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Seasonal population dynamics and trophic role of planktonic nanoflagellates in coastal surface waters of the Southern Baltic Sea

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Summary

We investigated the temporal dynamics and trophic role of different nanoflagellates in surface waters of the Gulf of Gdańsk (Baltic Sea) between April and October 2007. Two 18S rRNA gene clone libraries were constructed from samples collected in spring and summer, and weekly changes in the abundances of five phylogenetic groups were studied by fluorescence *in situ* hybridization with newly designed probes. Stramenopiles affiliated with MAST-6 and Pedinellales were most numerous in spring but rare in summer. Both groups formed short-lived blooms during a sudden drop of salinity due to riverine influx (from 7.1 to 6.2 practical salinity units). The analysis of food vacuole content suggested that MAST-6 nanoflagellates were herbivorous, whereas bacterivory was found both in plastidic and aplastidic pedinellid populations. Members of an uncultured lineage of aplastidic, bacterivorous cercozoans distantly related to *Ebria tripartita* were more abundant in summer when water temperatures exceeded 17°C. Multicellular trophonts and/or free-living single cell stages of two lineages of Group 1 parasitic Syndiniales (alveolates) were present in spring and early summer. One of these alveolate populations repeatedly peaked before and after the freshwater influx, but was conspicuously absent throughout the period of decreased salinity. Our results indicate that nanoflagellate populations in coastal surface waters may form short-lived blooms that can only be detected by high-frequency sampling, and that may be related both to seasonal development and to sporadic (e.g. mixing) events. In view of their trophic diversity we moreover suggest that nanoflagellates in eutrophic coastal waters should not be regarded as a single functional unit.

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Introduction

The unicellular eukaryotic plankton has been intensively investigated for over 150 years. So far, questions about diversity or seasonal successions of planktonic protists have been mainly addressed for the autotrophic microphytoplankton (Piwosz *et al.*, 2009) and for ciliates (Pfister *et al.*, 2002), probably due to their relatively large size and conspicuous morphological features. In addition, the picoplanktonic eukaryotes (< 2 μm) have recently also attracted increased attention (López-Garcia *et al.*, 2001; Moon-van der Staay *et al.*, 2001). By contrast, the population ecology of medium-sized, flagellated eukaryotes (2–10 μm) that are not or not exclusively phototrophic has received considerable less attention, although they represent an important link between the microbial loop and the herbivorous food chain in aquatic systems (Sherr and Sherr, 1992). One reason might be methodological problems: the traditional fixation protocols for phytoplankton samples often result in the loss of cell characteristics that would facilitate identification of nanoflagellate species, and PCR-generated 18S rRNA gene clone libraries may be dominated by sequence types from 'traditional' phytoplankton groups (e.g. green algae, diatoms, dinoflagellates) and metazoans (Diez *et al.*, 2001a; Countway *et al.*, 2005).

Thus, only few studies have focused on the diversity of eukaryotic nanoplankton and on the dynamics of individual populations within this 'black box' compartment of the microbial loop (Lim *et al.*, 1999; Chambouvet *et al.*, 2008). Moreover, such surveys on phagotrophic nanoflagellates have so far been exclusively carried out in oligotrophic habitats (Massana *et al.*, 2004; Not *et al.*, 2008; Rodríguez-Martínez *et al.*, 2009). However, an entirely different set of characteristic species might occur in eutrophic and/or brackish waters, such as the Baltic Sea. Its catchment area is inhabited by over 85 million people, which makes the sea vulnerable to anthropogenic

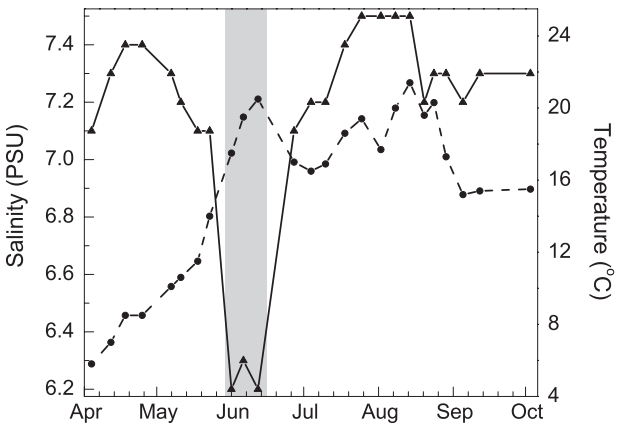

Fig. 1. Changes in salinity (solid line, triangles) and temperature (dashed line, circles) during the investigated period. The period of decreased salinity after freshwater inflow from the Vistula River is shaded in grey.

pollution, including eutrophication. In addition, salinity of surface waters in the Baltic Sea is generally lower and more variable than in other marine waters [20–1 practical salinity units (PSU)] owing to high runoff from rivers, as well as a narrow and shallow connection with the North Sea and the consequently reduced water exchange.

Even though numerous aspects of the Baltic Sea ecosystem and its planktonic biota have been extensively studied (Witek *et al.*, 1999; Riemann *et al.*, 2008), no information is as yet available about the phylogenetic identity and the population dynamics of nano-sized flagellates. We therefore investigated the seasonal occurrence of uncultured populations of nanoflagellates in surface water of the Gulf of Gdańsk. The dynamics of five monophyletic lineages were monitored at high temporal resolution during the most productive season (spring to early autumn) in the context of changes in temperature and salinity. Additionally, the feeding preferences of the investigated groups were addressed by tracer addition, microscopic inspection of food vacuole content and phylogenetic deduction.

Results

Environmental factors

Salinity was relatively constant through the investigated period (7.1–7.5 PSU), except for the short period from 1 June to 12 June, when it dropped to 6.2–6.3 PSU due to freshwater inflow from the Vistula river (Fig. 1). Salinity was restored to > 7 PSU after 4 weeks by a wind-driven local upwelling event. Water temperature gradually increased during the first 3 months, exceeding 20°C just before the upwelling in mid-June (Fig. 1). The subsequent influx of bottom waters caused a temperature decrease to 17°C. Thereafter, temperature varied with the changing weather in July and August and started to decrease to 15°C at the beginning of September.

Phytoplankton and aplastidic nanoflagellates

Sample collection started after the onset of the spring phytoplankton bloom. Dinoflagellates dominated throughout the late bloom period (Fig. 2A), but the most important species changed from *Pediniella catenata* to *Heterocapsa rotundata* and to unidentified naked nanoplanktonic dinoflagellates thereafter. Cyanobacteria (*Aphanothece* spp., *Oscillatoria* spp.) became very abundant at the period of lowered salinity and were replaced by cryptophytes and green algae only in the second half of August. At the end of summer phytoplankton numbers were low until an autumn diatom bloom in October (*Nitzschia longissima*).

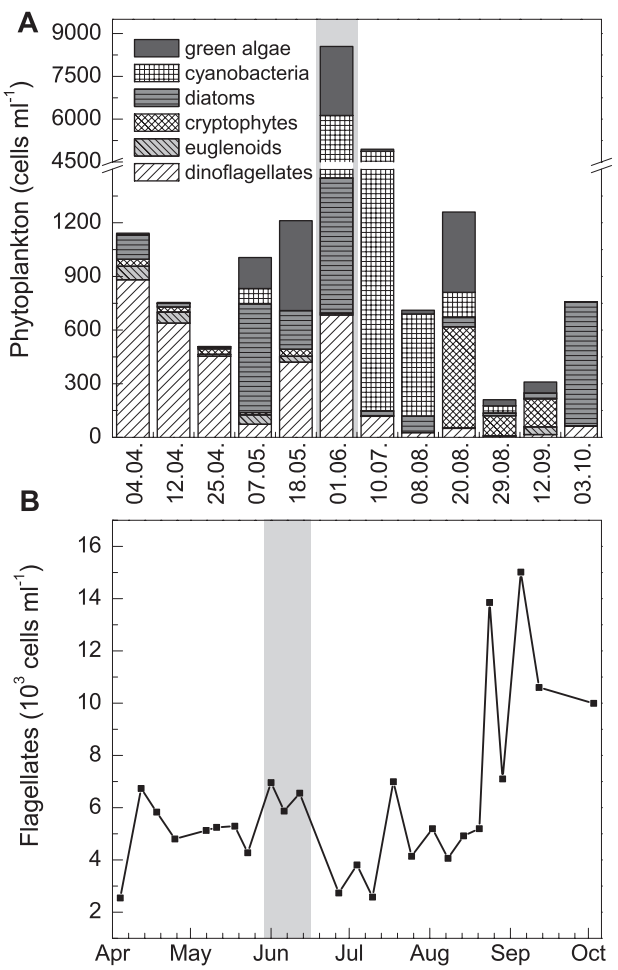

Fig. 2. Changes of (A) phytoplankton composition and (B) numbers of aplastidic nanoflagellates during the investigated period. The period of decreased salinity after freshwater inflow from the Vistula River is shaded in grey for reference. Note the break in the *y*-axis and the lower temporal resolution of A. The error ranges were estimated to be in a range of ± 20% (Lund *et al.*, 1958).

