen ratios compared to algae, making them a more efficient source of proteins and amino acids (e.g., Hitchcock 1982, Stoecker and Capuzzo 1990). Other methods used the lipids contained in a prey item to determine its food quality. Fatty acids are the principal form of stored energy in many organisms. Certain types of fatty acids such as ω3 polyunsaturated fatty acids (PUFAs), highly unsaturated fatty acids (HUFAs) and sterols, are considered essential as they cannot be easily synthesized by the organism and must be obtained in sufficient quantity from the food to maintain growth and survival (Sargent and Falk-Petersen 1988, Sanders and Wickham 1993, Brett and Müller-Navarra 1997 and references therein). Recent studies revealed that heterotrophic dinoflagellates are particularly

rich in fatty acids (Klein Breteler et al. 1999, Broglio et al 2003). On the contrary, *Phaeocystis globosa*, which is the main food source available during the spring bloom period in Belgian coastal waters, is considered to have a low nutritive value (i.e., absence of PUAFs; Claustre et al. 1990, Cotonnec et al. 2001). Our results indicate clearly that *P. globosa* was not an adequate food source for the two copepod species studied. These algae was generally strongly rejected and when significant clearance rates were measured these represented a very low ingestion compared to diatoms for instance. Suppressed feeding rates on phytoplankton during *Phaeocystis*-blooms were also reported in previous studies in southern North Sea (Daro 1985, Hansen and van Boekel 1991, Bautista et al. 1992, Breton et al. 1999, Gasparini et al. 2000). Recently, the intermediary dinoflagellate *Gyrodinium dominans* growing on *Phaeocystis globosa* has been shown to improve the nutritional quality of *P. globosa* for *Acartia tonsa*, as indicated by the much higher egg production rate of *A. tonsa* feeding on the dinoflagellate than of those feeding on *P. globosa* directly (Tang et al. 2001). Similarly, in laboratory grazing experiments performed with *P. globosa*, the oligotrichous ciliate *Strombidinopsis acuminatum* and *T. longicornis*, it was shown that *T. longicornis* greatly preferred the ciliates over *P. globosa* (Hansen 1995). Our *in situ* observations, of a preferential predation on dinoflagellates and ciliates during *P. globosa* blooms by *T. longicornis*, whereas *P. globosa* was selected against demonstrated the ability of copepods to adapt their feeding behaviour when faced with a nutritionally poor environment. Assuming that the major grazing pressure on *P. globosa* is caused by microzooplankton organisms (Admiraal and Venekamp 1986, Weisse and Scheffel-Möser 1990), which itself is the preferred food for the dominant copepods in Belgian coastal waters, these copepods would enhance the blooming of *Phaeocystis* (Fransz et al. 1992). This scenario is also demonstrated in the incubation experiments were *Phaeocystis* cell concentration was significantly higher in experimental bottles compared to control bottles at the end of the incubation leading to negative values of computed clearance rate. This was likely due to the selective predation by the copepods upon the microzooplankton, releasing the high microzooplankton grazing pressure on *P. globosa*. We cannot disregard, however, the possibility that these complex interactions within microbial community (trophic cascade; Nejstgaard et al. 2001, Broglio et al. 2004) taking place in the experimental bottles may have masked the simultaneous but weaker copepod grazing on *P. globosa* during the incubations.

In addition to cell size and food quality, some research on selective feeding has shown that prey motility can also influence the feeding behaviour of calanoid copepods (Jonson and Tiselius 1990, Saiz 1994, Saiz and Kiørboe 1995, Kiørboe et al. 1996, Kleppel et al. 1996,

Meyer-Harm et al. 1999). Copepods often have on the first antenna sensory hairs that work like mechanoreceptors. This is efficient to perceive the hydrodynamics disturbance associated with prey moving through the water (Tiselius 1990). The fact that for a given prey concentration clearance rates on diatoms were lower during *P. globosa* dominance than during the other periods, whereas clearance rates on microzooplankton remained unchanged is intriguing (Fig. V-9). It suggests that copepods were less hampered in their selection of microzooplankton than in their selection of diatoms during *P. globosa* dominance. Recent observations suggest a mean increase of viscosity of 250 % of the water during *P. globosa* bloom, with peak of up to 650 % (Dr Laurent Seuront, Marine station of Wimereux, pers. comm.). Although, an increase of viscosity may decrease the sensitivity of mechanoreceptors, motile protozoans are likely to remain better detectable by the copepods than non-motile prey. Further, in a more viscous environment the escape velocity of prey may decrease as well, which could favour copepod predation on motile prey.

Finally, all the factors affecting selection discussed above are not mutually exclusive and may have interacted with each other. *Temora longicornis* and *C. hamatus* may modify their diet according to the balance of these factors in the field through the seasons. Such feeding plasticity is consistent with the predictions of optimal foraging theory (reviewed in Pyke 1984) in the sense that prey selective behaviour by copepods should represent a strategy to optimize the predator's intake of energy in presence of an alternative prey.

