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Changes in microbial food web structure in response to changed environmental trophic status: A case study of the Vranjic Basin (Adriatic Sea)

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ABSTRACT

Vranjic Basin, in the eastern part of KaštelaBay (middle Adriatic Sea), received municipal wastewater until offshore submarine outfalls were finished in November 2004. To identify the responses of the microbial community to changes in the trophic status of the marine environment, two 4-year periods were compared: a eutrophic period (2001–2004) when the sewage waters entered the Basin and an oligotrophic period (2005–2008) after the outfalls were completed. The switch from eutrophic to oligotrophic conditions was accompanied by decreases in bacterial abundance, bacterial production and chlorophyll *a*, and increase in heterotrophic nanoflagellate (HNF) abundance and bacterial specific growth rate. Qualitative changes in the phytoplankton community manifested through dramatically decreased abundance of the diatom species *Skeletonema costatum* and Euglenophyta *Eutrepti*ella spp. during the oligotrophic period. Furthermore, the percent contribution of pico–nano phytoplankton chlorophyll to total chlorophyll increased from less than 40% during the eutrophic period to more than 60% during the oligotrophic period. Changes in seasonal patterns of phytoplankton, bacteria and HNF abundance were also observed, with summer maxima during the eutrophic period and spring and autumn maxima during the oligotrophic period. Significant changes in the microbial food web were also identified. During eutrophic conditions, bacteria were dominantly under the phytoplankton-mediated bottom-up control whereas HNF were dominantly controlled by ciliate grazing (top-down control). In contrast, during the oligotrophic period, predominantly top-down control of bacteria by strong HNF grazing was observed. At the same time, HNF were spared from strong ciliate predation pressure because the ciliates apparently switched their dominant prey from HNF to the pico-nano phytoplankton fraction during that period.

1. Introduction

The structure of the microbial food web and the microbial role in biogeochemical processes in aquatic ecosystems may vary considerably depending on environmental trophic status (Cotner and Biddanda, 2002; Berglund et al., 2007). Nutrient enrichment generally leads to an increased abundance and biomass of all components of the pelagic food web (Berninger et al., 1991) but the response of each group can differ strongly (Gasol and Vaqué, 1993; Jansson et al., 1996). Therefore, nutrient supply can influence the structure of the pelagic community and may have an effect on the interactions among the community components (Šolić et al., 2008). In the pelagic environment two contrasting trophic pathways have been identified as predominant, the classical chain (herbivorous food web) and the microbial food web (Legendre and Rassoulzadegan, 1995; Froneman, 2004). The former goes from large phytoplankton and zooplankton resulting in a shorter and simpler food web with a high carbon export potential, whereas the latter is a complex network of small prokaryotic organisms. Relative importance of the microbial food web decreases with increasing trophic status and nutrient recycling within the microbial web is of less significance at high nutrient loading (Cermeño et al., 2006; Vargas et al., 2007). Oligotrophic conditions are characterised by low nutrient concentrations with high proportions of dissolved rather than particulate carbon which favours prokaryotic heterotrophs over phagotrophic heterotrophs (Cotner and Biddanda, 2002). Further, the dominant producers in oligotrophic systems are small plankton (<5–10 μm), which are too small to be effectively ingested by mesozooplankton (Finlay and Roff, 2004; Vargas and Gonzalez, 2004). Therefore, heterotrophic and mixotrophic nanoflagellates may constitute an additional trophic link in oligotrophic systems (Unrein et al., 2007; Zubkov and Tarran, 2008). In contrast, in eutrophic systems the high relative concentrations of nutrients and particulate carbon along with increased

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particle sinking may decrease the importance of prokaryotic heterotrophs (Cotner and Biddanda, 2002). Agawin et al. (2000) showed that picophytoplankton dominated (>50%) the biomass and production in oligotrophic, nutrient poor, waters but represented <10% of autotrophic biomass and production in nutrient-rich waters. Reduced contribution of picophytoplankton in productive waters was suggested to result from increased loss rates, whereas the dominance of picophytoplankton in oligotrophic waters was attributed to the differential capacity to use nutrients as a function of differences in size and growth rates between picophytoplankton and larger cells (Marañon et al., 2003; Peréz et al., 2006). In oligotrophic systems, interactions between autotrophs and heterotrophs are tightly coupled because the dominant heterotrophs (bacteria and protozoa) have similar size, growth rates and nutrient composition as the dominant autotrophs (pico-nano phytoplankton).

Structural changes in the pelagic food web can result in a shift from bottom–up (BU) and top–down (TD) control of some groups of microorganisms. The BU control refers to the limitation of microorganisms by resources (food), and the TD regulation refers to the limitation of microorganisms below levels supportable by resources alone, because of predators. Several studies with very large data sets, which were performed over a large range of aquatic environments, suggest that bacteria seem to be more BU controlled in eutrophic systems, and more TD controlled in oligotrophic systems (Billen et al., 1990; Gasol, 1994; Gasol et al., 2002). However, the importance of the BU and TD regulation of bacteria may vary seasonally (Ducklow, 1992; Šolić et al., 1998) and even daily (Psenner and Sommaruga, 1992). Therefore, it seems that switches between two types of control are following changes in environmental conditions which occur on both spatial and temporal scales (Šolić et al., 2009).

The present study was performed in the eastern part of KaštelaBay (Vranjic Basin) in the coastal central Adriatic Sea. Until 2005, the Basin received high quantities of organic matter and nutrients due to the discharge of untreated sewage waters. The quantity of sewage entering the Basin was 4.4 million m^3 per year, which is about one-third of the total wastewater produced by the town of Split and smaller nearby cities (about 300,000 inhabitants) (Tudor et al., 1992). As a result, classical eutrophication effects occurred: an increase in primary production, oxygen supersaturation/hypoxia events, occurrence of monospecific blooms and so forth (Marasović and Pucher-Petković, 1991; Šolić et al., 1997). In November 2004, the sewage system was completed, comprising a network of pipelines, pumping stations, a tunnel, treatment plants and offshore submarine outfalls. Consequently, sewage discharge into Vranjic Basin suddenly stopped. This event provided a good opportunity to follow the responses of the microbial community to sudden changes in the trophic status of the marine environment, in a kind of *in situ* experiment. Most studies dealing with the effect of trophic status on the structure of microbial food webs have compared eutrophic and oligotrophic environments (very often between coastal areas and the open sea) (Sanders et al., 1992; Gasol et al., 2002). However, these environments differ in many other characteristics besides trophic status, such as depth, temperature, salinity, meteorological conditions and water column dynamics (Stenseth et al., 2006). In the present study, the changes in microbial community could be explained by changing trophic status with more certainty, because all other environmental factors should be more similar than when two different areas are compared.

In this paper, bacterial abundance, production and specific growth rate, abundance of HNF and ciliates as well as chl *a* (pico-nano and micro fractions) were measured. Changes in the abundance of these groups of microorganisms, their seasonal cycles and the mechanism of their control (bottom–up vs. top–down) were studied in response to changes in environmental trophic status.

