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Short-term changes in microbial communities in the water column around the fish farm in the Bay of Piran

Kratkotrajne spremembe mikrobne združbe v vodnem stolpcu okoli ribogojnice v Piranskem zalivu

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Abstract: A multidisciplinary approach was used to study the impact of fish farming on coastal bacterial communities in the inner part of the Bay of Piran (northern Adriatic). Differences in bacterial abundance, production and the occurrence of selected bacterial groups were studied in the water column around the cage and at different distances from the centre of the fish cage towards the open water, i.e., reference marine station. We also examined the effect of fish feeding on the surrounding system in a short-term *in situ* experiment based on the simultaneous collection of seawater samples from different locations around the fish cage before and after feeding of fish. Our study suggests that fish feeding has a moderate short-term effect on water column parameters, including bacterial abundance and production, only at a limited distance from the fish cages. The nitrifying, ammonia-oxidizing bacterial groups, as determined by the fluorescent *in situ* hybridization method, were represented at a higher percentage in the seawater samples in the middle and around the fish cages. β- *Proteobacteria*, γ-*Proteobacteria* and the *Cytophaga-Flavobacterium* group were represented to a higher percentage at sampling sites in the middle of the Bay of Piran and at the reference marine station. The *Vibrio* group was detected at all sampling sites. The accumulation of organically enriched fish food and waste products released into the seawater during the short-term experiment resulted in a significant increase in particulate matter, orthophosphate and ammonium. In response to the increase in inorganic nutrients, we observed a significant increase in bacterial production, while no significant differences were observed in bacterial abundance in such short time.

Keywords: aquaculture, bacterial abundance, bacterial community composition, bacterial production, fluorescent in situ hybridisation, pollution

Izvleček: Vpliv ribogojstva na bakterijske združbe smo proučevali z multidisciplinarnim pristopom v notranjem delu Piranskega zaliva (severni Jadran). Razlike v številčnosti bakterij, bakterijski produkciji in pojavljanju izbranih bakterijskih skupin smo preučevali v vodnem stolpcu okoli ribje kletke in na izbranih lokacijah od središča ribje kletke do odprtih vod. Spremembe pred in po hranjenju rib smo preučili tudi v kratkoročnem poskusu *in situ*, ki je temeljil na istočasnem vzorčenju morske vode na različnih lokacijah okoli ribje kletke. Naša študija kaže, da hranjenje rib kratkoročno zmerno vpliva na parametre vodnega stolpca, vključno s številčnostjo bakterij in pro-

dukcijo, in le na omejeni razdalji od ribjih kletk. Nitrifikacijske bakterije, ki oksidirajo amonij, določene s fluorescentno *in situ* hibridizacijsko metodo, so bile zastopane v višjem deležu v vzorcih morske vode v sredini in neposredni okolici ribje kletke. β-*Proteobakterije*, γ-*Proteobakterije* in skupina *Cytophaga-Flavobacterium* so bile zastopane v višjem odstotku na bolj oddaljenih vzorčnih mestih. Potencialno patogena vrsta *Vibrio* je bila prisotna na vseh mestih vzorčenja. Kopičenje organsko obogatene ribje hrane in odpadnih produktov, ki se sproščajo v morsko vodo v kratkem času po hranjenju, je povzročilo znatno povečanje koncentracij partikulatne organske snovi, ortofosfata in amonija. Kot odgovor na povečanje anorganskih hranil smo izmerili bakterijsko aktivnost, medtem ko razlik v biomasi v vodnem stolpcu ni bilo opaziti.

Ključne besede: akvakultura, bakterijska abundance, bakterijska produkcija, bakterijska vrstna sestava, fluorescentna in situ hibridizacija, onesnaženje

Introduction

In the last 20 years, aquaculture has expanded rapidly both globally and in the Mediterranean, with a focus on the production of sea bass *Dicentrachus labrax* and sea bream *Sparus aurata* (FAO 2018). The rapid expansion of aquaculture has raised increasing concerns about negative environmental impacts, given numerous well-documented cases (Bouwman et al. 2013). These are primarily the impacts of waste products from farming – organic matter, nutrients, chemicals and pharmaceuticals – and the transfer of genes, parasites and diseases between wild and farmed species (Hargrave et al. 1993, McGhie et al. 2000, Karakassis 2000, Kim et al. 2004, Armstrong et al. 2005, Bouwman et al. 2013, Cabello et al. 2013, Wang et al. 2020). The numerous documented negative environmental impacts of aquaculture are mainly related to the accumulation of uneaten feed and faeces in seawater and on the seabed beneath fish cages (Hargrave et al. 1997, Karakassis 2000, Pitta et al. 2006, Vezzulli et al. 2008, Reimers et al. 2013). Fish food consists largely of proteins and the main components of this organic enrichment affect biogeochemical processes and often lead to eutrophication, oxygen deficiency and hypoxia on the seabed (Hargrave et al. 1993, Karakassis 2000). The effects of high organic matter loading on sediment below aquaculture farms have been extensively studied, showing changes in the structure of benthic communities that affect the entire food web and lead to an overall decline in species diversity (Hargrave et al. 1997, Karakassis et al. 1998, Christensen et al. 2000, Grego et al. 2009, Mirto et al. 2012, Luna et al. 2013, Reimers et al. 2013).