The abundances of aplastidic nanoflagellates were lowest on the 1st sampling date, with 2.5×10^3 cells ml^{-1} (Fig. 2B). Typical error ranges of the counts were ± 20%, as determined in 11 samples during a previous study (Piwosz, 2009). As the phytoplankton bloom started to decline, the numbers of aplastidic nanoflagellates increased to 6.7×10^3 cells ml^{-1}. The freshwater inflow did not substantially influence their numbers, which remained relatively stable until the upwelling event. Thereafter their abundance decreased by more than twofold, from 6.7 to 2.7×10^3 cells ml^{-1}, but recovered to previous levels within 3 weeks. Distinct blooms of aplastidic nanoflagellates were observed in late August to early September, with estimated net community growth rate of up to 0.25 day^{-1}. High numbers of aplastidic nanoflagellates persisted until the end of the sampling.

Clone libraries

Restriction analysis of 18S rRNA gene clone libraries from 8 μm pre-filtered surface water yielded 286 clones with inserts of interest, from which only 171 partial 18S rRNA sequences of high quality could be obtained (62 in spring and 109 in summer). The numbers of operational taxonomic units (OTUs) at 97% similarity were 31 in spring and 14 in summer. Both libraries did not sample the diversity exhaustively, as assessed by the S_{Chao1} estimator (Fig. S1). Only the spring library was large enough to give stable and unbiased estimates of phylotypes richness. The summer library was substantially undersampled because of a strong bias towards sequence types affiliated with *Skeletonema* sp. ($n = 85$), although this diatom was of minor importance in the phytoplankton at that time (< 2 cells ml^{-1}). Therefore, no further comparison of eukaryotic diversity in spring and summer was performed. Details on the obtained sequence types and the results of the BLAST searches are given in Table S1. The closest relatives to most of the OTUs were retrieved from marine habitats with only few related sequence types of freshwater origin and none from terrestrial environments.

Only 9 out of 45 OTUs were related to uncultured, potentially phagotrophic or aplastidic nanoflagellates: 5 from the spring (stramenopiles and alveolates) and 4 from the summer clone library (cercozoans) (Fig. 3). The phylogeny of stramenopiles could be relatively well resolved, as reflected in high bootstrap values in maximum likelihood analyses (Fig. 3A). One of our sequence types grouped with two other sequences that form the MAST-6 clade (Massana *et al.*, 2004). Probe MAST-6 (Table 1) was designed to target this clade. The other OTU fell into the pedinellids clade (100% bootstrap support), for which we designed a group-specific probe Ped675 (Table 1).

Sequence types affiliated with cercozoans grouped within the free-living subphylum Filosa (Fig. 3B). Two OTUs clustered in a novel clade 2 (bootstrap value 62%) without any cultured and described representatives (Bass and Cavalier-Smith, 2004). Within this lineage the OTUs felt into separate subclades, supported by 99% and 61% bootstrap values. Neither of the subclades grouped with sequences from similar habitats, but rather from freshwaters and the marine water column, as well as from anoxic sediments. We designed two probes targeting the two subclades, but their cell numbers were very low or below detection limit (data not shown). The third cercozoan sequence type was distantly related to a clade harbouring *Ebria tripartita* (Fig. 3B). The monophyletic group of most closely related sequences targeted by the newly designed probe Cerc_Bal01 (Table 1) was supported by a bootstrap value of 93%. Within this clade our sequence type formed a well-separated sister branch to all other sequence types from anoxic, marine sediments (bootstrap value 100%).

The phylogeny of alveolates could be only partially resolved (Fig. 3C). Our OTUs fell into two well-separated subclades within the highly diversified Clade 1 of Group 1 of parasitic Syndiniales (Guillou *et al.*, 2008). We designed probe Alv_Bal01 for a small group of sequences

Table 1. Newly designed FISH probes.

Probe	Sequence (5′→3′)	Target group (number of targets)	Lowest weighted mismatch	% FA[a]
Cerc_Bal01	AGT AAA AGA TCT AAC TCG CC	Cercozoan subcluster (6)	1.7 (2.9)	35
MAST-6	CCA AAG CAG TTG GGA GTT AAC	MAST-6 cluster (3)	2.6 (2.9)	35
Ped675	TCA CAG TAA ACG ACA GGC GT	Pedinellales (21)	1.6 (2.8)	45
Alv_Bal01	AAG TGC AAT ACG CTC CCC TT	Subcluster of *Syndiniales* Group I Clade 1 (20)	1.4 (3.4)	50
Alv_Bal02	GAA ACC CGG GTT GGT TCA GT	Subcluster of Syndiniales Group I Clade 1 (34)	1.7 (3.3)	55
Non_Bal	CAA GGT ATT AAC CCG TGG GAT T	None	(4.2)	25

a. Percentage of formamide (FA) in hybridization buffer.
Numbers in parentheses in the column 'Target group' are the number of target sequences, based on NCBI (November 2008), including sequences from this study. Numbers in parentheses in the column 'Lowest weighted mismatch' are the values of the lowest weighted mismatch to eukaryotic sequence types (determined with the online ProbeCheck tool of the SILVA project).

