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Examples of high performance liquid chromatography (HPLC) application in marine ecology studies in the northern Adriatic

Vesna FLANDER-PUTRLE

Marine Biology Station, National Institute of Biology, Fornače 41, SI-6330 Piran; e-mail: flander@mbss.org

Abstract. Photosynthetic pigments have proved to be useful biomarkers of the abundance, composition and physiological status of the phytoplankton biomass in the marine environment. Using HPLC pigment analysis, we determined phytoplankton community structure in three different marine environments: in the area of a fish farm, in the area of sewage outlets, and in the mucilaginous aggregates. At the reference site we observed seasonal changes with prevalence of fucoxanthin-containing phytoplankton (i.e. diatoms) during winter/spring and autumn. In the fish farm area the concentration of chlorophyll *a* degradation products was higher, whereas in the locally enriched environment of sewage outlets we observed only small changes in taxonomic composition and phytoplankton biomass. The impact of season is more expressed than the impact of sewage discharge. With the use of HPLC pigment analysis we determined the development of phytoplankton community in different stages of mucilage aggregates. Phytoplankton biomass was composed primarily of diatoms, and as the aggregates aged, diatoms increased in the relative biomass. Our examples have proven the usefulness and suitability of HPLC pigment analysis in marine ecology studies.

Key words: pigments, HPLC, phytoplankton, Adriatic Sea, mucilage, fish farm, sewage outlets

Izvleček: PRIMERI UPORABE TEKOČINSKE KROMATOGRAFIJE VISOKE LOČLJIVOSTI (HPLC) PRI ŠTUDIJAH MORSKE EKOLOGIJE SEVERNEGA JADRANA – Fotosintezna barvila so se izkazala kot dobri kazalci abundance, sestave in fiziološkega stanja fitoplanktonske biomase v morskem okolju. Z uporabo HPLC-analize barvil smo določili strukturo fitoplanktonske združbe v treh različnih morskih okoljih: na območju ribogojnic, na območju komunalnih podvodnih izpustov in v sluzastih makroagregatih. Na referenčni postaji smo izmerili sezonske spremembe fitoplanktonske združbe s prevlado fukoksantin-vsebujočega fitoplanktona (t.j. diatomej) v zimsko-spomladanskem in jesenskem obdobju. Na območju ribogojnic smo izmerili višje koncentracije razgradnih produktov klorofila *a*, na lokalno obogatenem območju komunalnih izpustov pa smo opazili le manjše spremembe v taksonomski sestavi in biomasi fitoplanktona. Vpliv sezone je bolj izražen kakor vpliv podvodnih komunalnih izpustov. Z uporabo HPLC-analize barvil smo določili razvoj fitoplanktonske združbe v posameznih razvojnih stadijih sluzastih makroagregatov. V fitoplanktonski združbi sluzastih makroagregatov so prevladovale diatomeje in njihov delež k relativni biomasi je s starostjo agregatov še naraščal. Navedeni primeri kažejo na uporabnost in primernost HPLC-analize barvil v študijah morske ekologije.

Ključne besede: barvila, HPLC, fitoplankton, Jadransko morje, sluz, ribogojnice, komunalni izpusti

Introduction

In the late 1970's and early 1980's, HPLC (High Performance Liquid Chromatography) pigment analysis started to be used to determine phytoplankton community structure (Jeffrey et al. 1997). With the use of thin layer chromatography (Jeffrey 1974) it became evident that some photosynthetic pigments, above all carotenoids, are specific for particular phytoplankton taxonomic groups. Chlorophyll *b* was found only in green algae, while peridinin is characteristic for the majority of dinoflagellates (Jeffrey 1974, Jeffrey et al. 1975). Fucoxanthin is the main accessory photosynthetic pigment of diatoms (Stauber & Jeffrey 1988), but in considerable quantities it can also be found in some species of other phytoplanktonic groups (dinoflagellates, prymnesiophytes). The presence of its derivative 19'-hexanoyloxyfucoxanthin in samples indicate the presence of the microalgal group prymnesiophytes (Arpin et al. 1976, Wright & Jeffrey 1987), while 19'-butanoyloxyfucoxanthin is a biomarker for chrysophytes (silicoflagellates). Zeaxanthin is characteristic of cyanophytes (Guillard et al. 1985), although it can also be present in some representatives of prochlorophytes. In comparison to other techniques, the use of HPLC has proven to be the most appropriate to define the concentration of these pigments in phytoplankton community. For chlorophyll *a* measurements several different methods are used. The most popular in phytoplankton studies is fluorometric method, but spectrophotometric method is used as well. HPLC pigment analysis, together with characteristic ratios (K) between chlorophyll *a* concentration and biomarker pigment concentration (Tab. 1), enables researchers to estimate the relative contribution of the different phytoplankton groups to the total biomass (total chlorophyll *a*) (Everitt et al. 1990, Mackey et al. 1996). HPLC pigment analysis provides quantitative data on approximate 50 pigments from all algal classes (Wright et al. 1991), including picoplankton and fragile cell types that may be difficult to identify and count by microscopy or flow cytometry. The computer program CHEMTAX (Mackey et al. 1996) allows us to apportion the pigments between the various algal classes. Photosynthetic pigments have proved to be useful biomarkers of the abundance, composition and physiological status of the phytoplankton biomass in the marine environment, although they cannot be considered to be fully specific diagnostic markers of individual phylogenetic groups of phytoplankton, and their use should therefore be exercised with caution (Jeffrey et al. 1997). Several studies have demonstrated good agreement between chemotaxonomic assessments of phytoplankton and conventional microscopy (Carreto et al. 2003, Gieskes & Kraay 1986). Such a comparison performed in the Gulf of Trieste (Švagelj et al. 1996, Terzić 1996) confirmed the biomarker approach as a very promising tool to investigate phytoplankton in that area. The dynamics of the group-specific phytoplankton biomass in the northern Adriatic can be successfully described by the 7 most prominent biomarker pigments (Tab. 1). These pigments elucidate more than 90 % of the biomass associated with chlorophyll *a* in the northern Adriatic (Terzić 1996). The concentration of chlorophyll *a* is used to estimate the spatial and temporal changes in phytoplankton biomass as well as the abundance of phytoplankton. The chlorophyll *a* degradation products are indicators of physiological status of the phytoplankton community. Chlorophyllide *a* and pheophorbide *a* indicate the dying algae (Head et al. 1994). Pheopigments, above all pheophorbide *a*, are also indicators of zooplankton grazing and senescent algae (Jeffrey et al. 1997).