Marine microbes drive biogeochemical cycles in coastal areas (Kirchman 1994). They regulate carbon and energy transfer in the food web, the maintenance of water quality, and the health of marine ecosystems (Azam and Malfatti 2007). Marine microbes respond rapidly to environmental perturbations (Galand et al. 2016, Ape et al. 2019) and play an important role in the degradation and remineralization of organic matter. Several studies have shown that organic wastes from fish farms stimulate microbiological metabolism, most of which can be attributed to changes in enzymatic activity (Caruso et al. 2003, Vezzulli et al. 2004), nitrification, denitrification or sulphate reduction (McCaig et al. 1999, Christensen et al. 2000, Asami et al. 2005, Kondo et al. 2012, Dowle et al. 2015) ammonium concentrations, nitrification rates, and ammonia oxidizer most-probable-number counts were determined in samples of sediment collected from beneath a fish cage and on a transect at 20 and 40 m from the cage. The data suggest that nitrogen cycling was significantly disrupted directly beneath the fish cage, with inhibition of nitrification and denitrification. Although visual examination indicated some slight changes in sediment appearance at 20 m, all other measurements were similar to those obtained at 40 m, where the sediment was considered pristine. The community structures of proteobacterial β-subgroup ammonia-oxidizing bacteria at the sampling sites were compared by PCR amplification of 16S ribosomal DNA (rDNA. Increases in bacterial abundance (Mirto et al. 2000, Vezzulli et al. 2002, Bissett et al. 2007), virus-like

particles (Garren et al. 2008, Luna et al. 2013), and bacterial production (Navarro et al. 2008, Garren et al. 2008), as well as changes in bacterial community composition (Bissett et al. 2006, Garren et al. 2008, Castine et al. 2009, Kawahara et al. 2009, Quero et al. 2015, Galand et al. 2016, Martins et al. 2018, Kolda et al. 2020) have been detected in the sediments bellow fish cages.

Increased deposition of organic matter on the seabed below fish cages (Kovac et al. 2003, Lojen et al. 2005) has been confirmed for our study area and negatively affects the sediment chemical properties and the abundance and structure of meiofauna (Grego et al. 2009, 2020). The impact of aquaculture on microbes have been studied mainly in sediments, much less in the water column. In our study, we focused on the short-term effects of aquaculture activities on the bacterial community in the upper layer of the water column around the cage and at different locations from the centre of the fish cage to the open water. We mainly investigated the changes in nutrient concentrations, abundance and bacterial production before and after feeding the fish in a short-term *in situ* experiment based on the simultaneous collection of seawater samples from different sites around the fish cage.

Materials and methods

Study site and experimental setup

The study was conducted in October 2005 in the marine fish farm in the inner part of the shallow semi – enclosed Bay of Piran (Gulf of Trieste, northern Adriatic Sea) (Fig. 1). The bay is about 7 km long and 5 km wide and is characterised by limited hydrodynamics. The fish farm consisted of 20 floating cages with a total area of 2019 m^2, where sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus auratus*) were kept. The depth below the fish cages was about 13 m. The total annual production in 2005 was 100 tonnes. The fish were fed three times a day. The daily dietary intake was 12 kg, and consisted

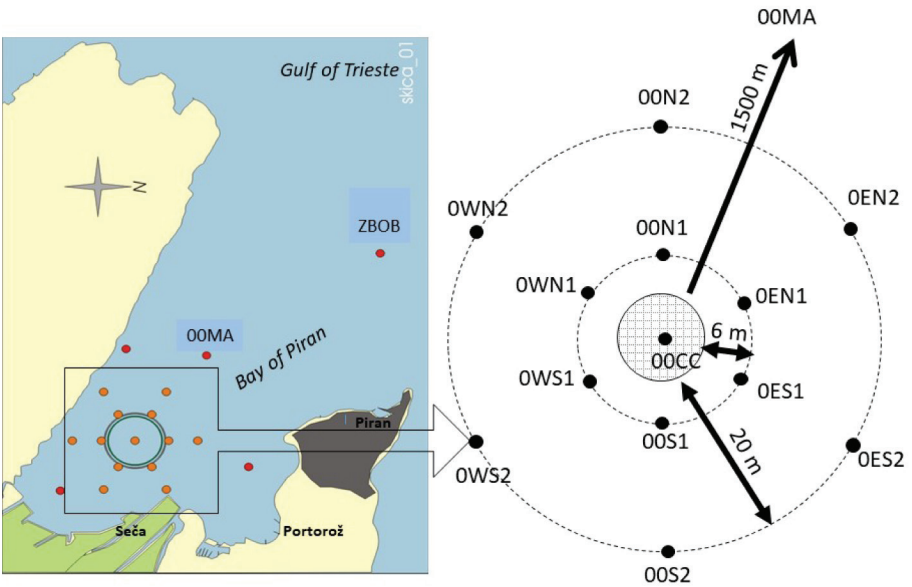

Figure 1: Sampling sites in the middle and around the fish cage at 6 and 20 m intervals, in the inner part of the Bay of Piran, and the reference station in the Gulf of Trieste (ZBOB).