2. Materials and methods

2.1. Study area

KaštelaBay, which has a surface area of 61 km^2 (15 km long and 6 km wide; Fig. 1) and an average depth of 23 m, communicates with the adjacent channel through an inlet 1.8 km wide and 40 m deep. The river Jadro, which discharges into the eastern part of the bay, is the most important freshwater source, with an average annual inflow of 10 $m^3\ s^{-1}$ (Orlić et al., 2007). Water circulation in the bay is generated mostly by the local wind, which is related to the passage of mid-latitude cyclones over the area (Gačić et al., 1987). The average water renewal time is about one month, while under strong wind conditions it can be as short as five days (Zore-Armanda, 1980). During the warm period of the year (July to September) wind forcing is relatively weak and the freshwater inflow low, due to which the renewal time is rather long. Vranjic Basin is a shallow, semi-enclosed eastern part of KaštelaBay, characterised by reduced water exchange with the rest of the Bay. Hydrographic properties are largely influenced by the River Jadro freshwater outflow. In the warmer part of the year weak water dynamics almost completely separate Vranjic Basin from the rest of KaštelaBay, and closed and circular water movements form within the Basin (Zore-Armanda, 1980).

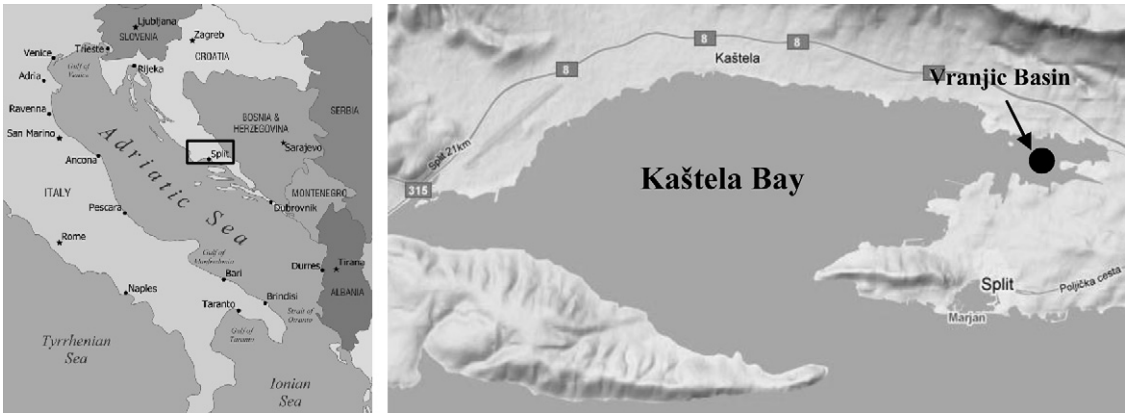

Fig. 1. Study area and the sampling station.

2.2. Data collection

Samples were collected on a monthly basis from January 2001 to December 2008 at depths of 0, 5, 10 and 15 m during daylight period using 5 l Niskin bottles. All samples employed for cell counts were poured into sterile acid washed glass bottles, fixed immediately with formaldehyde (final concentration 2%) and counted within 2 days after collecting. The same sampling and counting methods were used throughout the investigation period.

Bacteria and heterotrophic nanoflagellates (HNF) were enumerated by epifluorescence microscopy ("Olympus" BX50, 1000× magnification), using the standard DAPI staining technique (Porter and Feig, 1980). For biovolume estimates, the length and width of bacterial cells were measured with an eyepiece graticule (New Porton G12, Graticules, Ltd, UK). Biovolume was then converted to carbon biomass, assuming 0.220 pgC μm^{-3} (Bratbak and Dundas, 1984).

Bacterial cell production was measured from DNA synthesis based on incorporation rates of [^{3}H]thymidine (Fuhrman and Azam, 1982). [Methyl-^{3}H]thymidine was added to 10 ml samples at a final concentration of 10 nmol (specific activity 86 Ci $mmol^{-1}$). Triplicate samples and a formaldehyde-killed adsorption control (final concentration 0.5%) were incubated in dark at ambient temperature for 1 h. The incubations were stopped with formaldehyde (final concentration 0.5%). The thymidine samples were extracted with ice-cold TCA according to Fuhrman and Azam (1982). The TCA-insoluble fraction was collected by filtering the samples through a 0.2-μm pore size Sartorius filter. The conversion factors for bacterial cell production were calculated from bacterial cell number and [^{3}H]thymidine incorporation during bacterial growth in 1-μm prefiltered seawater (Riemann et al., 1987): $CF = (N_2 - N_1)/{}^3H$, where N_1 = number of bacteria in the beginning of the experiment, N_2 = number of bacteria at the end of the experiment, ^3H = integrated [^{3}H]thymidine incorporation rate during the experiment. From the estimates of bacterial production (BP) and bacterial biomass (BB), bacterial specific growth rate (SGR, d^{-1}) was computed as: $SGR\ (d^{-1}) = \ln(1 + BP/BB)$ (Gasol et al., 2002).

Ciliate samples were preserved in seawater containing 2.5% formaldehyde previously buffered with $CaCO_3$. We chose this fixative (instead of Lugol) because it does not stain the detritus (Fonda Umani and Beram, 2003), which can be abundant in the eutrophicated area of KaštelaBay. Since formaldehyde causes cell loss (Leakey et al., 1994), our data may be somewhat underestimated. Samples were sedimented (Utermöhl, 1958) for 48 h in plastic containers and decanted down to a volume of 2 l. This volume was poured into a cylinder and sedimented for 48 h. The excess volume was then reduced to 200 ml. Prior to microscopic analysis, the volume was further reduced to 20 ml. Decanting was carried out using a vacuum pump and a slightly curved pipette that removed water from the surface. The organisms were counted in glass chamber (76 × 47 × 6 mm) using an inverted microscope "Olympus" CK40 at magnifications of ×100.

Chlorophyll *a* measurements were performed using a Turner 112 fluorometer following acetone extraction (Strickland and Parsons, 1972). To distinguish producers which dominate in microbial food web from those in classical (herbivorous) food web chlorophyll *a* was measured from two phytoplankton size fractions: pico-nano fraction (<10 μm) and micro fraction (>10 μm) (Cushing, 1989; Legendre, 1990).

Temperature and salinity were measured with CTD multiparameter probes (Idronaut and SeaBird) with precision greater than ±0.01 °C and ±0.02 ppt, respectively. Dissolved oxygen concentration was determined by Winkler titration (Strickland and Parsons, 1972). Dissolved inorganic nutrients (NO_3, NO_2, NH_4, PO_4) were analysed on Bran + Luebbe as well as Seal AutoAnalyser using modified automated methods (Grasshoff, 1976). Total inorganic nitrogen and phosphorus were analysed as nitrate and orthophosphate, respectively after UV oxidation (Ace Glass Inc., USA).

2.3. Statistical analyses

All statistical analyses were performed using the statistical package StatSoft Inc. Statistica for Windows. The correlations between parameters were expressed as Pearson correlation coefficients. Regression analyses were used to explain relationship between bacterial production and bacterial abundance, and between concentration of chlorophyll *a* and bacterial production. Differences of the mean values of the studied parameters between the two periods were analysed by Student's *t*-test.