But there are also deficiencies regarding the use of HPLC method. One of the deficiencies of HPLC method, and of all other methods for phytoplankton pigments determination, is the variability of pigments' concentrations in the cell. This normally varies with different light quality and intensity, nutrients availability and physiological status of the cell. Not all biomarker pigments are fully specific for particular phytoplankton groups; on the other hand, not all species of particular phytoplankton group contain the same accessory pigment. Another deficiency of HPLC as a method for the determination of the phytoplankton community in comparison to the use of microscopy is that phytoplankton organisms cannot be determined to the species level. On the other hand, some phytoplankton species and groups are too small to be observed by microscope, but since they contain biomarker pigments they can be detected with HPLC. For this reason, the combination of methods, HPLC pigment analysis and microscopy, for the complete determination of the phytoplankton community structure in marine ecosystems is therefore recommendable.

In present paper we introduce three cases of HPLC pigment analysis approach in determining the composition and shifts in phytoplankton community structure, proving the HPLC method to be fast, precise and repeatable approach in marine ecology studies. Where possible, we compared the results of HPLC pigment analysis with references of phytoplankton counting using microscopy.

Table 1. Values of chlorophyll *a* : biomarker pigment ratios (K) in different phytoplankton groups, and their biomarker pigments.
Tabela 1. Vrednosti razmerja klorofil *a* : biomarkersko barvilo (K) pri različnih fitoplanktonskih skupinah, in njihova biomarkerska barvila.

Phytoplankton group	**Biomarker pigment**	**K**
diatoms	fucoxanthin	1.2[a]
prymnesiophytes	19'-hexanoyloxyfucoxanthin	1.1[a]
dinoflagellates	peridinin	1.5[a]
cyanophytes	zeaxanthin+lutein	1.7[b]
silicoflagellates	19'-butanoyloxyfucoxanthin	1.6[c]
cryptophytes	alloxanthin	1.85[d]
green algae (chlorophytes)	chlorophyll *b*	0.9[a]

[a] Terzić 1996, [b] Stransky & Hager 1970, [c] Everitt et al. 1990, [d] Hager & Stransky 1970

Materials and methods

All samples in the studies were collected in the Gulf of Trieste (Fig. 1). For the reference site we selected the sampling site 00OB, where the Oceanographic buoy is located, and sampling site F located close to the 00OB sampling site. The fish farm study area is situated in the inner part of the semi-enclosed Bay of Piran. We took water samples in six different directions at sampling sites at the distances 6 m and 20 m from the centre of the cage, respectively, and on the profile from the centre of fish cages outwards in a northern direction (Fig. 2). Fish cages were circular with 8 m diameter, and the depth of cages was 6 m. To study the impact of sewage discharged from a mechanical treatment plant into a shallow coastal sea, two submarine outfalls (sampling site 00PO in the area of Piran outfall and sampling site 00IO in the area of Izola outfall) were chosen as the area for extensive research. All mucilaginous aggregates were collected in the Gulf of Trieste, at the surface and in different depths of the water column.

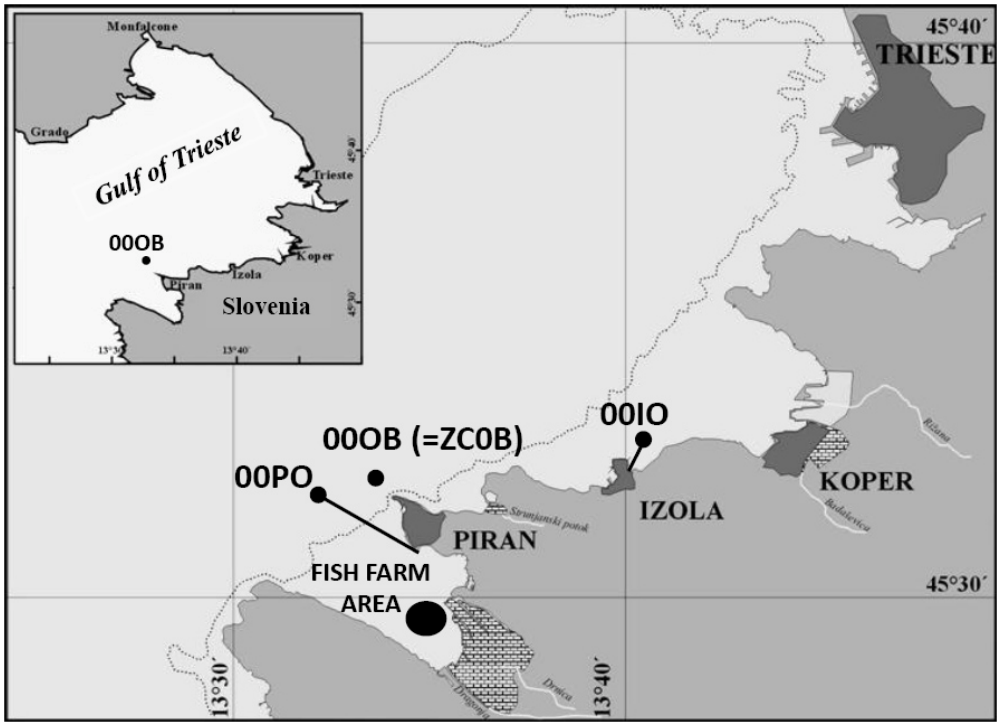

Figure 1. Sampling sites in the Gulf of Trieste.
Slika 1. Mesta vzorčevanja v Tržaškem zalivu.