Slika 1: Mesta vzorčenja morske vode v središču in okolici ribje kletke na razdalji 6 m in 20 m, v notranjem delu Piranskega zaliva, ter zunanji referenčni postaji v Tržaškem zalivu (ZBOB).

mainly of proteins (44 %) and lipids (22 %) and a smaller proportion of oligosaccharides (16 %), fibrins (2 %), ash (8 %) and vitamins, according to manufacturer.

To evaluate the impact of aquaculture on the pelagic bacterial community, two different studies were conducted. In the first study, seawater samples were collected at stations along the transect located 6 m, 20 m, 1500 m, and 5400 m from the centre of the fish cage (Fig. 1), at 5 m depth with Niskin samplers. At the same time, basic physical (temperature, salinity) and chemical parameters (total particulate matter, orthophosphate, total phosphorous and nitrogen, nitrate, ammonium) were analysed to determine the level of organic pollution.

For the second study, a system of anchored sampling bottles was constructed at different locations: in the centre of the cage (00CC), sampling points in a circle around the fish cage at a distance of 6 m (00N1, 0EN1, 0ES1, 00S1, 0WS1, 0WN1), and 20 m (00N2, 0EN2, 0ES2, 00S2, 0WS2, 0WN2) (Fig. 1). Each bottle (5 L) was attached to an underwater metal frame at 5 m depth. At the time of sampling, all bottles were opened simultaneously (by small boats and by divers). Seawater samples were collected at each sampling site before (bf) and 3 h after (af) fish feeding and the results for each parameter were expressed as the ratio K= C_{bf}/C_{af} in percentage.

Oceanographic parameters and nutrient analyses

Basic oceanographic parameters (temperature, salinity) were measured using a CTD fine –scale probe (Microstructure Profiler MSS90, Sea & Sun Technology GmbH), and 24-hour current measurements were made (not presented in this study). Total particulate matter (TPM) was measured gravimetrically, and inorganic nutrients (nitrate, ammonium, phosphate) were measured colorimetrically (Grasshoff et al. 1983) using a UV/VIS spectrometer, Perkin Elmer, Lambda 14.

Bacterial abundance and production

Seawater samples for total bacterial counts were fixed with formaldehyde (2% final concentration) and analyzed by the method of Porter and Feig (1980). Samples were stained with the fluorochrome 4',6'-diamino-2-phenylindole (DAPI, 1 μg mL^{-1} final concentration) and counted under an epifluorescence microscope (Olympus BX51).

Bacterial carbon production (BCP) was measured by incorporation of ^{3}H-leucine into newly synthesized proteins in bacterial cells (Simon and Azam 1989) using the centrifugation protocol of Smith and Azam (1992). Ultima Gold (PerkinElmer) was added to each sample and radioactivity was measured using a liquid scintillation counter (Canberra Packard TriCarb Liquid Scintillation Analyzer, model 2500 TR).

Table 1: List of oligonucleotide probes with sequence and target bacterial group.
Tabela 1: Seznam oligonukleotidnih sond s sekvenco in tarčno skupino bakterij.

Probe	Sequence	Target organisms
Nso 1225	5'- CGC CAT TGT ATT ACG TGT GA – 3'	nitrifying ammonia-oxidizing bacteria
NEU	5'- CCC CTC TGC TGC ACT CTA – 3'	nitrifying ammonia-oxidizing bacteria
EUBI,II,III	5'- GCT GCC TCC CGT AGG AGT – 3'	all bacteria
BET 42a	5' – GCC TTC CCA CTT CGT TT – 3'	β-proteobacteria
GAM 42a	5' – GCC TTC CCA CAT CGT TT – 3'	γ-proteobacteria
CF 319a	5' – TGG TCC GTG TCT CAG TAC – 3'	*Cytophaga-Flavobacterium* group of CFB phylum
GV	5' – AGG CCA CAA CCT CCA AGT AG – 3'	*Vibrio* group

Fluorescent in situ hybridization (FISH)

The abundance of selected phylogenetic groups was determined by the fluorescence *in situ* hybridization method using Cy3-labelled oligonucleotide probes (Thermo Electron Corporation). The sequences and target organisms of all 16S rRNA-targeted oligonucleotide probes are listed in Tab. 1.

Group specific probes were EUBI, II, III for all bacteria, BET 42a for β-*Proteobacteria*, GAM42 for γ-*Proteobacteria*, CF 319a *Cytophaga-Flavobacterium* cluster of *Cytophaga-Flavobacteria-Bacteroides* CFB phylum and GV for *Vibrio* group. The nitrifying, and ammonia-oxidizing bacteria groups were determined using the Nso 1225 probe specific for the ammonia-oxidizers and the NEU probe complementary to a signature region of most halophilic and halotolerant ammonia-oxidizers.