Ordination of samples by Principal Component Analysis (PCA) is a technique for mapping the samples in a low number of dimensions (usually 2) such that the distance between samples attempts to reflect (dis)similarity between them. In this study PCA was used to identify the main patterns of temporal fluctuations of the studied parameters. PCA ordination, based on a correlation matrix, was used to detect similarity between the fluctuations. To identify the main patterns of temporal fluctuations of the examined parameters PCA was carried out on the series consisting of the log-transformed monthly values data sets, separately for two studied periods.

2.4. Methods for discrimination relative importance of bottom–up and top–down control

In order to examine the regulation of bacteria by substrate availability (bottom–up control, BU) and by predation (top–down control, TD), data were analysed according to two different approaches based on empirical comparative data analyses (for more detailed explanation see Gasol et al., 2002).

The simultaneous observations of bacterial and HNF abundance are plotted on the graph which includes empirically determined maximum attainable abundance (MAA) and mean realised abundance (MRA) of HNF according to the framework proposed by Gasol (1994) (see Fig. 8). The points close to the MAA line indicate strong coupling between bacteria and HNF, which according to Gasol (1994) could be interpreted as strong TD control on bacteria and BU control on HNF. The MRA line balances the effect of both types of control. Thus, points which lay well below the MRA line indicate conditions when bacterial abundance was not controlled by HNF grazing (weak coupling between bacteria and HNF), which suggests domination of BU control on bacteria. At the same time, points below the MRA line suggest TD control of HNF. Therefore, *D* values (difference between maximal and realised HNF abundance at each bacterial concentration) could be a good indicator of the importance of HNF predators in controlling bacterial abundance, as a higher *D* value implies lower HNF predation pressure. Therefore, as a proxy for grazing pressure of HNF on bacteria, *D* values could be a good indicator of the relative importance of BU and TD control of bacteria and HNF.

Another empirical model for determining relative importance of BU and TD control of bacteria was proposed by Billen et al. (1990). These authors posited that bacterial production serves as a surrogate of nutrient supply, which is difficult to measure. If predatory mortality is low, all bacterial production can be converted into bacterial biomass. Alternatively, if grazing on bacteria is very high, bacterial biomass does not increase with increasing bacterial production. Therefore, a strong relationship (significant correlation and high positive slope) between bacterial production and bacterial biomass suggests predominantly BU control, whereas no relationship (no correlation or low slope) indicates TD control. Moreover, Ducklow (1992) suggested that the slope of a log–log regression

between bacterial production (independent variable) and bacterial biomass (dependent variable) would indicate the strength of BU control (b >0.6, strong; $b = 0.4–0.6$, moderate; $b < 0.4$, weak; and $b < 0.2$, none).

3. Results and discussion

3.1. Change of the trophic status

Comparing the concentrations of dissolved inorganic and organic nutrients (nitrogen and phosphorus) before and after the construction of the submarine sewage outfall it was observed that the concentrations were statistically significantly lower (Student's *t*-test; $P < 0.01$) in the latter period (Fig. 2A–D), suggesting the change of environmental trophic status (terms eutrophic and oligotrophic will be used in relative sense further on). Oligotrophic period was also characterised by lower ranges of nutrient concentrations. Furthermore, during the period after the submarine sewage outfall was constructed, an average oxygen saturation statistically significantly decreased in the surface layer and increased in the bottom layer (Fig. 2E–F), suggesting decrease in primary production in the euphotic layer and decrease in oxygen consumption (organic matter accumulation) in the bottom layer.

3.2. Temperature and salinity

Seasonal patterns of temperature and salinity were compared between eutrophic and oligotrophic periods (Fig. 3). During both

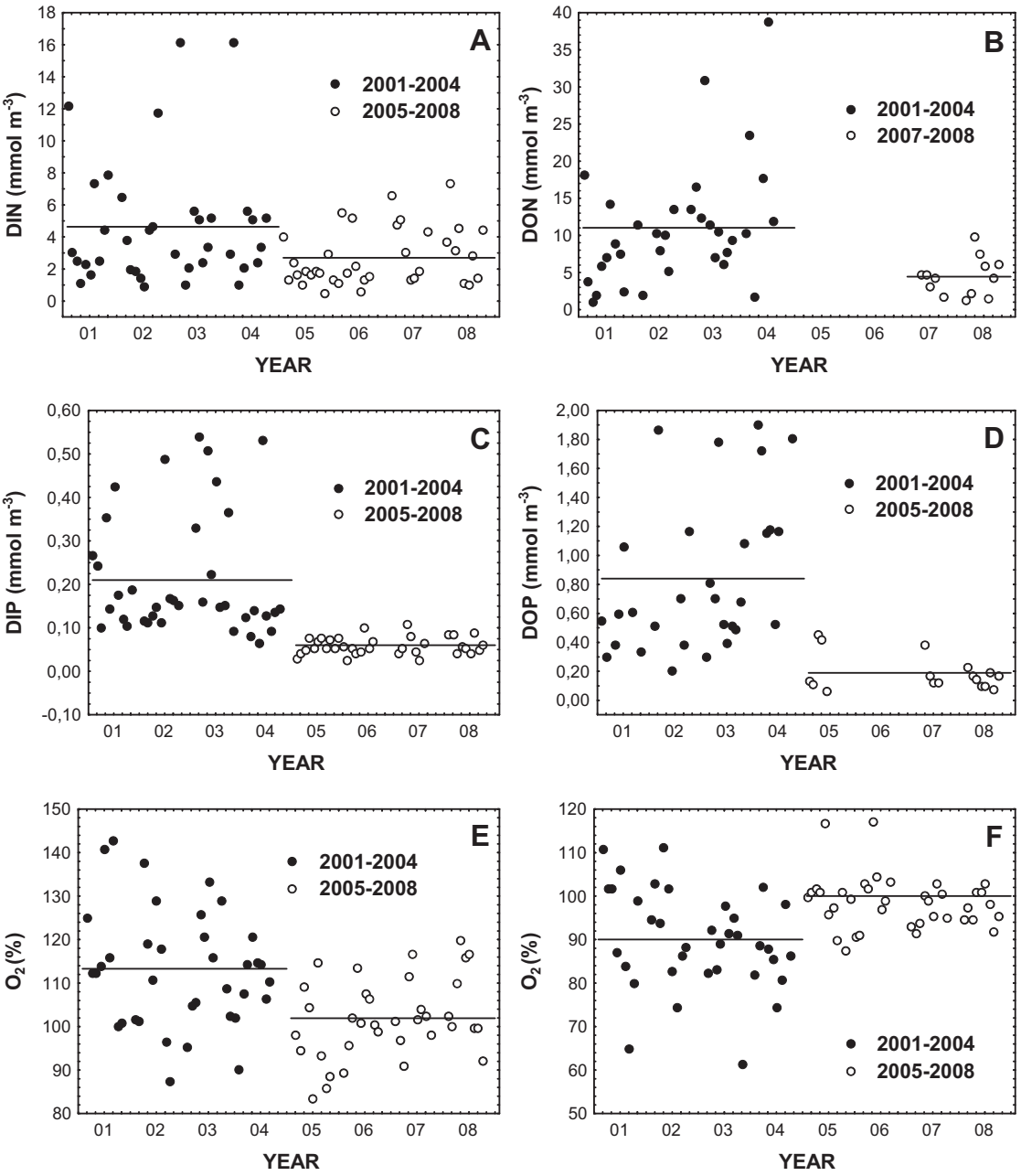

Fig. 2. Concentrations of dissolved inorganic nitrogen (DIN) (A), dissolved organic nitrogen (DON) (B), dissolved inorganic phosphorus (DIP) (C), dissolved organic phosphorus (DOP) (D) and oxygen saturation (%) in the surface (E) and bottom (F) layers. Average values (horizontal lines) for 2001–2004 and 2005–2008 (2007–2008 for DON) periods were statistically significantly different for all parameters (Student's *t*-test; $P < 0.01$).