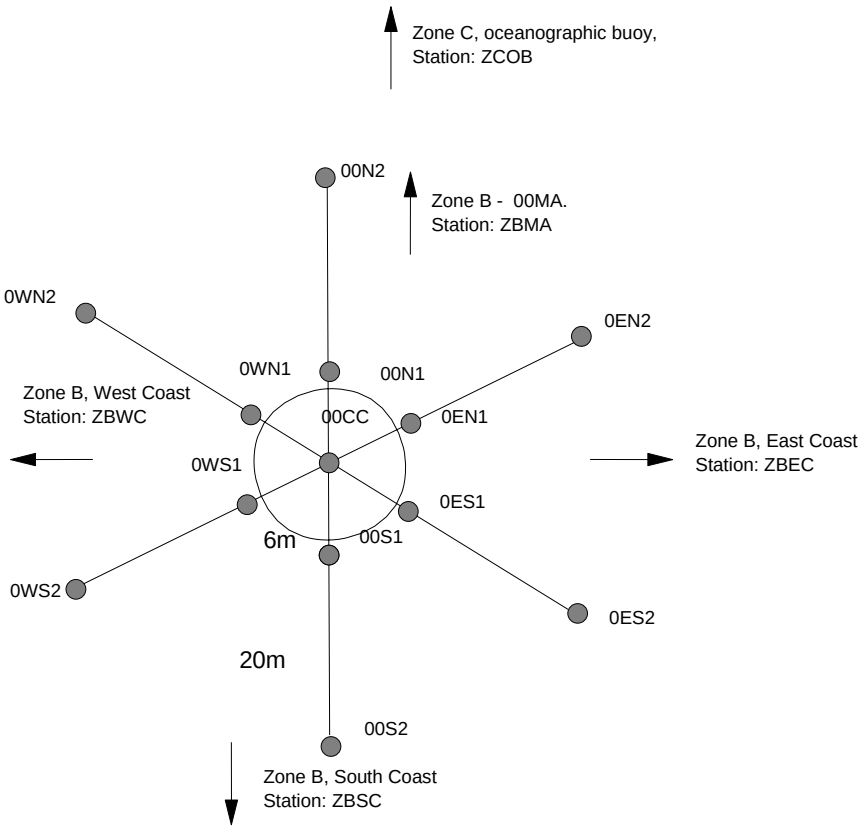

Figure 2. Scheme of sampling sites at fish farm area in October 2005. Circle represents a fish cage with 8 m diameter. Sampling sites are depicted as grey dots, located 6 m (00N1, 0EN1, 0ES1, 00S1, OWS1, 0WN1) and 20 m (00N2, 0EN2, 0ES2, 00S2, OWS1, 0WN2) from the centre of the cage (00CC).

Slika 2. Shema mest vzorčevanja na območju ribogojnic, oktobra 2005. Krog ponazarja kletko ribogojnice premera 8 m. Sive pike ponazarjajo mesta vzorčevanja od sredine kletke (00CC) navzven, na razdalji 6 m (00N1, 0EN1, 0ES1, 00S1, OWS1, 0WN1) in 20 m (00N2, 0EN2, 0ES2, 00S2, OWS1, 0WN2).

Seawater samples (1 l) were filtered through 47 mm Whatman GF/F filters that were immediately frozen until HPLC pigment analysis. Filters were extracted in 90 % acetone using sonication. Mucilage samples were condensed and the pellets extracted in 90 % acetone using sonication. After centrifugation for 10 min at 4,000 rpm, the supernatant was used in HPLC pigment analysis.

The only method used in this study was HPLC pigment analysis. An aliquot (300 µl) of clarified acetone extract was mixed with 300 µl 1 M ammonium acetate and injected into a gradient HPLC system. Pigments were analysed using the reverse-phase HPLC method (Barlow et al. 1993, Mantoura & Llewellyn 1983). The HPLC system with 200 µl loop was equipped with a single piston pump (Star 9010 Solvent Delivery System, Varian) and a reverse phase 3 µm C_{18} column (Pecosphere, 35×4.5 mm, Perkin Elmer) Solvent A consisted of 80 % methanol and 20 % 1 M ammonium acetate and solvent B 60 % of methanol and 40 % of acetone. A linear gradient from 0 % B to 100 % B for 10 min was followed by an isocratic hold at 100 % B for 6 min. The flow rate was 1 ml min^{-1}. Chlorophylls and carotenoids were detected by absorbance at 440 nm using a UV/Vis spectrophotometric detector (Spectra Physics, Model UV2000). Degradation products of chlorophyll *a* were detected by measuring fluorescence (420/672 nm) with a spectrofluorimetric detector (Spectra Physics, Model FL2000). Data collection and integration were performed by utilizing Agilent ChemStation software (Agilent Technologies). For HPLC analysis, we used gradient grade solvents for liquid chromatography from Merck KGaA, Germany, while for the identification of single pigments we used the pigment standards from DHI Water and Environment, Denmark.

To estimate the contribution of various phytoplankton groups, we multiplied the concentrations of individual biomarker pigments with published values of chlorophyll *a*: biomarker pigment ratios (K; Tab. 1). The relative contributions of different phytoplankton groups to total biomass (total chlorophyll *a*) was estimated using the equation: $X = K (C_{pig}/C_{chl\,a})$, where X is the relative contribution of different phytoplankton groups to total biomass, K is the chlorophyll *a*: biomarker pigment ratio for a certain phytoplankton group, C_{pig} is the concentration of biomarker pigment for that phytoplankton group, and $C_{chl\,a}$ is the concentration of chlorophyll *a* in the sample.

Results

We defined phytoplankton community based on HPLC pigment measurements in different environments: in the seawater at the reference sites, in the fish farm area, in the area of sewage outlets, and in the mucilaginous aggregates. The phytoplankton pigments were determined regarding their retention time at the column (Fig. 3).

Natural conditions (reference sites)

The regular monitoring of phytoplankton pigments gives an overview of seasonal changes in the phytoplankton community. Results obtained in the Gulf of Trieste (at the sampling site 00OB) with HPLC pigment analysis indicate, for example, increases of fucoxanthin concentration during the winter (in January 500 - 678 ng l^{-1}) and autumn months (in October 378 - 922 ng l^{-1}) (Fig. 4). This coincides with normal seasonal diatom blooms. In August-September we measured an increase in zeaxanthin concentration (13 - 155 ng l^{-1}) (Fig. 5). The most abundant phytoplankton group in the water column during spring and summer according to HPLC pigment analysis are prymnesiophytes (45.5 % ± 14.4 %), and diatoms (59.6 % ± 19.3 %) during autumn and winter (Fig. 6).