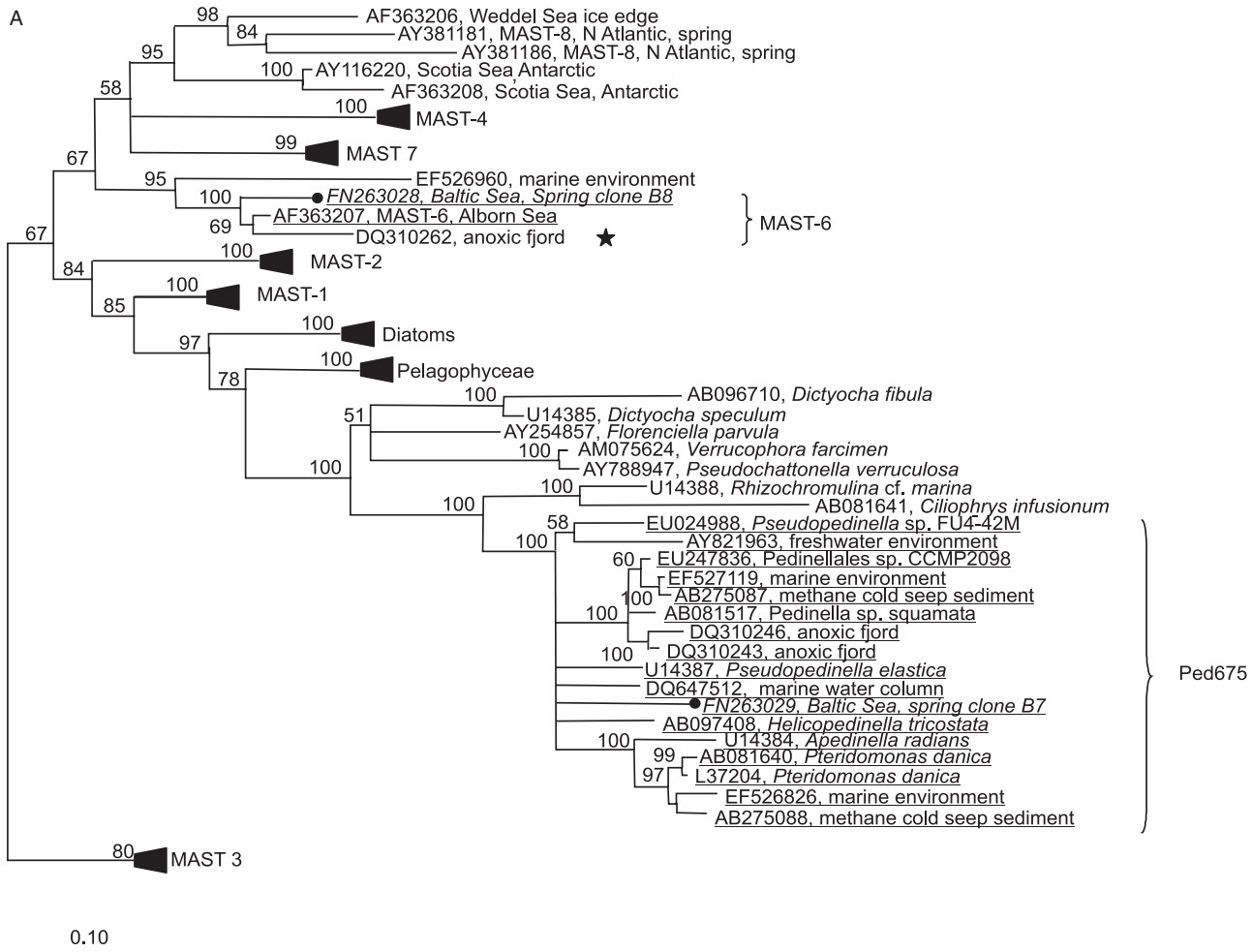

Fig. 3. Maximum likelihood (ML) trees of almost complete 18S rDNA sequences of different flagellate lineages: (A) stramenopiles; (B) Cercozoa; (C) Group I (Clade I) of parasitic Syndiniales (alveolates). Only bootstrap values > 50% (100 ML trees) are depicted, nodes with bootstrap values < 50% are collapsed into multifurcations. The naming of the collapsed groups (black trapeziums) follows the notations of Massana and colleagues (2004) for stramenopiles, Cavalier-Smith and Chao (2003) for Cercozoa, and Guillou and colleagues (2008) for alveolates, respectively. Sequences produced in this study are shown in bold italic fonts and marked by a dot at the branch ending. Groups of sequences targeted by the newly designed probes are underlined (asterisk in A indicates a case in which the probe target site has not been sequenced).

Table 2. Size spectra and food vacuole content of the studied populations of nanoflagellates.

		MAST-6		Ped675			
	Cerc_Bal01	Large morphotype	Small morphotype	Plastidic	Aplastidic	Alv_Bal01	Alv_Bal02
Dimensions							
Measured cells	113	101	102	101	100	100	101
Volume (μm^3)	248.0 ± 82.4	12005.4 ± 6056.6	1092.2 ± 385.9	1261.3 ± 760.1	438.8 ± 157.5	70.7 ± 62.7	98.1 ± 58.9
Length (μm)	4.7 ± 0.5	14.1 ± 2.4	6.4 ± 0.8	6.7 ± 1.2	4.8 ± 0.6	2.5 ± 0.6	2.8 ± 0.6
Width (μm)	3.5 ± 0.4	14.0 ± 2.4	6.4 ± 0.8	6.6 ± 1.0	4.7 ± 0.6	2.5 ± 0.6	2.8 ± 0.6
Food preference							
N samples	3	2	2	3	3	ND	ND
% Bacteria	62 ± 2	7 ± 1	19 ± 5	26 ± 4	48 ± 5	ND	ND
% Algae	9 ± 2	78 ± 2	73 ± 7	4 ± 3	13 ± 2	ND	ND
Feeding mode	Bacterivorous	Algivorous	Algivorous	Mixotrophic	Bacterivorous	Parasitic[a]	Parasitic[a]

a. Based on Guillou and colleagues (2008) and Skovgaard and Daugbjerg (2008).
Geometric average and standard deviation are given. Volume was calculated assuming cells to be in shape of a prolate spheroid. The feeding mode was deduced from the predominant type of prey item inside food vacuoles. ND, not determined.

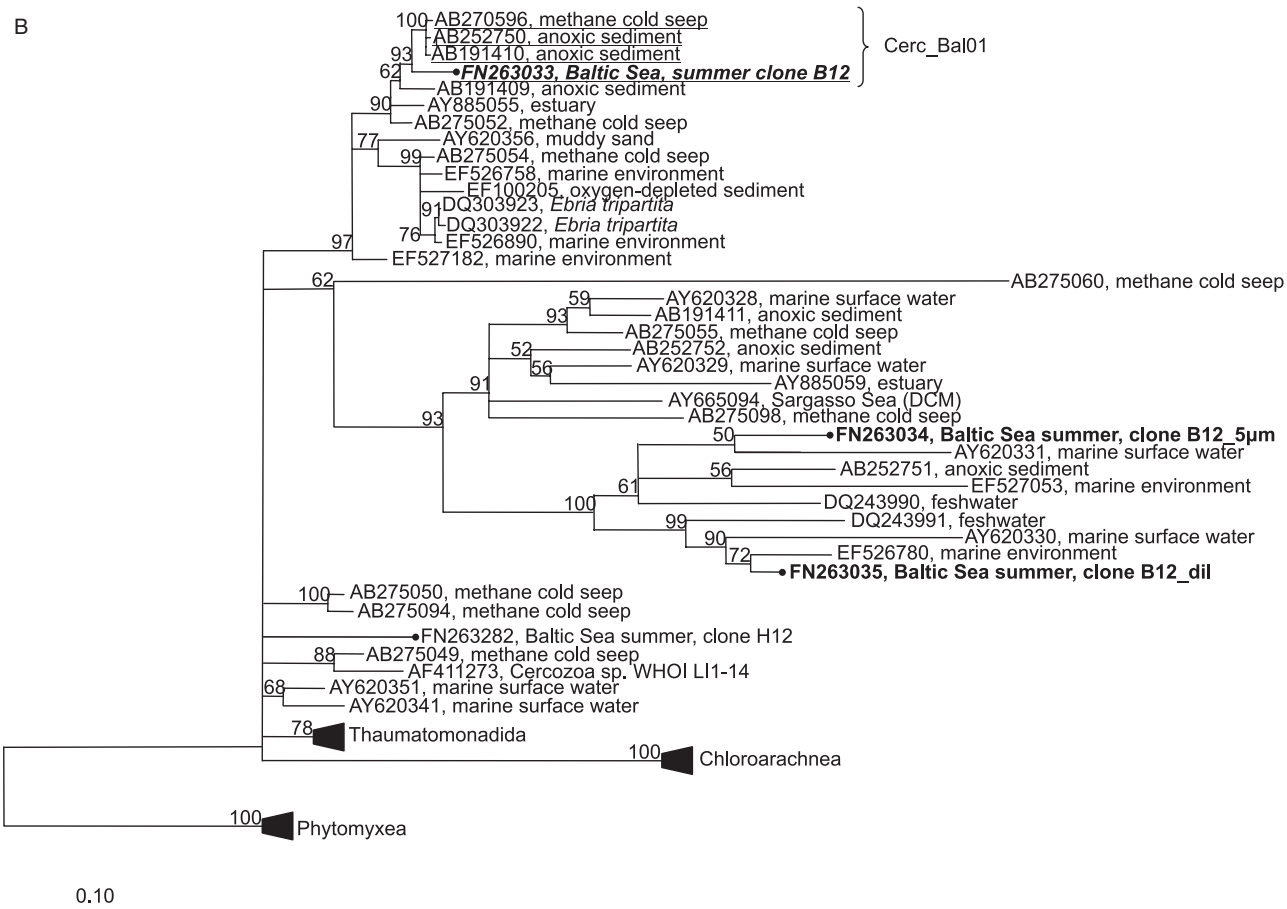

Fig. 3. *cont.*

with 100% bootstrap support and probe Alv_Bal02 for the second, larger subclade with 81% support.