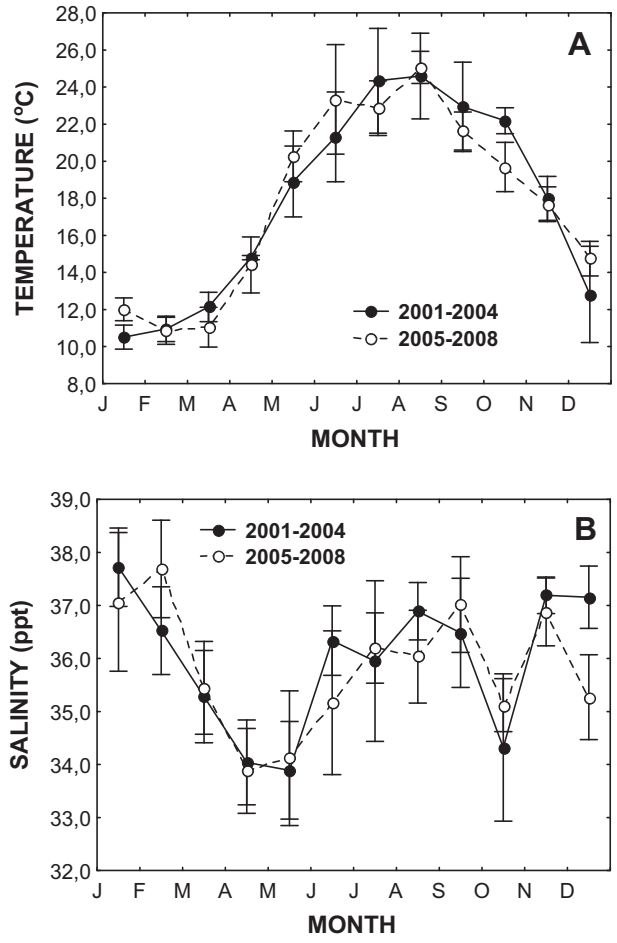

Fig. 3. Seasonal patterns of surface temperature (A) and salinity (B) during 2001–2004 and 2005–2008 periods (monthly means pooled for two periods ± SD).

periods the mean temperatures ranged from 10 to 11 °C in February to 25–26 °C in August and showed very similar seasonal patterns (Fig. 3A). Similar seasonal patterns were also established for salinity (Fig. 3B) showing the minimal values in spring (April–May) and autumn (October). Since hydrological properties of the Basin are largely influenced by the River Jadro freshwater outflow, the periods of the minimal salinity very probably coincided with the periods of the maximal water discharges. Therefore, it seems that there is no substantial change of hydrology between eutrophic and oligotrophic periods.

3.3. Quantitative and qualitative changes of the biological parameters

Comparison of the eutrophic period before the submarine sewage outfall was finished (2001–2004) to the oligotrophic period (2005–2008) after wastewater stopped entering the Basin showed a decrease in bacterial abundance, bacterial production and chlorophyll *a*, and an increase in HNF abundance and bacterial specific growth rate (SGR) (Fig. 4). During the eutrophic period, yearly means of bacterial abundance ranged from 4.79×10^6 to 6.31×10^6 cells ml^{-1}, with a mean value for entire period of $5.49 \pm 0.65 \times 10^6$ cells ml^{-1}. During the oligotrophic period, bacterial abundance ranged from 0.61×10^6 to 0.93×10^6 cells ml^{-1}, with a mean value for the entire period of $0.78 \pm 0.13 \times 10^6$ cells ml^{-1} (Fig. 4A). Similarly, the mean value of bacterial production decreased from 11.02 ± 0.90 μg C l^{-1} d^{-1} during the eutrophic period to 4.20 ± 1.24 μg C l^{-1} d^{-1} during the oligotrophic period (Fig. 4B). Chlorophyll *a* concentrations decreased from 1.76 ± 0.30 μg l^{-1} during the eutrophic period to 1.24 ± 0.26 μg l^{-1} during the oligotrophic period (Fig. 4D). On the other hand, SGR increased from 0.085 ± 0.011 d^{-1} during the eutrophic period to 0.210 ± 0.049 d^{-1} during the oligotrophic period (Fig. 4C), and HNF abundance increased from $0.57 \pm 0.03 \times 10^3$ cells ml^{-1} during the eutrophic period to $1.04 \pm 0.15 \times 10^3$ cells ml^{-1} during the oligotrophic period (Fig. 4E). Average values of all studied parameters statistically significantly differed (Student's *t*-test; $P < 0.05$ for chl *a*; $P < 0.01$ for all other parameters) in eutrophic (2001–2004) and oligotrophic (2005–2008) periods (Fig. 4).

Changes in total chlorophyll concentrations were accompanied by changes in the size composition of the phytoplankton. While large micro phytoplankton chl *a* fraction (CHL-M) decreased, small pico-nano phytoplankton chl *a* fraction (CHL-PN) showed a marked increase (Fig. 5). The percentage contribution of CHL-PN to total chlorophyll increased from less than 40% during the eutrophic period to more than 60% during the oligotrophic period. This result is in accordance with Bell and Kalff (2001), who reported that the contribution of the small phytoplankton fraction in the total phytoplankton community increased from <10% in the eutrophic conditions to >50% in the oligotrophic systems. The reason for this increase could be lower energetic costs associated with their biomass composition (Neidhardt et al., 1990) or their high affinity for inorganic nutrients (Button, 1986), which is also related to their small size. In spherical cells, as the cell gets smaller in diameter, the surface to volume ratio increases; therefore, there are fewer internal demands for nutrients and an increased relative capacity to supply those nutrients (Litchman et al., 2007).

The eutrophic period was characterised by intensive blooms of the centric diatom *Skeletonema costatum* (Greville) Cleve, ranging in abundance from 1.1 to 3.2×10^6 cells l^{-1} during the warmer part of the year. During the oligotrophic period, the abundance of *S. costatum* was up to 2.90×10^4 cells l^{-1} (Fig. 6). *S. costatum* is characterised by higher rates of nitrate uptake and growth (DeManche et al., 1979) that makes it a better competitor than many other diatoms under eutrophic conditions (Collos et al., 1997). In addition, high tolerance to ammonia makes this species more competitive in sewage-impacted waters (Lomas and Gelibert, 2000). Further, Euglenophytes are used as biological indicators of organic pollution in seawater (Stonik and Selina, 2001). Blooms of *Eutreptiella* spp., which were common in the Vranjic Basin during summer, did not occur in 2007 and 2008 (Fig. 6).