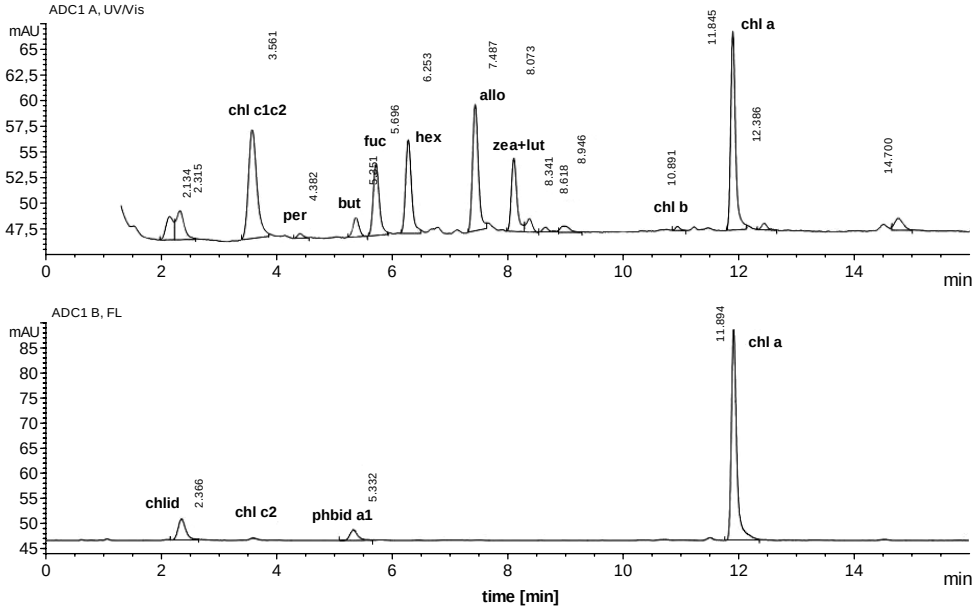

Figure 3. Chromatogram of chlorophylls and carotenoids with UV/Vis detector (upper record) and degradation products of chlorophyll *a* with spectrofluorimetric detector (lower record). From single peaks we can determine the quality (position of the peak) and quantity (the peak area) of phytoplankton pigments. (chl a – chlorophyll *a*, chl b – chlorophyll *b*, chl c1c2 – chlorophyll c_1 and c_2, per – peridinin, but – 19'-butanoyloxyfucoxanthin, fuc – fucoxanthin, hex – 19'-hexanoyloxyfucoxanthin, allo – alloxanthin, zea+lut – zeaxanthin and lutein, child – chlorophyllide and phbid – pheophorbide)

Slika 3. Kromatogram klorofilov in karotenoidov, posnet z UV/Vis detektorjem (zgornji zapis), in razgradnih produktov klorofila *a*, posnet s fluorescenčnim detektorjem (spodnji zapis). Iz posameznega pika lahko določimo kvaliteto (lega pika) in kvantiteto (površina pika) fitoplanktonskih barvil (chl a – klorofil *a*, chl b – klorofil *b*, chl c1c2 – klorofil c_1 in c_2, per – peridinin, but – 19'-butanoiloksifukoksantin, fuc – fukoksantin, hex – 19'-heksanoiloksifukoksantin, allo – aloksantin, zea+lut – zeaksantin and lutein, child – klorofilid and phbid a1– feoforbid a_1).

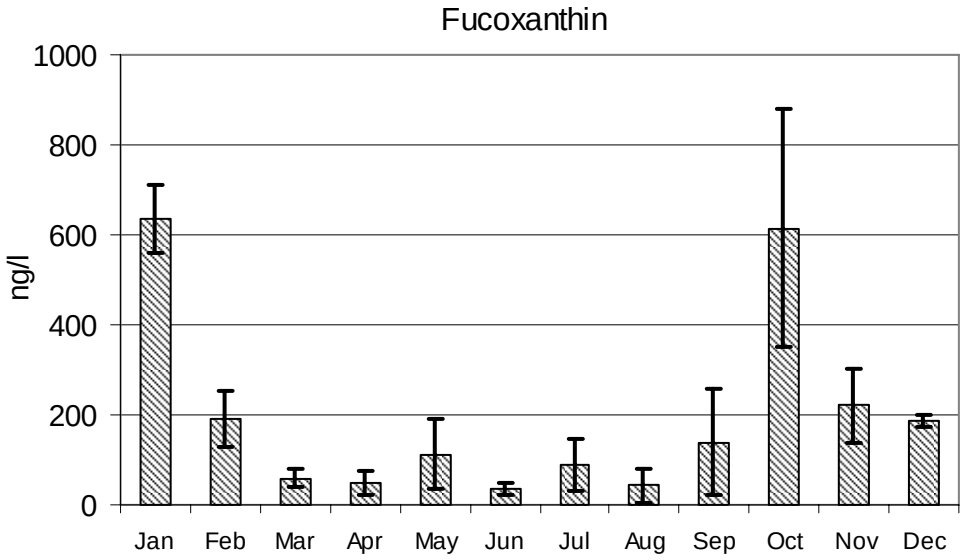

Figure 4. The results obtained in 2005 in the Gulf of Trieste with HPLC pigment analysis indicate increases of fucoxanthin concentration during seasonal blooms of diatoms.
Slika 4. Rezultati, pridobljeni s HPLC analizo barvil leta 2005 iz vzorcev iz Tržaškega zaliva, kažejo povečane koncentracije fukoksantina v obdobjih sezonskega diatomejskega cvetenja.

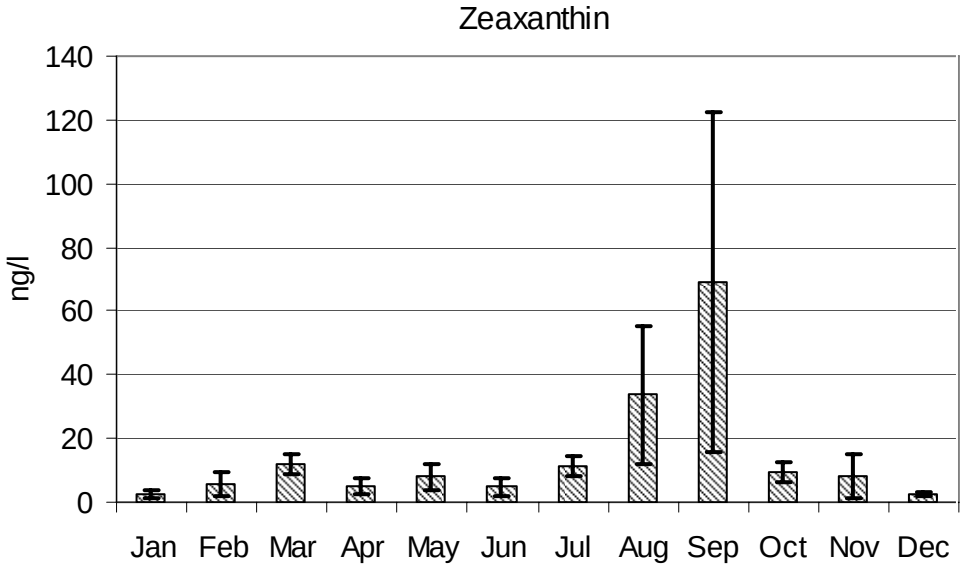

Figure 5. Results obtained in 2005 in the Gulf of Trieste with HPLC pigment analysis indicate increases of zeaxanthin concentration during August-September.
Slika 5. Rezultati, pridobljeni s HPLC analizo barvil leta 2005 iz vzorcev iz Tržaškega zaliva, kažejo povečane koncentracije zeaksantina v obdobju avgust-september.