Trophic characteristics and temporal dynamics of nanoflagellate populations

Free-living flagellates affiliated with group Bal01 cercozoans and with stramenopiles from the both studied lineages were observed to be phagotrophic (Fig. 4A–C, Table 2). Cercozoans proved to be mainly bacterivorous, and their aplastidic, oval cells (length 3.3–7.2 µm, width 2.0–4.4 µm), were often filled with 4′-6-Diamidino-2-phenylindole (DAPI) stained bacteria (Table 2). Phagotrophy of these flagellates was moreover qualitatively confirmed using fluorescently labelled bacteria (FLB) as tracers (Fig. 4A). At least 60% of pedinellids targeted by the probe Ped675 possessed three or six chloroplasts, and their cell sizes were in the range from 4.9 to 11.7 µm. Cells of aplastidic pedinellids were smaller, within a range of 3.3–6.2 µm (Table 2). Both plastidic and aplastidic pedinellids were likely able to ingest bacteria, as observed by DAPI staining (Fig. 4B, Table 2), but we did not observe FLB ingestion. The probe MAST-6 targeted two morphologically distinct, aplastidic populations, both capable of ingesting smaller algae and eukaryotes (Fig. 4C, Table 2). One MAST-6 morphotype was distinctly smaller, with cells sizes of 4.2–8.8 µm, whereas the size of the other morphotype ranged from 9.9 to 22 µm.

We observed pronounced temporal changes in the abundance of all studied groups (Figs 5 and 6) that clearly exceeded the known precision range of the method (± 20%, Lund *et al.*, 1958; Piwosz, 2009). The different stramenopile populations featured substantial but short-lived peaks in spring and were not present in countable numbers for period of more than 4 weeks (Fig. 5A and B). Small MAST-6 cells bloomed during the second week of May, increasing in number by more than an order of magnitude between successive sampling dates (estimated net growth rate 0.62 day^{-1}). This peak was followed by a rise of pedinellids with an aplastidic subpopulation of approximately 20% (Fig. 5B), which featured a maximal overall growth rate of 0.32 day^{-1}. Highest cell numbers in both stramenopile groups were observed shortly after the freshwater inflow. At that time point (1 June) pedinellids

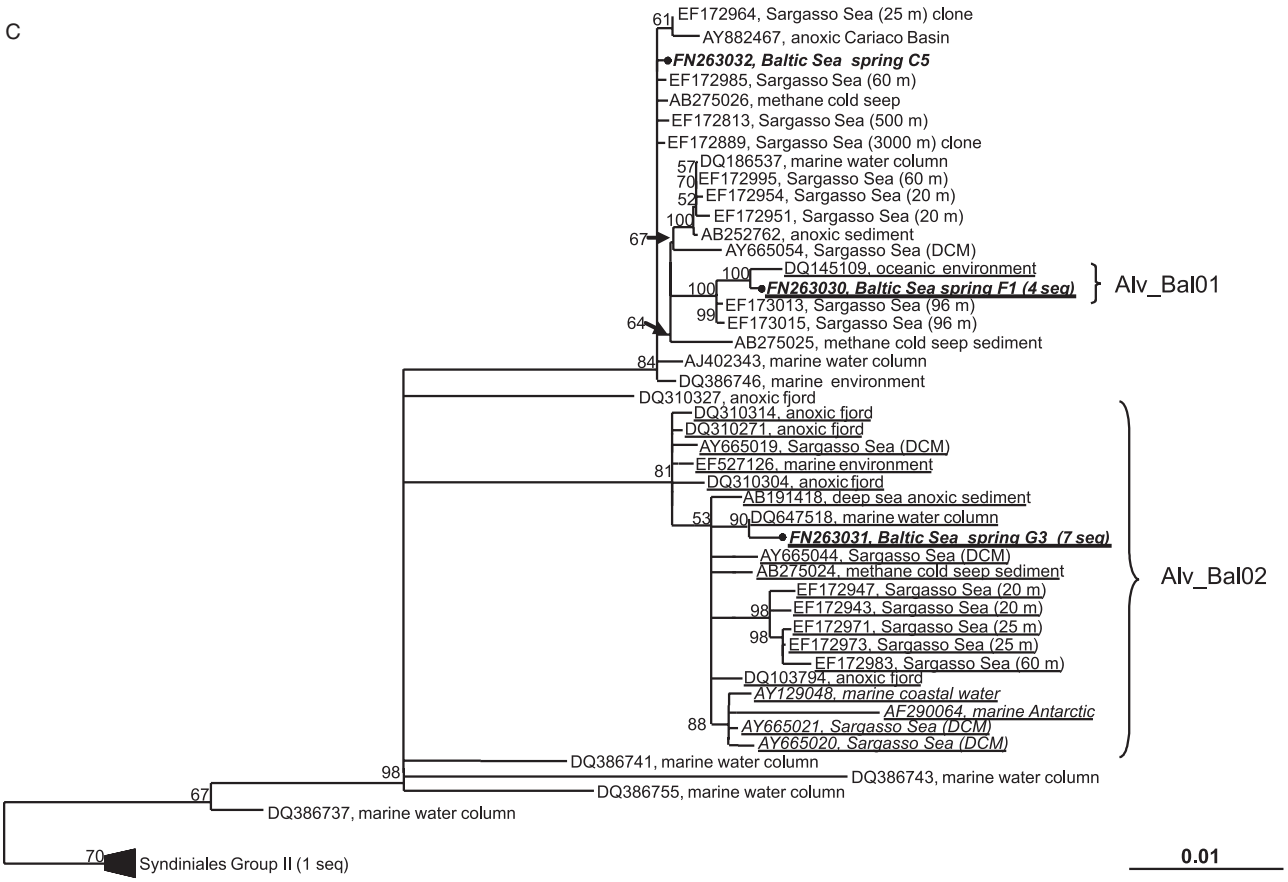

Fig. 3. *cont.*

(40% of them aplastidic) formed a maximum of > 1100 cells ml^{-1}, with a net growth rate of 0.22 day^{-1} (Fig. 5B). The concomitant peak of MAST-6 stramenopiles was mainly due to the large morphotype (Fig. 5A). Both morphological MAST-6 populations and pedinellids substantially declined during the subsequent weeks. They were generally not present in countable numbers thereafter, except for the small MAST-6 morphotype, which formed populations of < 100 cells ml^{-1} throughout the remaining season. Bacterivorous cercozoans targeted by the probe Cerc_Bal01 appeared in summer after the upwelling event and were present until the end of the investigation period at maximal densities of approximately 200 cells ml^{-1}. These flagellates declined in numbers when water temperature fell below 17°C (Fig. 5B).

Free living single cells of both populations of Syndiniales were morphologically very similar, around 2.5 μm long and with large nuclei (Table 2). These allegedly parasitic (Chambouvet *et al.*, 2008) flagellates formed densities of approximately 500–600 free-living single cells ml^{-1} (Fig. 6A and B). Alveolates detected by the probe Alv_Bal01 formed only one short-lived peak in early May, whereas several distinct maxima of cells from the phylogenetically wider group targeted by the probe Alv_Bal02 were observed. However, flagellates from the latter clade were conspicuously absent from the water column for the whole period of lowered salinity and only bloomed again after salinity increased above 7 PSU. Multicellular aggregates, presumably trophonts (2 to > 50 cells) were observed only for alveolates detected by the probe Alv_Bal02 (Fig. 4D). The occurrence of these trophonts mirrored the changes in the numbers of single cells, including the absence during the period of lower salinity (Fig. 6B).