3.4. Changes in seasonal cycles

Changes in seasonal cycles of chl *a*, bacterial abundance and HNF abundance are shown in Fig. 7. In an effort to follow the changes and facilitate comparison between different parameters, all data were standardised (shown as *z*-values). All studied parameters showed common changes in seasonal cycles between the two periods. During the eutrophic period, the parameters showed summer maxima (July–August for CHL *a*, July–September for bacterial abundance and June–August for HNF abundance). Maximal values of chlorophyll *a* ranged between 3 and 4 μg l^{-1}, bacterial abundance between 6×10^6 and 9×10^6 cells ml^{-1} and HNF abundance between 2×10^3 and 4×10^3 cells ml^{-1}. This pattern changed during the oligotrophic period, when the all followed parameters showed two peaks: the first in late spring (May–June) and the second in autumn (October). During this period maximal values of chlorophyll *a* ranged between 1.5 and 2 μg l^{-1}, bacterial abundance between 2×10^6 and 3×10^6 cells ml^{-1} and HNF abundance between 3×10^3 and 4×10^3 cells ml^{-1}. Reporting the increasing trend of eutrophication from 1960 to 1980

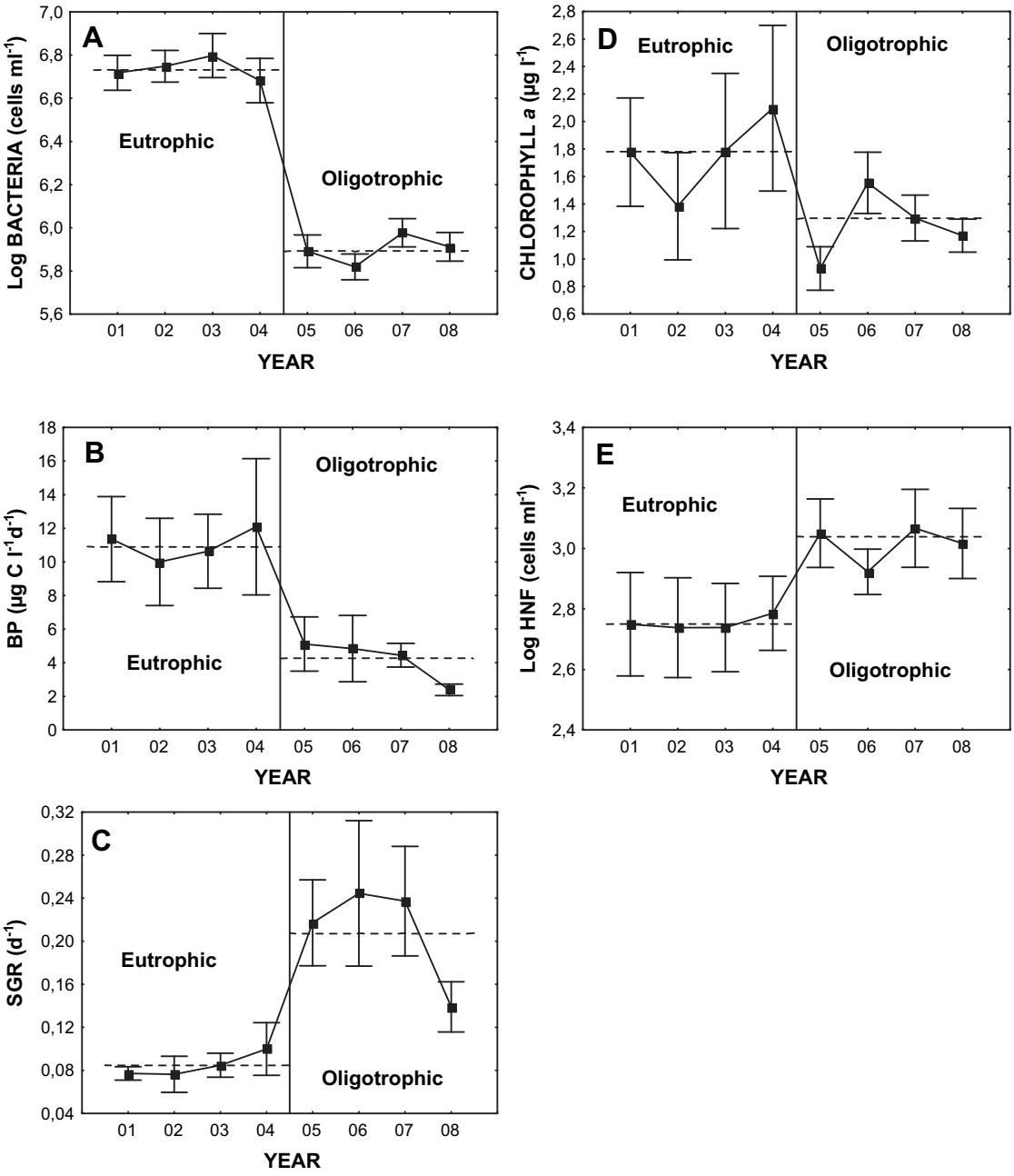

Fig. 4. Annual means (mean ± SE) of bacterial abundance (A), bacterial production (B), bacterial specific growth rate (SGR) (C), chlorophyll *a* (D) and HNF abundance (E) throughout the study period. Average values (dashed lines) statistically significantly differed (Student's *t*-test; $P < 0.05$ for chl *a*; $P < 0.01$ for all other parameters) in eutrophic (2001–2004) and oligotrophic (2005–2008) periods.

in the coastal Adriatic Sea, Marasović et al. (2005) also found that the increase in trophic status was followed by moving the phytoplankton peak from spring to summer period. Šolić et al. (1997) also reported a disturbance of the phytoplankton seasonal cycle due to eutrophication, arguing that in nutrient-unlimited conditions high phytoplankton abundance throughout the warmer period of the year could be the result of favourable temperature and light conditions.