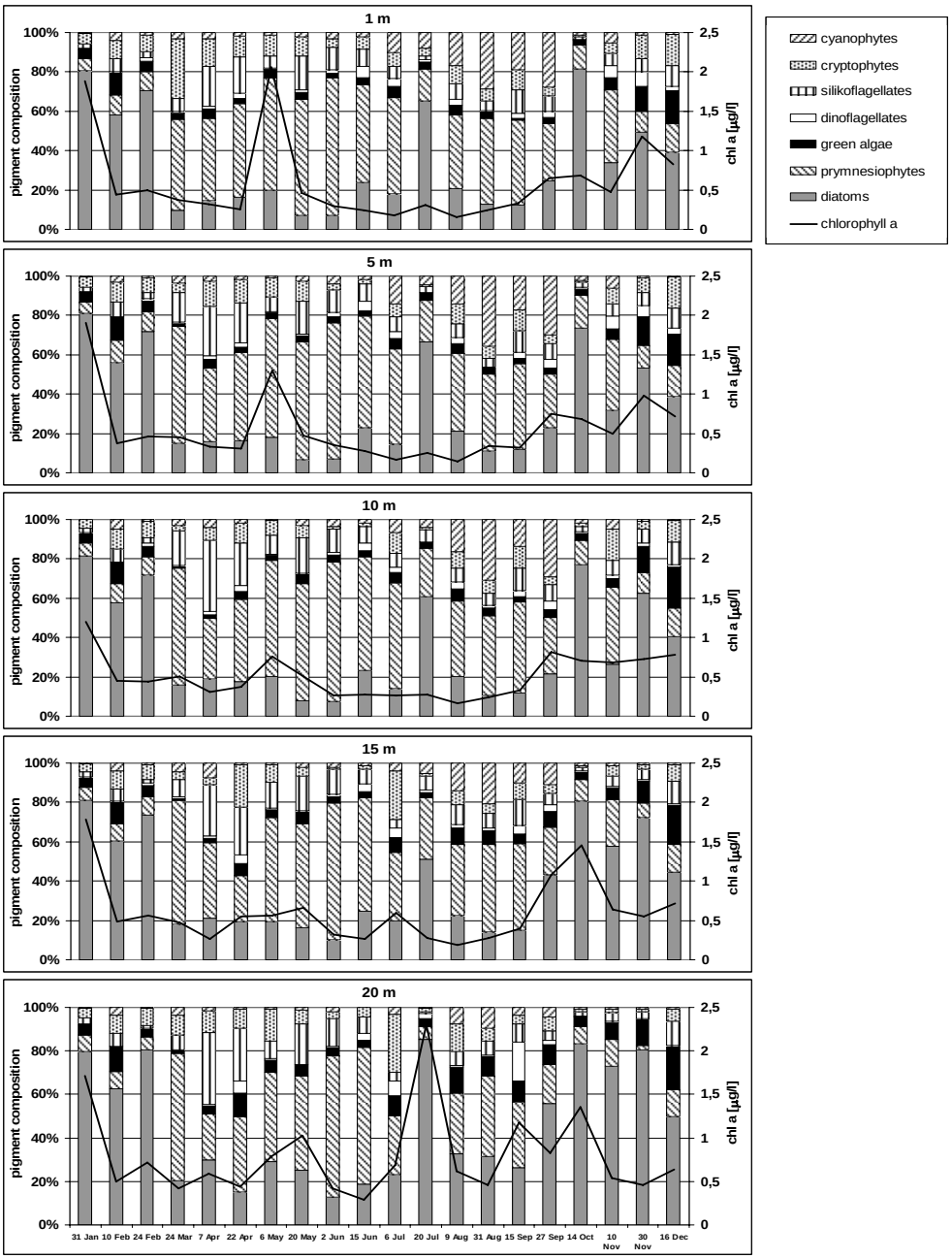

Figure 6. Phytoplankton composition and phytoplankton biomass, expressed as chlorophyll *a* concentration, obtained in 2005 at different depths at sampling site 00OB in the Gulf of Trieste.

Slika 6. Sestava fitoplanktonske združbe in fitoplanktonska biomasa, izražena s koncentracijo klorofila *a*, leta 2005 na različnih globinah postaje 00OB v Tržaškem zalivu.

Fish farm area

During the October 2005 sampling in the fish farm area we observed only minor differences in pigment concentrations and the phytoplankton groups' contribution to the total biomass. Chlorophyll *a* concentrations at the fish farm area were in the range from 895 up to 1568 ng l^{-1} (1149 ± 167 ng l^{-1}). Regarding phytoplankton pigments, the main phytoplankton groups were diatoms (66.3 – 77.9 %) followed by prymnesiophytes (13.7 – 21.2 %; Fig. 7). However, we noticed differences in the profile from the centre of the fish cage outward, mostly in the decrease of chlorophyll *a* degradation products concentration. The highest concentration of chlorophyll *a* degradation products was measured at sampling site 00N1 (A: before and B: three hours after feeding), situated 6 m from the centre of the fish cage towards the north, just outside the cage, where we observed the highest influence of fish farming, while 20 m from the centre of fish cage towards the north at sampling site 00N2 the influence was slightly lower, and the decline in the direction outward from the fish cage was nicely expressed at sampling site ZBMA, situated 1,166 m from the centre, and sampling site ZC0B, situated 6,825 m from the centre towards the north (Fig. 8). In the centre of fish cage (00CC) the concentration of chlorophyll *a* degradation products was lower than at distances of 6 and 20 m from the centre. The main concentration of chlorophyll *a* degradation products at the fish farm area was due to concentrations of chlorophyllide *a* (226 – 708 ng l^{-1}) and pheophorbide a_1 (166 – 515 ng l^{-1}). A similar trend was observed for pheophorbide a_2, but here the concentrations were very low (8 – 21 ng l^{-1}).

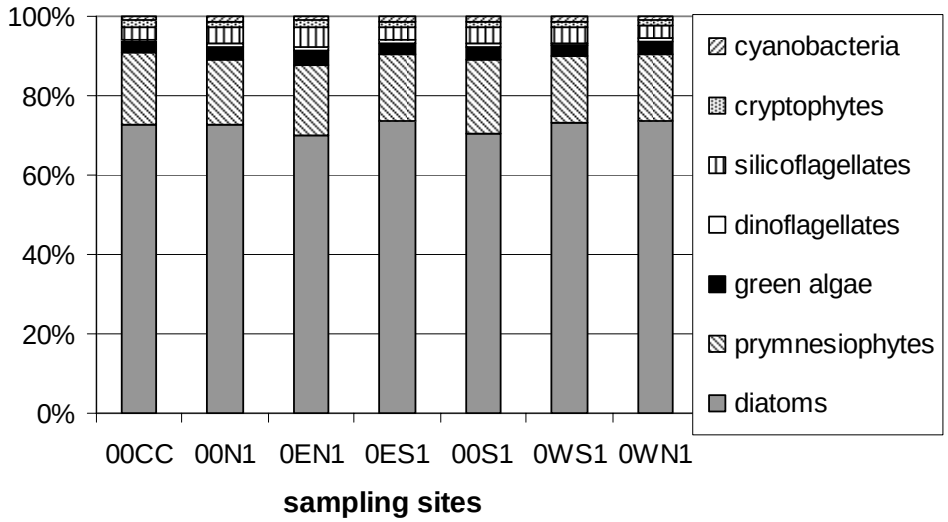

Figure 7. The phytoplankton community structure at the fish farm area (in the centre of fish cage – 00CC and 6 m from the centre in six different directions – 00N1, 0EN1, 0ES1, 00S1, 0WS1, 0WN1).
Slika 7. Sestava fitoplanktonske združbe na območju ribogojnice (v središču ribje kletke – 00CC ter 6 m od centra v šestih različnih smereh – 00N1, 0EN1, 0ES1, 00S1, 0WS1, 0WN1).