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Conclusions and future perspectives

The results obtained during this study improved our understanding of the adaptive feeding behaviour of copepods with respect to phytoplankton species and microzooplankton seasonal succession in Belgian coastal waters. The experimental strategy consisted in measuring and comparing, along a seasonal cycle, the feeding activity of the main copepods on the ambient food resources in order to detect food-related seasonal changes in their feeding behaviour. Considering the known flexibility of copepod feeding behaviour different experimental approaches were used to better appraise trophic interactions with their potential available prey, diatoms, *Phaeocystis globosa*, flagellates and ciliates.

Chloropigment gut content analyses of the common southern North Sea calanoid copepod *Temora longicornis* (chap IV), showed maximum phytoplankton ingestion during the early spring bloom of small-chain forming diatoms in February-March and decreasing phytoplankton ingestion during the *P. globosa* bloom and the following nanoflagellate growth in April-May. Gut pigment analysis by HPLC revealed that diatoms were the main phytoplankton group ingested, whereas chlorophyll c_3, the biomarker of *P. globosa* in our area, was not detected in *T. longicornis*' guts. Although this method as the advantage to provide data directly from the field and does not required incubations, uncertainty about pigment destruction during digestion may have lead to some underestimation of phytoplankton ingestion. Further, lack of information about the transformation pathways of pigments in copepods' body tissues can also induces erroneous interpretation of selectivity between phytoplankton groups based on biomarker pigments (chap III). Pigment composition of a strain of *P. globosa* isolated from Belgian coastal waters (chap II), also highlighted (1) the importance of carefully characterizing pigment patterns from the main algal species of a studied area and (2) the need of knowledge of mechanisms underlying changes affecting pigment ratios in phytoplankton species, to improve the usefulness of HPLC pigment analysis in identifying taxonomic composition of phytoplankton assemblages.

The comparison of phytoplankton carbon ingestion estimated from gut fluorescence method with the total carbon ingestion estimated from egg production (chap IV) revealed that the contribution of herbivory to *T. longicornis*' diet was more important in fall and winter than during spring and summer. Phytoplankton spring bloom, either during diatom dominance or during *P. globosa* dominance, did not enhance the contribution of herbivory to the diet. Actually, the increase of carbon requirements for egg production in the entrance of the growing season was accompanied by a decrease of herbivory contribution to the diet. It

appeared then that above a certain level of carbon requirements (ca. 2.7 µgC fem.$^{-1}$ d^{-1}), *T. longicornis* females needed non-phytoplanktonic food resources to meet their energetic demands for egg production. Further, except for the early-spring period, the reproductive potential of *T. longicornis* was never achieved, including during maximum food biomass. These results indicate that (1) food quantity and quality may have limited egg production rate, and (2) ingestion of heterotrophic food resources did not allow compensating for food limitation. Further investigations are needed to understand and predict changes in growth and reproduction of copepods in the field due to food quality. Emphasis should be placed on determining the biochemical requirements of copepod in terms of essential elements (carbon and nitrogen), major organic compounds (protein, lipid ...) and vitamins during growth period and to support fecundity. These results would be compared with the nutritional quality of their potential prey available *in situ*.

Feeding incubation experiments performed with *T. longicornis* and *Centropages hamatus* to estimate diet composition and prey selection among the natural plankton assemblage (chap V) confirmed that diatoms were the main food source for both species, representing generally more than 50 % of the ingested carbon. However, during *P. globosa* and nanoflagellate dominance, dinoflagellates and ciliates contributed to the major part of the diet. *Phaeocystis globosa* never represented more than 3 % of the total carbon ingestion and the nanoflagellates represented a significant portion of the diet only in fall. In most of the cases, however, the total carbon ingestion measured on the natural prey community (Phytoplankton + microzooplankton) was not sufficient to cover egg production carbon requirements (Fig. V-14). Future research on copepod diet composition should then consider other possible sources of carbon in addition of those examined in this study and in particular the potential contribution of detritus and of larger prey (e.g., copepod eggs).

Our results showed that copepods were not only omnivorous but highly selective as well, with ciliates < 18 µm being the preferred prey category whereas *P. globosa* and nanoflagellates were the most avoided prey. Although diatoms represented an important contribution to the diet, they were often ingested in a lower rate than expected from their availability *in situ*. Prey selectivity appeared to be not size-dependant for both copepod species studied. In future experiments, identification of ciliate and dinoflagellate species and determination of their feeding mode (auto-, hetero- or mixotrophic) should help in understanding what are the main factors driving feeding selective behaviour (food quality, prey motility...). Further analysis on selectivity among the diatom species identified in this

study will certainly give some clues to understand the variable selectivity observed upon this prey category.

Finally, the results obtained in this study highlight the necessity for the biogeochemical MIRO model to take into account that copepod ingestion rate is the resultant not only of prey availability but also of a selective feeding behaviour and that preferential feeding among the available prey may vary according to changes in their physiological needs (e.g., growth, fecundity...).