3.5. Changes in interactions within the microbial food web

In order to examine the regulation of bacteria and HNF by substrate availability (bottom-up control, BU) and predation (top-down control, TD), simultaneous observations of bacterial and HNF abundance are plotted in Fig. 8, according to the framework proposed by Gasol (1994). The most points sampled during the eutrophic period lay above the MRA line, whereas most samples from the oligotrophic period are below the MRA line (Fig. 8). Consequently, the average values of *D* (the distance between the maximal and actual measured HNF abundance) were statistically significantly lower (Student's *t*-test: *t*-value = 8.27; df = 63; $P < 0.01$) during the oligotrophic period compared to the eutrophic period. This pattern suggests stronger coupling between bacteria and HNF (TD regulation of bacteria and BU control of HNF dominating) during the oligotrophic period. In the eutrophic period, HNF predation pressure on bacteria was lower suggesting a greater importance for BU control of bacteria and TD control of HNF. Several studies with very large data sets performed over a large

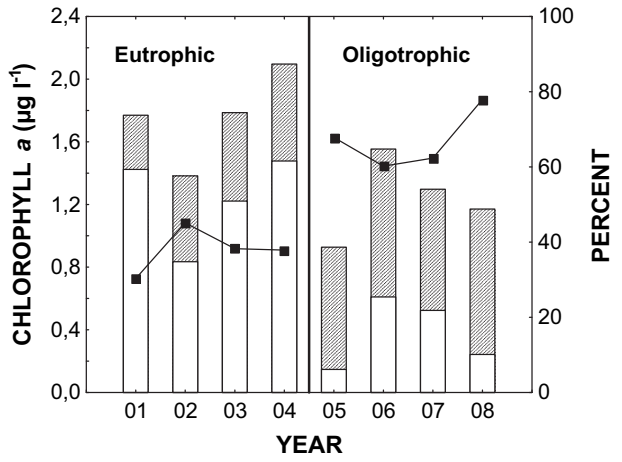

Fig. 5. Changes in chlorophyll *a* concentration of the micro (white bars) and the pico-nano (dashed bars) fraction of phytoplankton, and contribution of the pico-nano chlorophyll *a* fraction in the total chlorophyll *a* (lines) throughout the study period.

range of aquatic environments suggest that there is a general tendency of increased predation pressure on bacteria in oligotrophic environments, and a relaxation of this in more nutrient-rich environments (Billen et al., 1990; Gasol, 1994; Gasol et al., 2002). This pattern has also been verified in experiments with simulated oligotrophic and eutrophic environments (Weisse and Scheffel-Möser, 1991).

To determine if BU control of bacteria, which obviously dominated during the eutrophic period, switched to TD control during the oligotrophic period, the empirical model proposed by Billen et al. (1990) was applied. Application of this empirical model to our data revealed a strong positive relationship between bacterial biomass and production ($r = 0.825$; $b = 0.882$; $P < 0.001$) during the eutrophic period, indicating strong BU control of bacteria, and no relationship (insignificant correlation; $P>0.1$) during the oligotrophic period, suggesting the importance of TD control (Fig. 9A). Furthermore, a strong relationship between bacterial production and chl *a* during the eutrophic period was established ($r = 0.648$; $b = 0.453$; $P < 0.001$), suggesting BU control of bacteria was mediated by phytoplankton (Findlay et al., 1991; Gasol and Duarte, 2000; Kirchman et al., 2009) (Fig. 9B). This relationship was not found during the oligotrophic period (insignificant correlation; $P > 0.1$).

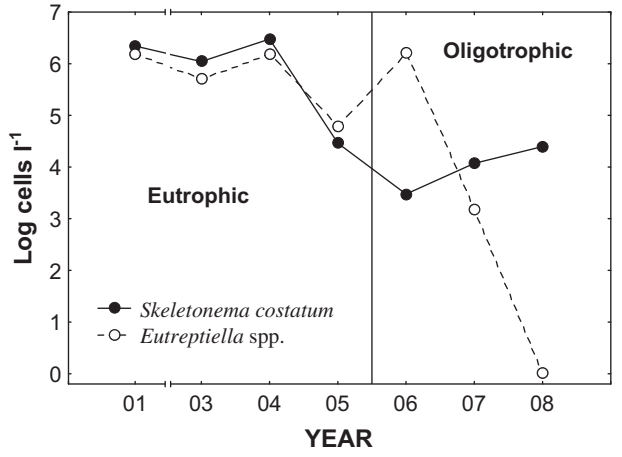

Fig. 6. Maximum abundance of *Skeletonema costatum* and Euglenophyte algae *Eutreptiella* spp. recorded during the warmer part of the year (May–September) throughout the study period (data for 2002 are missing).

To identify similarities in the temporal fluctuations of the examined parameters, Principal Component Analysis (PCA) was carried out on the time series data; analyses were conducted separately for the eutrophic and oligotrophic periods. Fig. 10 shows the ordination of the first two principal components (PC1 and PC2). These components can be regarded as the two best possible single

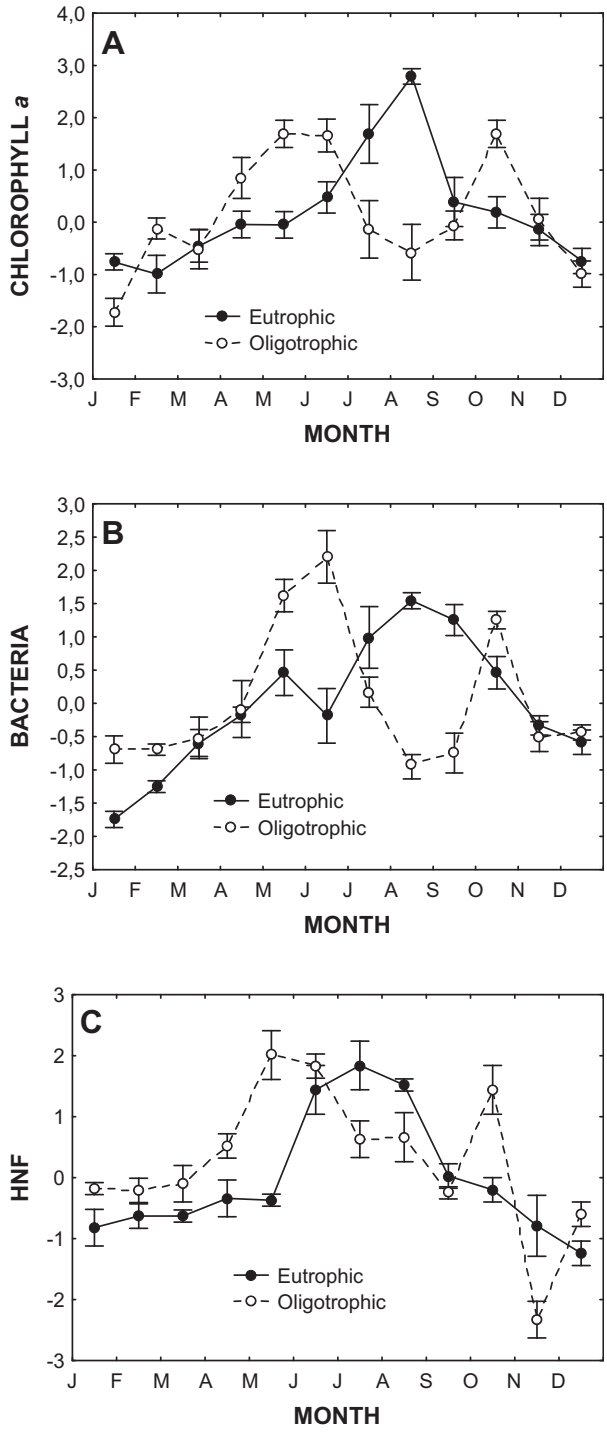

Fig. 7. Comparison of the seasonal patterns of phytoplankton biomass (expressed as chlorophyll *a*) (A), bacterial abundance (B), and HNF abundance (C) between eutrophic (2001–2004) and oligotrophic (2005–2008) periods. All data are shown as *z*-values (a normalised value created from a member of a set of data by expressing it in terms of the standard deviation from the mean, using the equation $z = (x - \mu)/s$, where x is an item of data, μ is the mean of the data, and s is the standard deviation; the mean and standard deviation of the set of such z values are 0 and 1, respectively).