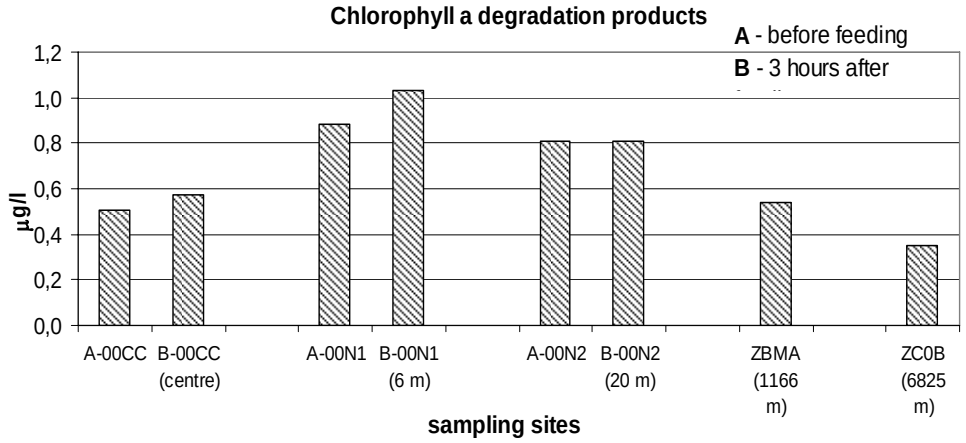

Figure 8. Chlorophyll *a* degradation products concentration in the profile from the centre of the fish cage (00CC) outward (00N1 - 6 m from the centre, 00N2 - 20 m from the centre, ZBMA, ZC0B - in the direction outward to the open sea); A – before feeding, B – three hours after feeding.

Slika 8. Koncentracija razgradnih produktov klorofila *a* v različni oddaljenosti od območja ribogojnic (00CC – središče ribje kletke, 00N1 – 6 m, 00N2 – 20 m, ZBMA – 1166 m in ZC0B – 6825 m od središča ribje kletke proti odprtemu morju); A – pred hranjenjem, B – tri ure po hranjenju.

Sewage outlet area

Samples were taken in the area of two sewage outlets in different seasons during the same year. We observed small changes in taxonomic composition and phytoplankton biomass in a locally enriched environment. Chlorophyll *a* concentrations were different in different seasons. The highest concentrations of chlorophyll *a* in the water column were measured in autumn; 472 ± 78 ng l^{-1} at 00PO in November 1999, and 1387 ± 243 ng l^{-1} at 00IO in October 2000. The concentrations of chlorophyll *a* from submarine outfall area (00PO) were in the range of results from reference site F (Fig. 9) situated close to 00OB site in the Gulf of Trieste. Fucoxanthin concentrations measured at 00PO (45 – 447 ng l^{-1}; 179 ± 87 ng l^{-1}) and 00IO (57 – 1333 ng l^{-1}; 399 ± 383 ng l^{-1}) were the highest among all biomarker pigments. Concentration of 19'-hexanoyloxyfucoxanthin were the lowest during the summer sampling except in the bottom layer. Higher concentrations were measured during the autumn sampling, particularly in the upper 14 m. The chlorophyll *b* concentrations were higher in deeper layers of the water column. Increased concentrations of zeaxanthin were observed during the November sampling in the upper 10-m layer of the water column. The phytoplankton composition determined on the basis of phytoplankton pigments also vary among different seasons with the highest contribution of diatoms during winter, summer and autumn, and prymnesiophytes during spring.

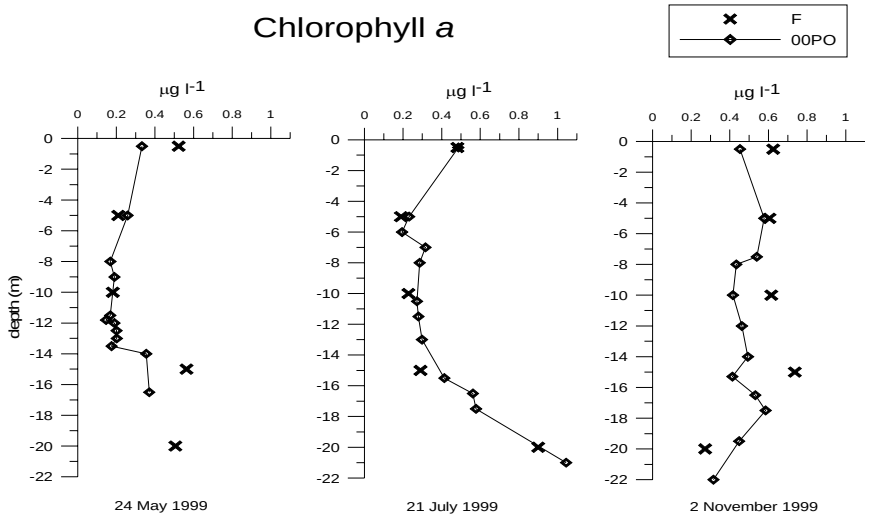

Figure 9. Chlorophyll *a* concentration measured in the water column during the May, July and November 1999 samplings in the Piran sewage outlet area (00PO) and on reference site (F).
Slika 9. Koncentracija klorofila *a*, izmerjena maja, julija in novembra 1999 v vodnem stolpcu na območju piranskega komunalnega izpusta (00PO) in referenčne postaje (F).

Mucilaginous aggregates

Using HPLC analysis, the development of phytoplankton assemblages in mucilaginous aggregates was observed during mucilage event in 2000. Phytoplankton biomass measured as chlorophyll *a* concentration per dry mass was very high in mucilaginous aggregates (390.82 ± 101.16 µg g^{-1} of dry mass). In the early loose stage of mucilage the phytoplankton community was heterogeneous, as indicated by the diversity of pigments. Our HPLC analysis showed that although the phytoplankton biomass was composed primarily of diatoms, prymnesiophytes, dinoflagellates, cyanobacteria and green algae were also present (Fig. 10). The number of phytoplankton groups showed some tendency to decrease as the aggregates aged, while diatoms increased in the relative biomass. The fucoxanthin concentration indicated more or less the same trend as chlorophyll *a* concentration (Fig. 11). The concentration of 19'-hexanoyloxyfucoxanthin was higher in the early loose mucilaginous aggregates, and decreased with the age of aggregates.