From each sampling station, 10 ml of sample was fixed overnight with 37% formaldehyde. 5 ml subsamples were filtered onto 0.2 µm polycarbonate filters (47 mm, Poretics) and rinsed with 3 x PBS, 1x PBS solution, and distilled water and stored at -20 ºC. For each probe, a filter piece was placed on a glass slide covered with Parafilm and overlaid with 20 µl hybridization probe prepared by mixing 2 µl probe and 18 µl hybridization solution. The hybridization solution contained 360 µl NaCl (5 M), 40 µl Tris-HCl (1M, pH 7.2-8), 2 µl 10% SDS, the optimal concentration of formamide, 400 µl blocking solution (Roche), and 500 µl distilled water. The filters were incubated in the hybridization oven at 42 ºC for 3h. After hybridization, filters were washed in wash solution (1000 µl Tris/HCl (1M), 700 µl NaCl (5 M), 500 µl EDTA, 25 µl 20% SDS and 47.8 ml distilled water) for 20 min at 48 ºC. Washed and dried filter pieces were stained with 1 µg mL^{-1} 4',6'- diamino-2-phenylindole (DAPI). Cells were counted by epifluorescence microscopy (Olympus BX51). The abundance of each bacterial group detected was expressed as the percentage of each group in the total bacterial count.

Statistical analysis

A three-way ANOVA was conducted to test the main interactive effects of bacteriological and chemical parameters (orthophosphate, total phosphate, ammonium, bacterial abundance and carbon production), feeding activity (before and after feeding), and sampling sites (distance – 0 m, 6 m, 20 m; and directions – south, north, east, west).

Table 2: The results (means and standard deviations) of total particulate matter (TPM), nitrate, ammonium, orthophosphate, total phosphorus (Tot P) and nitrogen (Tot N), bacterial biomass and bacterial carbon production rate (BCP) in seawater samples from different sites as a function of distance from fish cages.

Tabela 2: Rezultati (srednja vrednost in standardni odmik) celokupne partikulatne snovi (TPM), nitrata, amonija, ortofosfata, celokupnega fosforja (Tot P) in nitrogen (Tot N), bakterijske biomase in bakterijske produkcije (BCP) v vzorcih morske vode na različnih merilnih mestih glede na oddaljenost od ribiških kletk.

		Distance from the fish cage (m)				
Parameter	**Cage**	**6**	**20**	**200**	**1500**	**5400**
TPM (mg L^{-1})	2.40	2.12 ± 0.56	2.55 ± 1.79	2.09 ± 0.35	1.67	1.04
Nitrat (µM L^{-1})	0.95	0.33 ± 0.07	0.34 ± 0.12	0.38 ± 0.18	0.51	0.20
Ammonium (µM L^{-1})	0.68	0.59 ± 0.12	0.57 ± 0.04	0.82 ± 0.28	0.76	0.52
Tot N (µM L^{-1})	31.13	33.80 ± 4.86	31.72 ± 3.92	42.46 ± 9.20	45.68	37.01
Orthophosphate (µM L^{-1})	0.12	0.10 ± 0.01	0.11 ± 0.04	0.11 ± 0.01	0.15	0.11
Tot P (µM L^{-1})	0.32	0.29 ± 0.02	0.30 ± 0.04	0.29 ± 0.06	0.33	0.31
Bacterial biomass (µg C L^{-1})	43.95	53.21 ± 12.3	44.15 ± 5.62	37.38 ± 10.3	27.28	21.37
BCP (µg C $L^{-1}day^{-1}$)	10.31	9.27 ± 2.72	10.89 ± 2.26	1.65 ± 1.08	1.34	3.9

Results

Effects of fish farm activity on nutrient and microbial variables along the pollution gradient

Results of chemical and microbiological analyses of the upper layer of the seawater column did not differ significantly among sampling sites, although, higher concentrations of total particulate matter, nitrate, and bacterial carbon production were measured near the fish cage (Tab. 2).

Total particulate matter values ranged from 2.12 to 2.55 mg L^{-1} at sites near the fish cage and were twice the values measured at the site 5400 m from the fish farm. The highest nitrate concentration was at the cage site and was three times lower at all other sites (Tab. 2). Concentrations of ammonium, orthophosphate, total phosphorus (Tot P), and nitrogen (Tot N) in the upper layer of the water column did not differ among stations, or concentrations were slightly higher at the station in the middle of the Bay of Piran.

Bacterial carbon production differed significantly ($F=7.932$, $p < 0.001$) between sites and was on average up to 5-fold higher near the fish cage compared to values measured at the sampling site 1500 m away, and 3-fold higher compared to bacterial production measured at the reference site (Tab. 2, Fig. 2).

Similar trends were observed for bacterial abundance, but the difference was not statistically significant. The average bacterial abundance in the vicinity of the fish cage (0, 6, and 20 m) ranged from 2.4 to 3.1 x 10^9 cells L^{-1} and decreased with increasing distance from the cage. Bacterial abundance was more than twice as high at the fish farm as at the sampling sites at 1500 m and 5400 m (Tab. 2, Fig. 2).