The numbers of flagellates affiliated with the MAST 1–4 lineages, as assessed by fluorescence *in situ* hybridization (FISH) with the published probes for these groups (Massana *et al.*, 2002; 2006a) were below detection levels throughout the study (data not shown).

Discussion

Are there unique nanoflagellate assemblages in shallow brackish waters?

Previous studies about the phylogenetic affiliation of small planktonic eukaryotes have generally focused on olig-

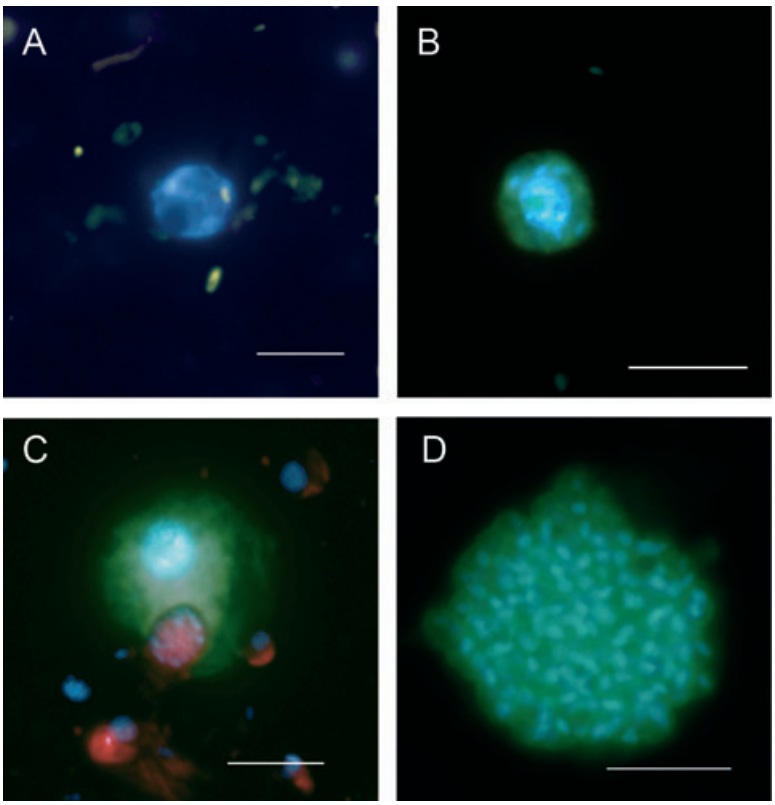

Fig. 4. Photomicrographs of different nanoflagellates.
A. Cercozoan cell detected by probe Cerc_BAL01 and Alexa350 labelled tyramides (blue) with ingested fluorescently labelled bacterium (yellow).
B. Pedinellid (probe Ped675, green) with DAPI stained nucleus and ingested bacteria (blue).
C. Cell detected by probe MAST-6 (green) with ingested algal cell (red) and DAPI-stained nuclei (blue).
D. Aggregated cells (trophont) of alveolates detected by probe Alv_Bal02 (green) and DAPI-stained nuclei (blue). Scale bars are 5 μm in A, 10 μm in B and C, and 20 μm in D. All depictions are false-colour overlays of greyscale images from the same respective microscopic fields obtained at different excitation wavelengths. The original greyscale images used for the overlays are shown in the Fig. S4.

otrophic sites (Marie *et al.*, 2006; Not *et al.*, 2007). The brackish, eutrophic waters of the Gulf of Gdańsk appear to provide an entirely different habitat for eukaryotic microorganisms. For one, the flagellate groups depicted in Figs 5 and 6 might be classified as euryhaline, because they apparently occur both in marine and brackish waters (Takishita *et al.*, 2005; 2007; Behnke *et al.*, 2006). By contrast, some groups of typical marine nanoflagellates appeared to be rare or even missing in the Gulf of Gdańsk, e.g. members of the Novel Marine Stramenopiles (MAST) lineages 1–4 that are common in oceanic and coastal temperate waters (Massana *et al.*, 2006a) were not found either in our clone libraries (Table S1) or by direct microscopic analysis with the available FISH probes (Massana *et al.*, 2002; 2006a).

A number of sequence types targeted by the probes Ped675, MAST-6, Cerc_Bal01 and Alv_Bal02 have been retrieved from anoxic environments (Fig. 3). However, at a closer look most of these sequences were collected at the oxic/anoxic boundary [anoxic fjord (Behnke *et al.*, 2006) or Canaco Basin (Stoeck *et al.*, 2006)]. Therefore, it is conceivable that these sequences may have originated from sedimenting cells from the upper water layers. Alternatively, it is possible that some flagellates may occur in both oxic and anoxic habitats. The ability of cyst formation that is widespread within, e.g. cercozoans (Hoppenrath and Leander, 2006) might explain the observed grouping of sequence types from anoxic sediments and the water column in our phylogenetic analysis (Fig. 3). On the other hand, the planktonic cercozoan population targeted by the probe Cerc_Bal01 in the shallow waters of the Gulf of Gdańsk might have originated from benthic habitats, i.e. it might be tychoplanktonic.

Temporal dynamics of different nanoflagellate groups

The total numbers of aplastidic nanoflagellates in the Gulf of Gdańsk ($2.5–15.0 \times 10^3$ cells ml^{-1}, Fig. 2B) were high compared with oligotrophic regions (70–1500 cells ml^{-1}) (Diez *et al.*, 2001b; Not *et al.*, 2008). They were, however, only slightly above values from the Bothian Bay of the northern Baltic Sea (688–3470 cells ml^{-1}) (Kuosa, 1991). Similarly high numbers have been previously found at the site of our study (Rychert, 2006).

Incubations of differently processed seawater suggest that some aplastidic nanoflagellates are able to rapidly respond to environmental changes (Massana *et al.*, 2006b). However, it is unknown if this growth potential is also realized *in situ*; so far all seasonal investigations on nanoflagellate populations dynamics have been based on monthly – or longer – sampling intervals (Massana *et al.*, 2002; Rodríguez-Martínez *et al.*, 2009). This may result in missing the apparently short-lived blooms of some

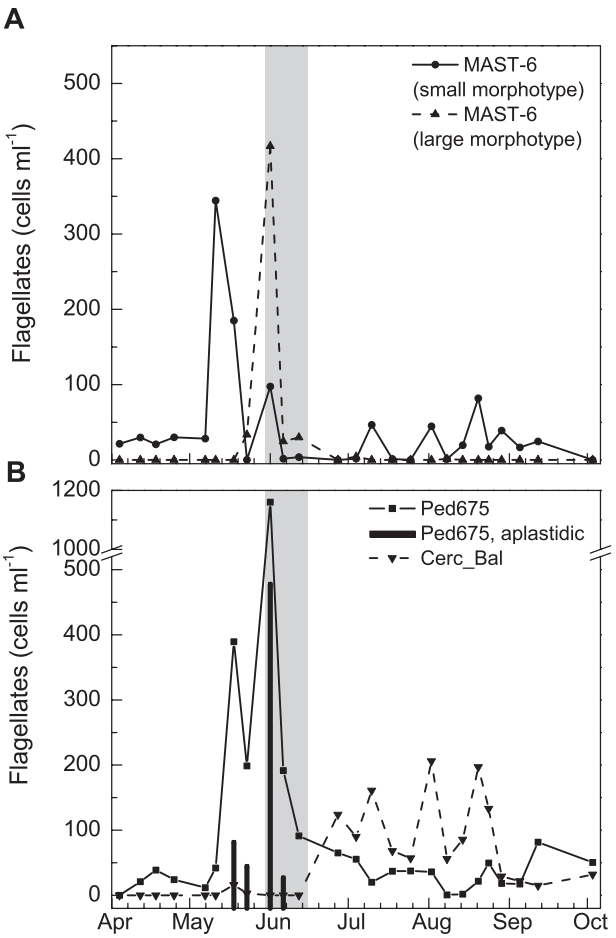

Fig. 5. Abundance (symbols and lines) of free-living nanoflagellates affiliated with (A) MAST-6 (probe MAST-6) and (B) pedinellids (probe Ped675) and group Bal01 cercozoans (probe Cerc_Bal01). Note that the *y*-axis scale differs between the upper and lower panels and the break in the *y*-axis of B. Black bars in B depict the numbers of aplastidic pedinellids. The period of decreased salinity after freshwater inflow from the Vistula River is shaded in grey for reference.

nanoflagellate groups (Figs 5 and 6). The observed rapid increase in abundance within periods of 1 week indicates that some flagellates were able to quickly respond to environmental fluctuations, and their rapid disappearance suggests that this growth potential could have been matched by the response of their predators (Calbet and Landry, 1999) or parasites (Massana *et al*., 2007).