Based on HPLC pigment analysis, the fucoxanthin concentrations revealed that diatoms were the main contributor to the phytoplankton biomass in the mucilaginous aggregates (22.5 – 92.7 % of total chlorophyll *a* biomass). Significant contributions were also made by zeaxanthin- (cyanobacteria: 1.6 – 19.0 %), peridinin- (dinoflagellates: 0.6 – 62.2 %), 19'-hexanoyloxyfucoxanthin- (prymnesiophytes: 0.0 – 25.3 %), and chlorophyll *b*- containing (green algae: 3.0 – 30.1 %) phytoplankton groups.

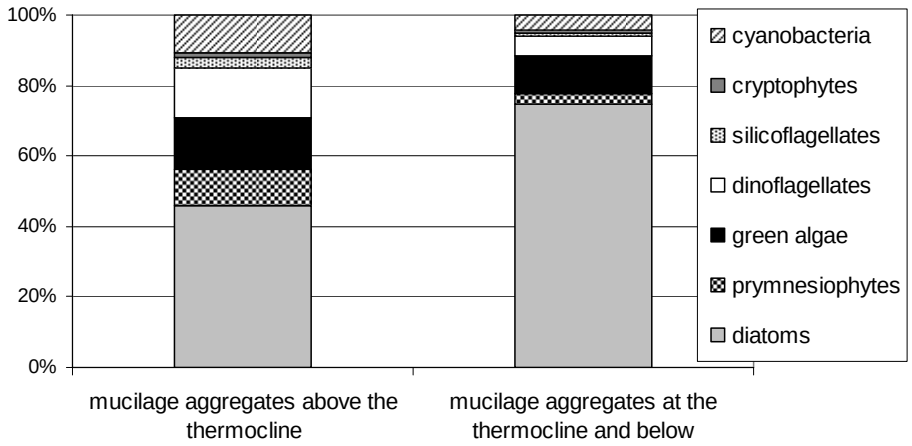

Figure 10. The phytoplankton groups' contributions to the total biomass in mucilaginous aggregates collected above the thermocline, as well as at and below thermocline during the 2000 mucilage event.
Slika 10. Prispevek posameznih fitoplanktonskih skupin k celokupni biomasi v agregatih sluzi, nabranih nad, ter na in pod termoklino med pojavom sluzi leta 2000.

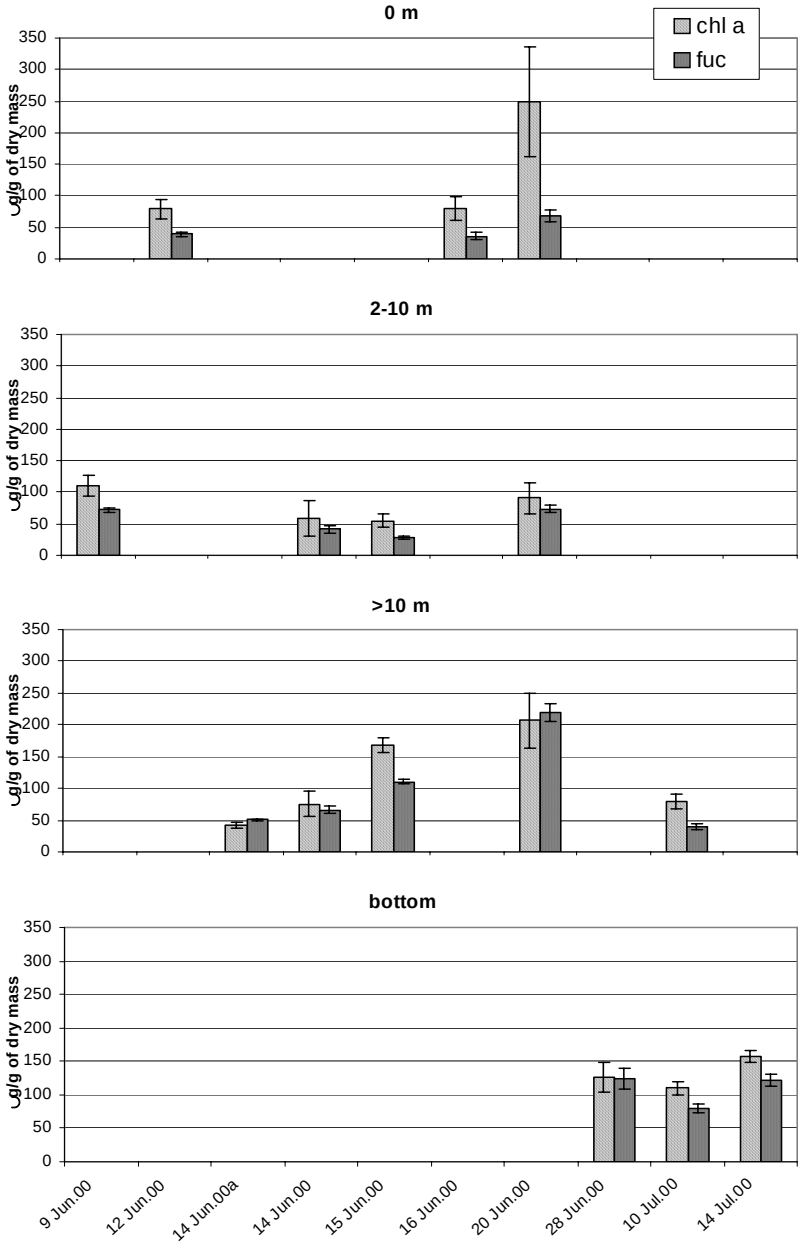

Figure 11. Chlorophyll *a* and fucoxanthin concentrations in the mucilage aggregates collected at different depths during the 2000 mucilage event.

Slika 11. Koncentracije klorofila *a* in fukoksantina v agregatih sluzi, nabranih na različnih globinah med pojavom sluzi leta 2000.

Discussion and conclusions

Natural conditions (reference site)

A regular monitoring of phytoplankton pigments gives an overview of seasonal changes in the phytoplankton community, in which prymnesiophytes are the most abundant during spring and summer, while during autumn and winter the prevalence of diatoms was observed. With seasonal diatom blooms observed with the use of microscopy (Mozetič et al. 1998) as increased diatom abundance, the increase of fucoxanthin concentration during the winter/spring and autumn months is connected. Increase in zeaxanthin concentration during August-September coincides with the seasonal cyanobacteria peak observed using microscopy (Turk et al. 2001) as well.