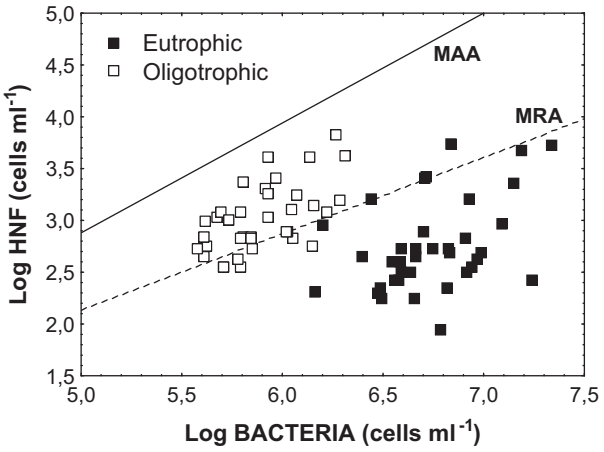

Fig. 8. Relationship between bacterial and HNF abundance at study stations, plotted in a theoretical model (Gasol, 1994) (MAA = maximum attainable abundance; MRA = mean realised abundance) during eutrophic (2001–2004) and oligotrophic (2005–2008) periods.

representations of month-to-month fluctuations for all studied parameters, explaining 71% of variability for both eutrophic and oligotrophic periods. During the eutrophic period, HNF and ciliates showed similar patterns of temporal fluctuations. Another group of parameters, with mutually similar patterns of fluctuations that differed from the first group, consisted of bacterial abundance, bacterial production and total chl *a* (Fig. 10A). During the oligotrophic period, the parameters grouped in a different way. One group consisted of bacterial abundance and HNF abundance, and another group consisted of ciliate abundance and chl *a* (both total and pico-nano fractions) (Fig. 10B).

These results are supported by the correlation analyses between the studied parameters, which were also conducted separately for the eutrophic and oligotrophic periods (Table 1). This analysis showed a strong relationship between bacteria (both abundance and production) and chl *a*, suggesting strong phytoplankton-mediated BU control of bacteria during the eutrophic period. Furthermore, the high correlation between HNF and ciliates (strong TD control of HNF) could explain the weak relationship between bacteria and HNF through the 'trophic cascade effect'; thus grazing pressure on HNF by ciliates resulted in reduced HNF grazing on bacteria. During the oligotrophic period, the strong predation pressure on HNF by ciliates ceased. Consequently, a strong relationship between bacteria and HNF was established, suggesting predominantly TD control of bacteria and BU control of HNF. On the other hand, a high correlation between ciliates and pico-nano chl *a* fraction (CHL-PN) suggests that the dominant prey of ciliates switched from HNF during the eutrophic

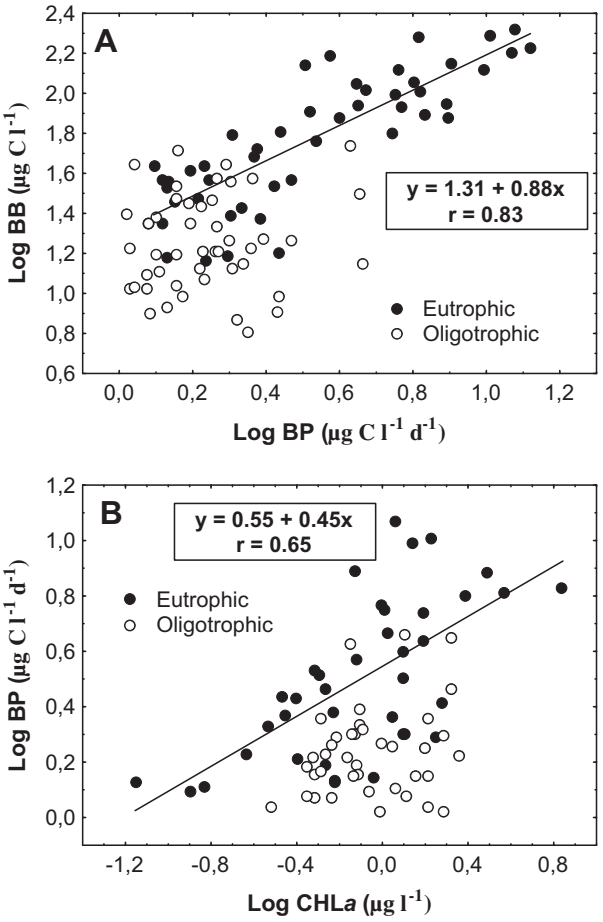

Fig. 9. Relationships between bacterial production (BP) and bacterial biomass (BB) (A), and between chlorophyll *a* (CHL *a*) and bacterial production (BP) (B) during eutrophic (2001–2004) and oligotrophic (2005–2008) periods. Only significant regressions are plotted.

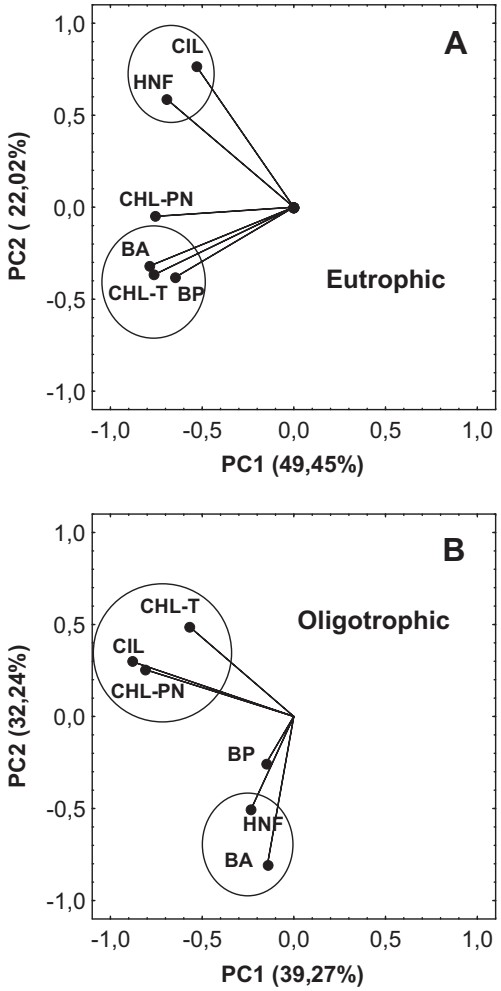

Fig. 10. Principal Component Analysis (PCA) plots based on log-transformed biological parameters (BA = bacterial abundance, BP = bacterial production, CHL-T = total chlorophyll *a*, CHL-S = chlorophyll *a* from the small phytoplankton fraction, HNF = heterotrophic nanoflagellates, CIL = ciliates) for eutrophic (2001–2004) (A) and oligotrophic (2005–2008) (B) periods.