Molecular analyses of targeted ribosomal RNA using the FISH method and various oligonucleotide probes revealed differences in the composition of bacterial groups in seawater along the transect (Fig. 3). Based on the literature data, we selected specific probes for the bacterial groups expected in an environment polluted by aquacultures.

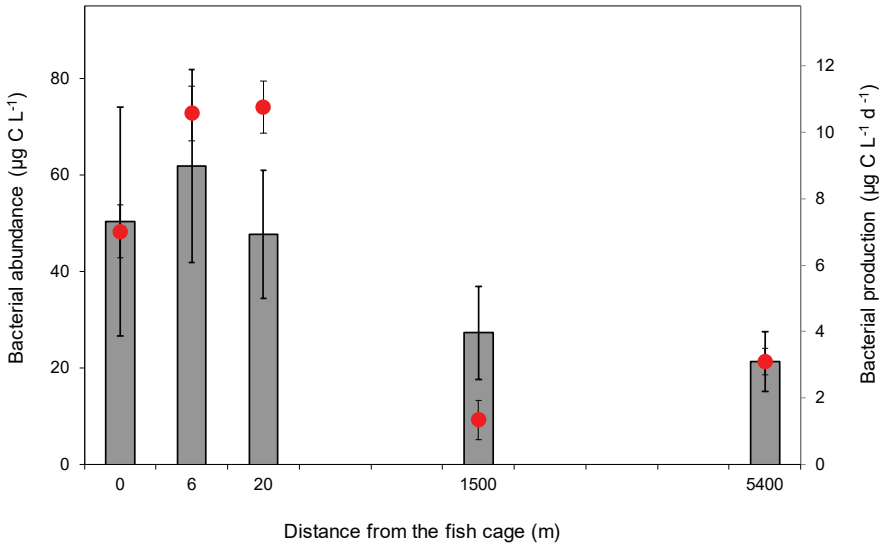

Figure 2: Bacterial biomass (■) and bacterial production (●) at sampling sites at different distances from the fish cages in the Bay of Piran.

Slika 2: Bakterijska biomasa (■) in bakterijska produkcija (●) na merilnih mestih različnih razdalj od ribjih kletk v Piranskem zalivu.

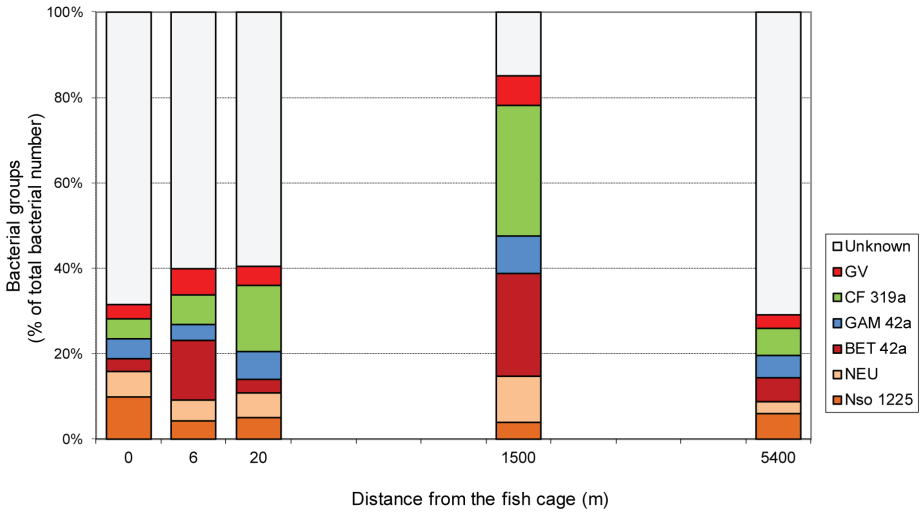

Figure 3: Percentage of bacterial groups determined by the fluorescent *in situ* hybridization method (FISH) at sampling sites at different distances from fish cages in the Bay of Piran.

Slika 3: Procent posamezne skupine bakterij določene z metodo Fluorescentne *in situ* hibridizacije (FISH) na merilnih mestih, ki so različno oddaljeni od ribjih kletk v Piranskem zalivu.

The various groups were represented differently. In the middle and immediate vicinity of the fish cage, all identified bacterial groups accounted for 30-40% of the total number of bacterial cells in the seawater samples, and the composition of the remaining portion was not determined. Within the identified groups, the nitrifying and ammonia-oxidizing bacterial groups Nso 1225 and NEU were represented with a higher percentage, 10 and 6 %, respectively. At other sampling sites, the β-*Proteobacteria*, γ-*Proteobacteria* and the *Cytophaga-Flavobacterium* group of the CFB strain (*Cytophaga-Flavobacteria-Bacteroides*) predominated (Fig. 3). At the sampling site 1500 m from the fish cage, the identified bacterial groups of all selected oligonucleotide probes accounted for 85 % of the total bacterial count. Compared to the other stations, there was a higher percentage of all bacterial groups, especially the β-*Proteobacteria*, and *Cytophaga-Flavobacterium* groups, except for the ammonia-oxidizers of the β-*Proteobacteria* subgroup (the Nso 1225 probe), which was represented with the highest percentage at station 00CC. At the reference marine site, all bacterial groups were evenly distributed, however accounted for only 30 % of the total bacterial count and most of the community composition was unknown, possibly due to untargeted bacterial groups of the α-*Proteobacteria* that may predominate in coastal, more oligotrophic waters. The *Vibrio* group (up to 6% of the total bacteria count) was detected at all sampling sites.