Fish farm area

Aquaculture has a serious impact on the environment and may subsequently affect populations of phytoplankton differently (Arzul et al. 2001). Regarding phytoplankton pigments, the phytoplankton composition with prevalence of diatoms followed by prymnesiophytes did not vary from the normal seasonal community in the Gulf of Trieste (Mozetič et al. 1998).

Chlorophyll *a* degradation products are good indicators of the physiological state of phytoplankton and show that fish farming influences the phytoplankton population, since we observed higher concentrations in the fish farm area. Chlorophyll *a* degradation products indicate a higher level of decaying, dead and decomposing phytoplankton biomass, and increased grazing. The main contribution of chlorophyll *a* degradation products in the fish farm area was due to the presence of chlorophyllide *a*, which is a biomarker of senescent phytoplankton cells, mostly diatoms (Jeffrey et al. 1997). The significant higher pheopigment concentrations in the fish farm area (< 3 nautical miles from fish farm zone) was also measured in the Aegean Sea (Pitta et al. 2005), indicating an intensive grazing on phytoplankton biomass by heterotrophic organisms.

Phytoplankton pigments can be also used to assess the trophic state of the coastal sea using the F_p pigment index (Claustre 1994). The F_p index is determined as $F_p=(fuc+per)/(fuc+per+hex+but+zea+chlb+allo)$, where *fuc* is fucoxanthin, *per* is peridinin, *hex* is 19'-hexanoyloxyfucoxanthin, *but* is 19'-butanoyloxyfucoxanthin, *zea* is zeaxanthin, *chlb* is chlorophyll *b* and *allo* is alloxanthin; the values of the F_p ratio from this equation are in a range from 0 to 1. The single pigment index, F_p, is used to capture the trophic status of an ecological province and is also used as a measure of phytoplankton biomass supporting new production (Claustre 1994). Calculated values of F_p trophic index were higher in the fish farm area compared to the control site (Flander-Putrle & Malej 2003), again showing a strong influence of the fish farm on the environment.

Sewage outlet area

To study the impact of sewage discharge into the shallow coastal sea, microscopic (Mozetič et al. 2001) and HPLC analyses were used to identify the taxonomic composition and ratios among major phytoplanktonic taxa. Both methods indicated small changes in taxonomic composition and phytoplankton biomass in a locally enriched environment, which would be difficult to follow without a small-scale vertical sampling strategy.

We observed that the season had a greater impact on phytoplankton community composition than the sewage outlet area. Concentration of 19'-hexanoyloxyfucoxanthin determined using HPLC analysis indicated the occurrence of some prymnesiophytes, which are part of the group of (unidentified) microflagellates, and the occurrence of *Emiliania huxleyi*, the most abundant coccolithophorid (Mozetič et al. 2001). A significant correlation was observed between diatoms and their characteristic biomarker – fucoxanthin (Mozetič et al. 2001). Increased concentrations of zeaxanthin were observed during the November sampling in the upper 10-m layer of the water column, when cyanobacteria (zeaxanthin as biomarker) were also abundant. A distinctive feature was the increase of chlorophyll *b* concentrations throughout the water column. Higher concentrations were measured in deeper layers of the water column. Higher chlorophyll *b* concentrations may be due to the increase of green algae biomass in the nutrient-enriched water layers. Chlorophyll *b*-containing flagellates (chlorophytes and particularly euglenophytes) are common in coastal inshore waters, brackish waters, and also in polluted estuaries with high nutrient loads and large organic content (Mozetič et al. 2001).

Mucilaginous aggregates

Massive occurrences of mucilaginous aggregates have been observed seasonally during summer in the northern Adriatic Sea, when the water column is stratified. Mucilaginous episodes of different intensity have been occurring in the northern Adriatic at irregular intervals for over 270 years. Prior to our study (Flander-Putrle & Malej 2008), the phytoplankton composition of mucilaginous aggregates was most often assessed microscopically (Stachowitsch et al. 1990, Totti et al. 2005). These data were mostly qualitative and did not distinguish among the different mucilage forms and stages. An additional problem with aged mucilaginous masses is that microscopic identification of the organisms is very difficult since they can be badly damaged (Pistocchi et al. 2005). Using HPLC analysis, the development of phytoplankton assemblages in mucilaginous aggregates was observed. Based on HPLC pigment analysis, the fucoxanthin concentrations revealed that diatoms were the main contributor to the phytoplankton biomass in the mucilaginous aggregates. A similar composition, with diatom prevalence followed by dinoflagellate contribution, was reported using microscopic observations of mucilaginous aggregates (Revelante & Gilmartin 1991, Stachowitsch et al. 1990, Monti et al. 1995, Baldi et al. 1997). Weekly or more frequent observations during the summers of 1997, 2000, 2002 and 2004 indicated that the phenomenon passed through a characteristic »life cycle«, with identifiable stages of development. The analyses suggest a progress from fresh mucilaginous aggregates

(with prymnesiophytes and silicoflagellates) to intermediate stages (heterogeneous phytoplankton community) to »aged« mucilaginous aggregates (when diatoms prevail) (Flander-Putrle & Malej 2008). Comparisons of the succession of phytoplankton groups in seawater showed differences between mucilaginous and non-mucilaginous years. The former were characterised by a higher contribution of prymnesiophytes in spring (April-May; in mucilaginous years 38.9 % ± 17.6 %, and in non-mucilaginous years 19.6 % ± 12.8 %), and the prevalence of diatoms in summer (July; in mucilaginous years 64.2 % ± 15.6 %, and in non-mucilaginous years 40.3% ± 13.6 %) (Flander-Putrle & Malej 2008). The F_p index showed a clear difference between mucilaginous and non-mucilaginous years. In April-May (1997 and 2000), before the appearance of mucilage, F_p ratios were lower (0.25±0.10 and 0.27±0.10), respectively, indicating a smaller fraction of old production compared to the non-mucilaginous years (0.40±0.15 and 0.39±0.15). In contrast, when mucilage occurred in June-August, F_p ratios were higher (0.62±0.25 and 0.54±0.20) than during non-mucilaginous years (0.45±0.18 and 0.38±0.17) (Flander-Putrle & Malej 2008).

Conclusions

The use of HPLC pigment analysis proves to be very useful in marine ecology studies. Only using HPLC pigment analysis without any microscopy, we can get a clear insight into what is going on in different ecological conditions. It is a very fast, precise and repeatable method. Some phytoplankton species and groups are too small to be observed by microscope, but since they contain biomarker pigments, they can be detected with HPLC. On the other hand, phytoplankton organisms can be determined to the species level using microscopy, while this is impossible with the use of HPLC pigment analysis. We are well aware that HPLC pigment analysis cannot fully replace microscopy. For this reason, the combination of methods, HPLC pigment analysis and microscopy, for the complete determination of the phytoplankton community in marine ecosystems is recommendable.

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