Table 1
Pearson correlation coefficients between studied parameters during eutrophic and oligotrophic periods.

Correlation between	Eutrophic period	Oligotrophic period
Bacterial abundance : HNF abundance	0.367*	0.584*
Bacterial production : HNF abundance	n.s.	0.352*
Bacterial abundance : Ciliate abundance	n.s.	n.s.
Bacterial production : Ciliate abundance	n.s.	n.s.
Bacterial abundance : Chl *a*-T[a]	0.576*	n.s.
Bacterial production : Chl *a*-T	0.648*	n.s.
Bacterial abundance : Chl *a*-PN[b]	0.370*	n.s.
Bacterial production : Chl *a*-PN	n.s.	n.s.
HNF abundance : Chl *a*-T	n.s.	n.s.
HNF abundance : Chl *a*-PN	n.s.	n.s.
HNF abundance : Ciliate abundance	0.723*	n.s.
Ciliate abundance : Chl *a*-T	n.s.	0.401*
Ciliate abundance : Chl *a*-PN	n.s.	0.746*

*$P < 0.01$.
[a] Total chlorophyll *a*
[b] Chlorophyll *a* (pico-nano fraction).

period to pico-nano phytoplankton during the oligotrophic period (Fig. 10; Table 1).

Therefore, our results showed marked changes in the trophic relationship within microbial community between eutrophic and oligotrophic conditions. Eutrophic environment was characterised with tight coupling between bacteria and phytoplankton (phytoplankton-mediated bottom-up control) and strong top-down control of HNF by ciliate grazing. On the other hand, in oligotrophic conditions, top–down control of bacteria by HNF grazing was observed, and ciliates switched their dominant prey from HNF to the pico-nano phytoplankton fraction.

Tight coupling between bacteria and phytoplankton in eutrophic conditions could result from the fact that organic carbon is less available to bacterioplankton in eutrophic systems due to reduced relative phytoplankton exudation and/or increased sedimentation (Gasol and Duarte, 2000). Baines et al. (1994) found that the fraction of primary production lost to sinking increased with productivity in marine systems. Furthermore, there is considerable evidence indicating that prokaryotic heterotroph growth is often limited by inorganic nutrients in many different kinds of ecosystems (Toolan et al., 1991; Pomeroy et al., 1995; Cotner et al., 1997), even in relatively productive environments. Nutrient limitation (very often phosphate, but occasionally nitrogen) has been reported for the coastal Adriatic Sea (Vukadin and Stojanoski, 2001). This can be explained by the rapid rate of nutrient accumulation into particulate forms, as well as its removal from the water column by settling of organic matter to the seabed (Marasović et al., 2005). Finally, strong coupling between bacteria and phytoplankton during the eutrophic conditions could also result from a higher proportion of high nucleic acid (HNA) bacteria in comparison to low nucleic acid bacteria (LNA) in eutrophic areas (Šolić et al., 2009), because, as suggested Scharek and Latasa (2007), HNA bacteria are more dependent on phytoplankton substrates than LNA bacteria.

Another characteristic of the eutrophic period was strong TD control of HNF by ciliates (Table 1, Fig. 10A), suggesting indirect control exerted by ciliates on bacterial biomass through HNF removal (via the trophic cascade). Analysis of the size composition of ciliates in eutrophic KaštelaBay showed that the smallest size categories (<40 μm) dominated the overall population (Bojanić et al., 2006). This is supported by Urrutxurtu et al. (2003), who found that the size structure of ciliate assemblages was related to the trophic status of the water, with small organisms dominating in eutrophic conditions. These small ciliates have been found to be the most important grazers of HNF (Rassoulzadegan et al., 1988; Bojanić et al., 2005). Therefore, during eutrophic conditions, the factors balancing bacterial growth and mortality could be ciliate grazing and viral lysis rather than grazing by HNF. Previous studies in KaštelaBay have shown that ciliates contribute about 20% of total grazing on bacteria (Šolić and Krstulović, 1995; Bojanić et al., 2006), and viral lysis has been found to increase across the trophic gradient in the Adriatic Sea (Wienbauer and Peduzzi, 1995).

In contrast, a significant relationship between bacteria and phytoplankton was not found during the oligotrophic period. If bacteria are controlled by substrates which are not directly exuded by phytoplankton, a significant relationship between them would not be expected (Findlay et al., 1991). Either the nutrient-limited phytoplankton was not able to provide enough EOC to meet bacterial requirements, or the phytoplankton-produced DOM was of low quality (Obernosterer and Herndl, 1995) so as to preclude a significant relationship between chl *a* and bacterial activity.

Although bacterial abundance and production were lower in the oligotrophic period than in the eutrophic period, the bacterial specific growth rate (SGR) showed a marked increase during the oligotrophic period (Fig. 4C). Density-dependent logistic growth implies that bacterial SGR is low when bacterial abundance is close to the carrying capacity (the maximal abundance of bacteria that the particular environment can sustain), while bacterial SGR is high when abundance is smaller than the carrying capacity. When bacterial abundance is close to the carrying capacity, bacterial growth is limited by resource availability, and when bacterial abundance is far from the carrying capacity, bacterial growth could be limited by predators (Wright and Coffin, 1984). HNF are the most important bacterial predators in the coastal Adriatic (Šolić and Krstulović, 1994), and strong coupling between bacteria and HNF during the oligotrophic period (Table 1, Fig. 10B) is consistent with the statement that HNF control bacterial standing stock by direct cropping of bacterial production (Gonzalez et al., 1990; Sherr et al., 1992). These authors suggest that bacterivorous protozoa crop production rather than simply the standing stock of bacteria because of higher grazing pressure on more actively growing and dividing cells.

Furthermore, during the oligotrophic period, HNF were not under the strong ciliate predation pressure, which could explain the increase in HNF abundance (Fig. 4E). It seems that ciliates switched their dominant prey from HNF to small phytoplankton (Table 1, Fig. 10B). This switch could be because the proportion of small chl *a* fraction in the total chl *a* increased during oligotrophic conditions (Fig. 5), but also could be a result of changing qualitative and size composition of ciliates in oligotrophic conditions (Bojanić, 2001).

The present study pointed to some changes which were observed in microbial community when the trophic status of the studied area changed from eutrophic to oligotrophic. The switch from eutrophic to oligotrophic conditions was accompanied by quantitative (decrease in bacterial abundance, bacterial production and chlorophyll *a*, and an increase in HNF abundance and bacterial specific growth rate), structural (phytoplankton species composition, phytoplankton size structure, seasonal cycles) and functional (trophic relationships, bottom-up vs. top-down control) changes within microbial community. However, there are many other parameters important for better understanding of microbial food web functioning in different trophic conditions which were not followed in this study. The role of viruses, grazing measurements (by heterotrophic and mixotrophic protozoa) and better insight in species composition and size structure of bacterial, phytoplankton and protozoan assemblages should be included in further studies.

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