Effects of fish feeding on nutrient levels, bacterial abundance and production in the water column in a short period of time

The results of chemical and bacteriological analyses of seawater, sampled simultaneously at all sampling sites before and 3 hours after feeding, showed changes in the concentrations of several parameters. The results of the relative difference (%) of total particulate matter, orthophosphate and ammonium concentrations after fish feeding in relation to before fish feeding at the sites around the fish cage (in the middle, 6 m and 20 m away) are shown in Fig. 4.

With few exceptions, all values increased 3 hours after feeding compared with results before fish feeding. Overall, concentrations of total particulate matter, increased from 2.12 ± 0.5

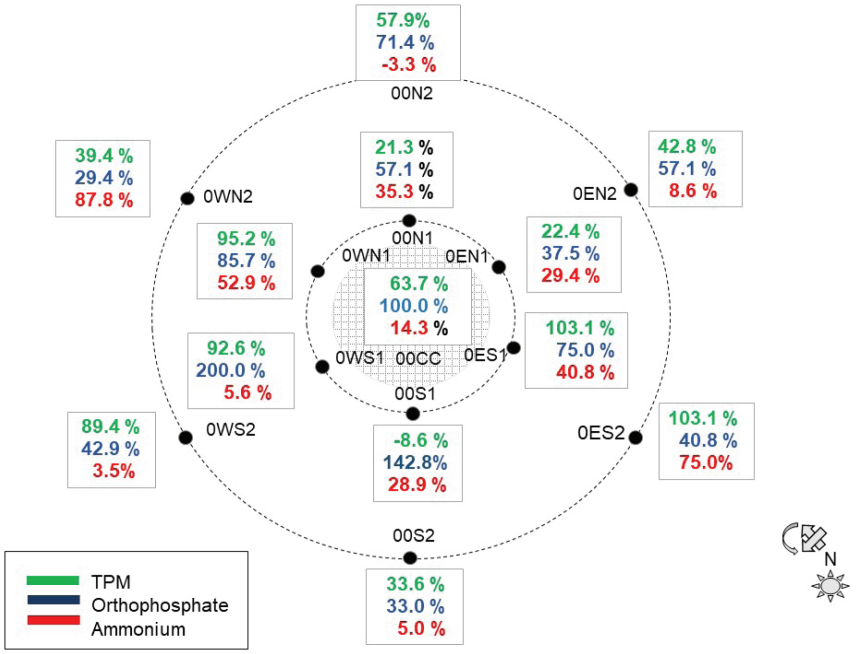

Figure 4: Results of relative difference (%) of total particulate matter (TPM) (green), orthophosphate (blue) and ammonium (red) concentrations after fish feeding in relation to before fish feeding at the sites around the fish cage (in the middle, 6 m and 20 m away).

Slika 4: Rezultati relativne razlike (%) v koncentracijah totalne partikulatne snovi (TPM), ortofosfata in amonija po hranjenju rib glede na vrednosti pred hranjenjem v okolici ribje kletke (v centru, 6 m in 20 m stran).

mg L^{-1} to 5.65 ± 1.1 mg L^{-1}. A similar trend was observed in the concentrations of orthophosphate, which doubled from 0.07 ± 0.01 µmol L^{-1} to 0.14 ± 0.04 µmol L^{-1}, ammonium increased from 0.59 ± 0.1 µmol L^{-1} to 0.77 ± 0.1 µmol L^{-1}, and nitrate from 0.33 ± 0.07 µmol L^{-1} to 0.77 ± 0.2 µmol L^{-1}. A three-way ANOVA showed a significant increase in concentrations of orthophosphate (F=25.09, $p < 0.0001$), total phosphorus (F=37.08, $p < 0.001$), ammonium (F=8.61, $p < 0.009$) after the feeding. But we found no significant difference between distance and direction of sampling sites.

During the experiment, bacterial carbon production increased at almost all stations around the cage at radius of 6 m and 20 m. The average rate of bacterial carbon production was significantly higher 3 hours after feeding (F=18.81, $p < 0.001$) within the 20 m radius and an overall increase from 9.66 ± 2.8 µg C L^{-1} d^{-1} to 14.54 ± 2.9 µg C L^{-1} d^{-1} was recorded. The highest increase was observed towards the south and southwest, up to 163% at the station 00S2 and 145% at station 0WS1 and in the middle of the cage up to 125% (at the station 00CC) (Fig. 5 A).