It should be noted that the apparently temporal fluctuations in the abundances of different nanoflagellates could in parts also be due to spatial heterogeneity (Lovejoy *et al*., 2001). The hydrology in the Gulf of Gdańsk is very dynamic and water is exchanged with the open Baltic Sea within approximately 14 days (Witek *et al*., 1999). Moreover, planktonic nanoflagellates might also have been washed in by riverine water masses. Nevertheless, the observed changes in cell densities (Figs 5 and 6) were generally so extreme that they most likely also reflected truly temporal aspects of population development.

The studied populations formed high cell densities only at particular seasons. By contrast, no clear seasonality was found for marine nanoflagellates affiliated with the MAST-4 lineage (Massana *et al*., 2002; Rodríguez-Martínez *et al*., 2009). However, those studies targeted the whole phylogenetic cluster (> 50 sequences) of the MAST-4 lineage. The apparent lack of seasonality might thus have been due to successions of different subpopulations. This might also explain the multiple population maxima of nanoflagellates detected by the phylogenetically relatively broad probe Alv_Bal02 (Figs 3C and 6B). By contrast, organisms targeted by the narrower probe Alv_Bal01 formed a single peak only (Fig. 6A).

All populations that were present at the time of the freshwater inflow reacted strongly to this event, even though the decrease in salinity was not substantial (< 1 PSU). The initial stimulation of growth in the algivo-

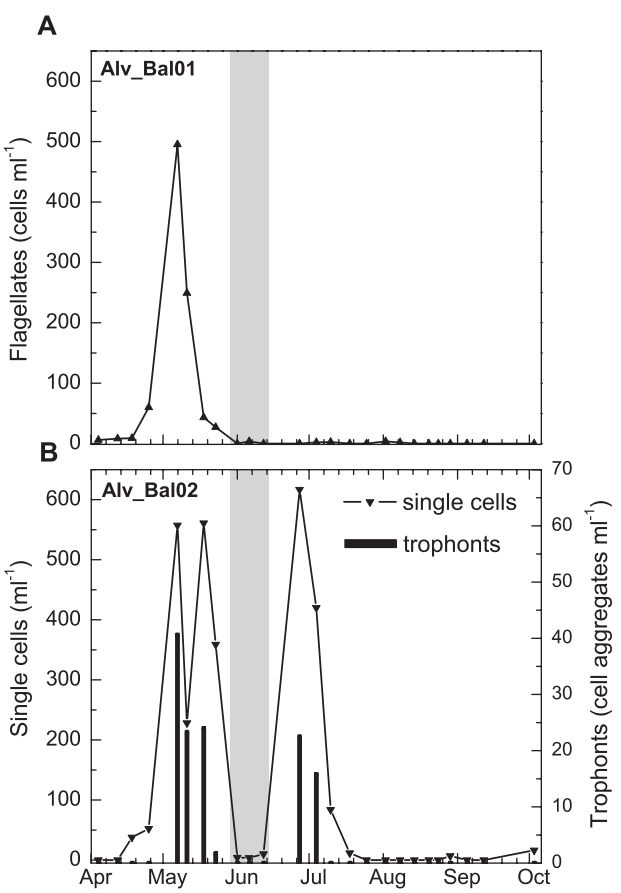

Fig. 6. Abundance of single cells (symbols and lines) of nanoflagellates affiliated with the (A) Bal01 (probe Alv_Bal01) and (B) Bal02 (probeAlv_Bal02) lineage of Group I parasitic Syndiniales. The bars in B show the numbers of cell aggregates (2→50 cells), likely representing trophonts. The period of decreased salinity after freshwater inflow from the Vistula River is shaded in grey for reference.

rous MAST-6 stramenopiles (Figs 4C and 5A) could have been due to changing food availability, as suggested by high abundance of unicellular cyanobacteria and green algae (Fig. 2), whereas pedinellids might even have been directly stimulated by lower salinity (Suikkanen *et al.*, 2007). The rapid subsequent decline of both stramenopile lineages might have been related to the rise of predatory ciliates (*Holophyra* spp., *Strombidium* spp.) and rotifers (*Synchaeta* spp.) during that period (data not shown). Again, genotypic shifts within these probe-defined nanoflagellate groups cannot be excluded, as, e.g. indicated by the rise of a distinctly larger MAST-6 morphotype and the changing ratio of plastidic to aplastidic pedinellids (Fig. 5). The impact of decreasing salinity on alveolates targeted by the probe Alv_Bal02 was as drastic as on stramenopiles (Fig. 6B). This group of nanoflagellates is most closely related to genotypes only from marine environment (Fig. 3C), and it (or its presently unknown host species) might exist at its distribution limit with respect to salinity in the Gulf of Gdańsk. In fact, a salinity around 6 PSU is known to represent a boundary that strongly separates freshwater and marine species (Remane, 1934), including protists (Tikhonenkon *et al.*, 2006). Unfortunately, we could not thoroughly test this hypothesis statistically due to the very low abundances of the studied nanoflagellates during most of the investigated period (i.e. the changes in the numbers of cells during the period of low abundances likely were more related to the precision of the counting approach than to a response of the populations to environmental parameters).

Trophic role of different nanoflagellate groups

Knowledge of the temporal dynamics of different nanoflagellate groups is a crucial first step to understand their specific role in the environment. However, this should be accompanied by information on their respective role in the food web. Initially aplastidic nanoflagellates were mainly considered as grazers of prokaryotes in the context of the microbial loop model (Sherr *et al.*, 1987). However, cross-system comparisons of bacterial and flagellate numbers soon challenged this view (Gasol and Vagué, 1993). One explanation for the observed weak correlations between the numbers of aplastidic nanoflagellates and bacteria was the possibility of alternative carbon sources for the former. We found strong indications that the studied genotypic groups also differed in their respective feeding mode (Fig. 4, Table 2). For example, the two groups of alveolates studied herein should not be regarded as bacterivores at all as they were likely parasitic (Chambouvet *et al.*, 2008; Guillou *et al.*, 2008).

Heterotrophic stramenopiles harbour cultivable bacterivorous genera that are often rare in environmental samples, e.g. *Cafeteria* or *Paraphysomonas* (Lim *et al.*, 1999), whereas the numerically dominant groups in marine plankton (MAST-1 and -4) are not cultured (Massana *et al.*, 2006a,b). In contrast to the other known MAST organisms that were shown to be bacterivores (Massana *et al.*, 2009), the population targeted by the probe MAST-6 appeared to exploit unicellular algae as a food source (Fig. 4C, Table 2). Thus they formed part of the herbivorous food chain (Sherr and Sherr, 1992) rather than of the microbial loop, as might have been erroneously deduced from their cell size and phylogenetic affiliation. The other stramenopile group in the Gulf of Gdańsk, Pedinellales, harbours culturable plastidic and aplastidic species (Swale, 1969). Plastidic pedinellid species are bacterivorous and can selectively discriminate against non-bacterial particles (Swale, 1969). This might explain why DAPI-stained bacteria-like particles but no FLB could be detected inside the food vacuoles of pedinellids (Fig. 4B). Such avoidance behaviour has also been observed in freshwater flagellates (Boenigk *et al.*, 2001).

Species of free-living Cercozoa (Filosa) (Cavalier-Smith and Chao, 2003) are important bacterivores in soils and sediments (Park *et al.*, 2003). In the water column they might also form a part of the pelagic microbial food web, e.g. the population targeted by the probe Cerc_Bal01 was shown to graze on bacteria (respectively FLB, Fig. 4A, Table 2) in the Gulf of Gdańsk, regardless of the fact that it might by tychoplanktonic rather than a constant component of the pelagic community.

Concluding remarks

The composition of nanoplanktonic flagellates in coastal brackish waters of the Baltic Sea appears to be very complex, not only in terms of phylogenetic diversity, but also in the respective trophic roles of different genotypes. Our study showed that flagellate groups with different feeding modes (bacterivores, algivores, parasites) form very short-lived peaks. Therefore, the importance of nanoflagellate bacterivory and herbivory might change substantially not only between seasons, but also from one week to another. These results indicate that estimates of bacterivory based on total numbers of aplastidic nanoflagellates should be avoided in ecological studies as they might be both too high and too low: the aplastidic but not phagotrophic forms that were identified by our probes (parasitic Syndiniales) contributed up to 22% of the total number of aplastidic nanoflagellates in the Gulf of Gdańsk, while a part of bacterivory was due to mixotrophic groups such as pedinellids. In summary, it appears that aplastidic nanoflagellates are not an ecologically as meaningful unit as, e.g. the phytoplankton, and that different populations have contrasting functions in aquatic systems.

Experimental procedures

Study site

The Gulf of Gdańsk is an open bay at the southern coast of the Baltic Sea, in Polish territorial waters. The water exchange is mainly with the open sea, but the hydrodynamics and chemistry are also influenced by the Vistula River and local upwelling events (Kowalewski and Ostrowski, 2005). The salinity of the surface layer ranges from 6 to 8 PSU. The ecosystem of the Gulf of Gdańsk is protected in the framework of the European NATURA 2000 network and the Baltic Sea Protected Areas under recommendation of the Helsinki Commission.

Sampling

Sampling was generally performed at weekly intervals from 4 April to 3 October 2007. Twenty litres of surface seawater was collected from the jetty with a clean, open plastic container prerinsed three times with the sampling water. The collected seawater was transported to the laboratory within 15 min in a closed plastic container that had been cleaned with 10% HCl for 2 days, thoroughly washed with deionized water and rinsed with the sampled seawater. Temperature was measured *in situ* with a thermometer, and salinity (conductivity) was determined in the laboratory with an InoLab probe (WTW).

Phytoplankton composition

Phytoplankton samples were collected on 4, 12, 25 April; 7, 18 May, 1 June, 10 July, 8, 20, 29 August, 12 September and 3 October. Two hundred and fifty millilitres of seawater was fixed with alkaline Lugol's solution, followed by formalin to a final concentration of 1%. Samples were stored in dark, plastic bottles at 4°C. Phytoplankton cells in 20 ml subsamples were counted according to Uthermöhl (1958).

Aplastidic nanoflagellates numbers

For the determination of the numbers of aplastidic nanoflagellates, 25–50 ml of water sample was fixed as described above for phytoplankton. Samples were then decolourized with 3% $Na_2S_2O_3$ (Sherr *et al.*, 1987) and filtered on polycarbonate membrane filter (pore size 0.8 μm, diameter 25 mm, Isopore, Millipore). They were subsequently washed with sterile deionized water (MilliQ, Millipore), dried and stored at −20°C until further processing. To count aplastidic nanoflagellates, filter sections were embedded in glycerol mountant that contained DAPI (1 μg ml^{-1}). At least 200 aplastidic flagellates (based on fluorescence) per sample were counted at 1250× magnification in at least 20 microscopic fields by epifluorescence microscopy (AxioImager.M1, Carl Zeiss, Germany).

DNA extraction, clone libraries and probe design

Water samples for the construction of 18S rDNA clone libraries were collected on 7 May and on 20 August. Two to three litres of the untreated seawater was pre-filtered through membrane filters (8 μm pore size, diameter 47 mm, Isopore, Millipore) at pressure of approximately 0.26 bar and cells were harvested on cellulose acetate filters (pore size 0.45 μm, diameter 47 mm, Satorius). Filters were stored at −20°C until further processing (< 6 months).

Genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen). Cells were removed from the filters by scraping them with a sterile scalpel into excess sterile MilliQ water and were concentrated to pellets by two subsequent centrifugations at 16 600 *g* for 30 min and 1 h. Afterwards the pellets were frozen at −20°C for 15 min. The next steps were performed according to the manufacturer's instruction, but increasing the volume of buffers and RNase A by 50% in the two steps (steps 7 and 9 in the protocol). DNA was eluted with the elution buffer included in the kit. DNA concentration was measured with Quanti-iT PicoGreen dsDNA kit (Molecular Probes).

We used published general eukaryotic primers (Moon-van der Staay *et al.*, 2000) to amplify almost complete 18S rRNA genes (approximately 1.8 kbp). Five reactions per sample were pooled for a clone library construction to account for the random PCR bias. Platinum PCR SuperMix High Fidelity Taq (Invitrogen) was used to minimize incorrect nucleotide incorporation. The amount of template DNA in the reaction tube ranged from 17.5 to 84 ng. 25 PCR cycles (94°C→30 s; 55–60°C→30 s; 68°C→2 min, final elongation 68°C→10 min) were performed. Annealing temperature was 60°C for the spring and 55°C for the summer samples. The temperature for the spring sample was increased because unspecific PCR products were still detected by gel electrophoresis at the lower annealing temperature. Purified PCR products (QIAquick PCR purification kit, Qiagen) were immediately cloned into the pCR 2.1 TOPO vector (TOPO TA cloning kit, Invitrogen). Plasmids from the transformants were screened for properly sized inserts (approximately 1.8 kbp) by restriction with EcoR-I (10 U μl^{-1}) (2 h at 37°C, inactivation of the enzyme for 30 min at 65°C). The inserts were partially sequenced with primer Euk528F (Elwood *et al.*, 1985) on an ABI 3730 system (Applied Biosystem) using the BigDye Terminator Mix. The quality of sequences was assessed by the Sequencing Analysis Software v5.2 (Applied Biosystem). To find the closest relatives, we performed BLAST searches with the WU-BLAST2 algorithm (Lopez *et al.*, 2003). From each group of highly similar sequence types one was selected for full sequencing with vector primers M13F and M13R (supplied with the cloning kit), applying additionally the internal primers 345F (Zhu *et al.*, 2005) and 1055F (Vaulot, 2006) whenever necessary. Partial sequences were assembled using the ContigExpress Software, a component of Vector NTI Advance v10.3 (Invitrogen). Sequences were deposited in the EMBL nucleotide database under accession numbers FN263028 – FN263035 (full-length sequences shown in Fig. 3), FN263250 – FN263273 and FN263275 – FN263285. The coverage of the diversity of eukaryotic microbes in the clone libraries was estimated with the S_{Chao1} estimator (Chao, 1987) using the online input form provided by Kemp and Aller (Kemp and Aller, 2004).

Partial and full-length sequences were aligned online with the SINA web aligner against the SILVA database (Pruesse *et al.*, 2007). A first phylogenetic analysis of almost full-length rRNA gene sequences was performed using the software

ARB (Ludwig *et al*., 2004). Maximum parsimony phylogeny was inferred using > 8800 almost complete protistan sequences. Bootstrapped (100 repetitions) maximum likelihood trees were then calculated using the RaxML algorithm on a subset of most closely related sequences (517 for cercozoans, 124 for stramenopiles and 485 for alveolates) on a dedicated web server (Stamatakis *et al*., 2008). The resulting trees were imported into ARB, reduced to a subset of most closely related sequences, and nodes with bootstrap values < 50% were collapsed into multifurcations.