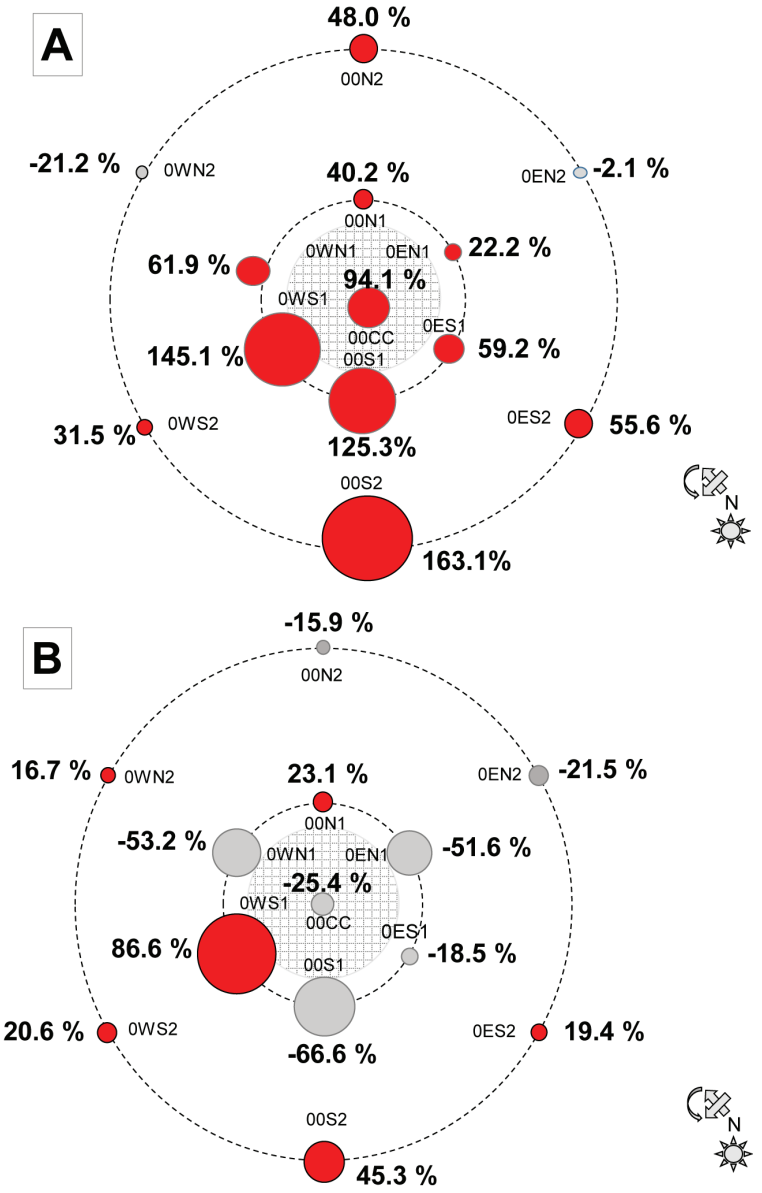

Figure 5: Relative difference (%) in bacterial carbon production **(A)** and bacterial biomass **(B)** measured before and after fish feeding at the sites around the fish cage (in the middle, 6 m and 20 m away). The size of the circle shows the increase (red color) or decrease (grey color).

Slika 5: Relativna razlika (%) v izmerjeni bakterijski produkciji ogljika **(A)** in bakterijski biomasi **(B)** pred in tri ure po hranjenju rib v okolici ribje kletke (v centru, 6 m in 20 m stran). Velikost kroga ponazarja % povečanja (rdeč krog) ali znižanja (siv krog).

Bacterial biomass, recalculated from bacterial counts using the 19.8 fg C cell^{-1} conversion factor (Lee and Fuhrman 1987), changed between sites, but no significant differences in concentrations were observed after feeding. Mean values decreased from 48.8 µg C L^{-1} ± 9.8 to 46.2 ± 19.7 µg C L^{-1}, showing an increase after feeding only at some sites in the southwest direction. No changes or even a slight decrease was observed at all other sites (Fig. 5 B). The production/biomass (P/B) ratio changed from 0.20 ± 0.06 to 0.38 ± 0.2 after feeding and was on average up to 4.5 times higher near the fish cages than at other sampling sites, reflecting higher daily biomass production.

Discussion

Fish farming changes the load of organic and inorganic nutrients according to the increase of organic wastes (uneaten food, faeces, soluble excreta), which potentially negatively affects the marine environment, especially shallow areas, gulfs and semi-enclosed bays. Our study suggests that fish farming has moderate impact on water column parameters, including bacterial abundance and production, but only at a limited distance from fish cages (< 20 m), while at other distances no effects were detectable using our approach. Direct effects of nutrient pollution from fish farms on water quality and secondary effects on production and bacterial communities have also been reported in previous studies in the region (Turk and Malej 2003) and in other coastal areas (Price et al. 2015, Šestanović et al. 2016). In this study, we only analysed the effects on the pelagic microbial community, while the effects of increased organic matter on the benthic microbial community were not analysed in detail. Uneaten food and faeces lead to the accumulation of a large amount of organic matter on the seabed below the cages in our study area (Lojen et al. 2005) since the sedimentation rates in the vicinity of the fish cages were significantly higher (eight times higher) than background values for Piran Bay (Kovac et al. 2003). Accumulation of organic and inorganic nutrients showed significant changes in redox potential and concentrations of chlorophyll *a* and phaeopigments in the sediment (Štrukelj 2008), as well as structure and abundance of meiofauna (Grego et al. 2009, 2020). The negative influence of fish farm on the phytoplankton and bacterioplankton in immediate proximity was confirmed with in situ experiment with incubation in dialysis bags (Štrukelj 2008).