Full-length sequences and extensive phylogenetic analysis were the basis for designing oligonucleotide probes for FISH. We developed DNA probes for monophyletic groups of cercozoans (Cerc_Bal01), stramenopiles (MAST-6 and Ped675) and alveolates (Alv_Bal01 and Alv_Bal02) (Table 1). The specificity of the probes was tested *in silico* using the ARB Probe_Match function and the SILVA online ProbeCheck tool against the NCBI database. Subsequently, environmental samples were hybridized with these probes at increasingly stringent conditions to establish specificity, and the highest formamid concentration was chosen at which the brightness of hybridized cells was still unaffected. Negative control organisms for experimentally testing probe specificity were available cultures of *Cryptomonas* sp. (Cryptophyta), *Bodo* sp. (Kinetoplastida), *Poterioochromonas* sp. strain DS. (Synurophyceae) and *Cyclidium* sp. (Ciliophora) fixed by the same procedure as the environmental samples. The test organisms were also hybridized with the general eukaryatic probe Euk1209R (Giovannoni *et al*., 1988) to exclude false negative results due to inaccessibility of the cells to catalysed reported deposition (CARD)-FISH probes after fixation (Fig. S2). In addition, simultaneous cross-hybridizations with two probes designed for closely related groups of Syndiniales (Alv_Bal01 and Alv_Bal02) were performed on field samples using tyramides labelled with $Alexa_{488}$ and $Alexa_{633}$.

FISH

The samples for determination of the numbers of specific flagellate groups targeted by the newly designed probes were collected exactly as described for the counts of aplastidic nanoflagellate numbers. Staining of flagellates by FISH and CARD was generally based on the standard protocol for bacteria (Pernthaler *et al*., 2004), but omitting the enzymatic permeabilization step. In short, filters were embedded in 0.1% agarose to minimize cell loss and incubated 20 min in 0.01 M HCl to inactivate endogenous peroxidases. Samples were not dehydrated in ethanol in order to maintain chlorophyll fluorescence. Filter sections were hybridized at 35°C for 3 h, and washed at 37°C for 30 min. The respective concentration of formamide (Calbiochem) in hybridization buffer for different probes is listed in the Table 1. Besides analysis with the newly designed probes, published probes for groups of Novel Marine Stramenopiles were also employed (Massana *et al*., 2002; 2006a).The published concentrations of formamide for these probes were increased by 20% to establish stringent conditions at the lower hybridization temperature of our protocol. Amplification with tyramides (Sigma) labelled with fluorescein (Molecular Probes, Invitrogen) was performed at 37°C for 30 min. Negative controls were performed in the samples with the highest abundances of the investigated flagellates: (i) by FISH with a probe Non_Bal that has at least 4.2 weighted mismatches to all available eukaryotic sequences (Table 1) at conditions of low stringency (25% formamide) and (ii) by CARD without the preceding hybridization step.

The samples were counterstained with DAPI (1 μg ml^{-1}) and mounted in glycerol medium [5:1 mix of Citifluor AF1 (Linaris, Switzerland) and Vectashield (Vector Laboratories, Canada)]. Preparations were store at –20°C until further processing. At least 200 hybridized flagellates were counted in 20 or more microscopic fields by epifluorescence microscopy (AxioImager.M1, Carl Zeiss, Germany) at blue/UV excitation. If the densities of target cells were too low, the complete filter piece was screened (> 170 microscopic fields). For discrimination between aplastidic and plastidic Pedinellales (samples 18 May to 6 June) targeted by the probe Ped675, > 170 hybridized cells (except for sample from 6 June: 71 cells) were examined for the presence of chloroplasts at 590 nm excitation by epifluorescence microscopy (AxioImager.Z1, Carl Zeiss, Germany). This fluorescence was visualized with an AxioCam MR3 camera (Carl Zeiss, Germany).

Size measurements

At least 100 hybridized cells were photographed after visualization by epifluorescence microscopy (AxioImager.Z1, Carl Zeiss, Jena, Germany) with an AxioCam MR3 camera (Carl Zeiss). Photographed cells were measured using the contour (Spline) tool of the AxioVision software (Carl Zeiss). The shape of the cells was assumed to be a prolate spheroid, and their volume was calculated according to the formula $V = [4\pi \times (\text{width})^2 \times \text{length}]/3$.

Feeding modes

The food vacuole content of at least 100 hybridized flagellate cells from the samples with the highest abundance was examined by epifluorescence microscopy (AxioImager.M1, Carl Zeiss) at green/blue/UV excitation. The presence of bacteria in food vacuoles was assessed based on their DAPI signal at UV excitation, and the presence of algae at green excitation (chlorophyll a autofluorescence). Prey items were counted only if they were in the focal plane of a flagellate cell to ensure that they had not settled onto the surface of the examined cell or did not shine through from underneath (Fig. S3).

Uptake of FLB

Fluorescently labelled bacteria were used as one tool to qualitatively assess phagotrophy of investigated flagellates. Environmental bacteria were harvested on polycarbonate membrane filters (pore size 0.22 μm, diameter 47 mm, Isopore, Millipore) from approximately 6 l of sequentially prefiltered seawater (10, 5, 1.2 μm). Cells were gently transferred from the filters into 10 ml of a sterile 0.85% NaCl solution buffered with 0.05 M Na_2HPO_4 (pH 9.0), and further stained with 5-([4,6-Dichlorotriazin-2-yl]amino)fluorescein hydrochloride (Sigma) as previously described (Sherr *et al*., 1987).

Fluorescently labelled bacteria were added to 300–500 ml of triplicate environmental sample at final concentrations of 15–20% of native bacterial numbers. Samples were collected and processed like samples for CARD-FISH counts. Samples with high abundance of targeted flagellates were hybridized with the corresponding probe as described above, but the subsequent signal amplification was performed using tyramides labelled with $Alexa_{350}$ (Beardsley *et al*., 2005). Filter section were mounted in DAPI-free glycerol medium and inspected by epifluorescence microscopy for the presence of FLB in food vacuoles.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Predicted number of phylotypes based on the S_{Chao1} estimator, versus size of subsamples of the clone libraries. Each point is the mean of 10 replicate subsamples of the library. Phylotype-richness estimate reached asymptote for the spring library, indicating that it was large enough to yield stable and unbiased richness estimate. S_{Chao1} estimator did not reach an asymptotic maximum in summer, indicating that the phylotype richness was underestimated.
Fig. S2. Examples of photomicrophotographs of the negative control organism *Poterioochromonas* sp. strain DS. (A) Cells stained with DAPI shown in UV excitation and the same microscopic field in blue excitation (B) *Poterioochromonas* sp. strain DS cells hybridized with the general eukaryotic probe Euk1209R (C) *Poterioochromonas* sp. strain DS cells stained with DAPI and the same microscopic field in blue excitation (D) showing no signal from the cells hybridized with the Ped675 probe. The depictions at the blue excitation were taken in the LUCIA G\F software with identical acquisition settings of Gain (1.42), Offset (0) and Gamma (1.00), but with different exposure times (10 ms for Euk1209R – B and 70 ms for Ped675 – D). The settings for the pictures at UV excitation (A and C) were adjusted automatically. Scale bars correspond to 10 μm.
Fig. S3. Photomicrographs from a *Z*-stack of a DAPI-stained pedinellid cell (from above focal plane, z-distance, 1–2 μm). Ingested bacterial cells are only in focus in (C), slightly above the z-layer of the in-focus position of the nucleus (D). Scale bar, 10 μm
Fig. S4. Photomicrographs of the different channels of composite pictures that are depicted in false colour overlay in Fig. 4. A–D: FISH fluorescence; E: FLB fluorescence, or F–H: DAPI fluorescence; I: chlorophyll a autofluorescence.
Table S1. Summary of the 18 rDNA clone libraries from the Gulf of Gdańsk. Numbers of sequences per OTU (> 97% similarity) are given. Closest relatives are based on the BLAST search, with accession number in parentheses.

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