In addition, we investigated the effects of feeding of the sea bass and sea bream on bacterial abundance and production in a short-term *in situ* experiment based on the simultaneous sampling of seawater from different sites around the fish cage before and after fish feeding (Fig. 5). Feeding fish with organically enriched fish food resulted in significant increases in total particulate matter, dissolved organic and inorganic nutrients in the seawater of the cage and surrounding area. Increased concentrations of ammonium and orthophosphate could have triggered intense bacterial metabolism, which might be reflected in the recorded increase of the bacterial production. The observed higher P/B ratio reflects higher daily biomass production near the fish cages as compared to other sampling sites, although no significant differences in bacterial abundance were observed over the 3 h period. Previous results from the P-limited northern Adriatic showed that dissolved phosphorus concentrations in fish farms were elevated in the water column compared to reference stations (Matijević et al. 2009), resulting in changes in abundance and production of phytoplankton and bacterioplankton (Šestanović et al. 2016). In our study area, correlations between the bacterial community and orthophosphate concentration have been documented (Sjöstedt et al. 2013, Malfatti et al. 2014, Tinta et al. 2015), as well as rapid turnover and uptake of orthophosphate (Turk et al. 1992). Similar effects of organic material input from fish farms on bacterial activity, especially enzymatic activity, have been described in different aquaculture areas (Vezzulli et al. 2002, Caruso et al. 2003).

The effects of dissolved wastes (inorganic and organic nutrients) depend on the rate at which these nutrients are diluted before being assimilated by the pelagic ecosystem. It is assumed that in cases where the flushing time (i.e., the time required to exchange the local water volume with new coastal water) is shorter than the typical microbial generation time and the risks of an increase in nutrient concentrations caused by the fish farming do not result in a measurable increase in local microbial

biomass (Black 2001). Smaller pelagic organisms such as bacteria near fish farms have access to highly available organic nutrients and have short generation times (Kirchman 2016). Because the generation time of heterotrophic bacteria in the Bay of Piran ranges from 5 to 82 hours (Turk et al. 2020), we did not detect differences in bacterial abundance in our 3-hour experiment. Changes in the concentrations of inorganic and organic nutrients mainly affect bacterial production. During the field experiment currents near the fish cage showed north-westerly to south-westerly direction with a velocity of 7 cm s^{-1} (Malačič and Forte 2003), which could influence the higher concentrations of inorganic nutrients and bacterial production at sampling sites located in this direction.

The method of fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes is widely used for cultivation-independent identification, quantification, and visualization of microbes in different environments (Amann et al. 1990). Nitrifying bacteria are particularly difficult to culture, because they have a long generation time and poor counting efficiency. Analysis with oligonucleotide hybridization probes specific for different ammonia-oxidizing bacteria (probes Nso 1225 and NEU) showed that at least two different species of ammonia-oxidizing bacteria were present with a higher percentage in the seawater samples in the middle and around the fish cage. The importance of nitrifying bacteria in the context of fish culture has been studied previously (McCaig et al. 1999, Bissett et al. 2006). McCaig et al. (1999) demonstrated the importance of sediment contamination in fish farms on nitrification rates and the composition of ammonium-oxidizing bacteria. Using 16S rDNA clone library analysis, Bissett et al. (2006) demonstrated the presence of ammonium-oxidation *Nitrospira* associated clones and differences in the microbial community as a function of sediment pollution level. The groups of β-*Proteobacteria*, γ-*Proteobacteria* and *Cytophaga – Flavobacterium -Bacteriodes* reached a higher percentage at more distant sampling sites. The importance and seasonal changes in these bacterial communities has been demonstrated in recent studies but only on benthic communities using next -generation sequencing methods (Garren et al. 2008, Fodelianakis et al. 2014, Quero et al. 2015, Martins et al. 2018, Duarte et al. 2019, Ape et al. 2019, Kolda et al. 2020, Roquigny et al. 2021). Species belonging to the genus *Vibrio* are heterotrophic bacteria that increases rapidly when nutrients are available (Thompson et al. 2006). Some *Vibrio* species are known pathogens of fish (Austin and Austin 2016), and the presence of *Vibrio* near the fish cages could be an important reservoir for fish pathogens that could spread over large geographic areas.

Conclusions

Given the rapid development of aquaculture, concerns remain about the environmental impact of intensive aquaculture on water and sediment quality, particularly in shallow and semi-enclosed bays. Our study suggests that aquaculture has moderate effects on water column parameters, and can cause changes in bacterial abundance, production and community composition, but only at a limited distance from fish cages. However, we did not follow change in the sediment where in fact these effects might be more pronounced. We also confirmed that concentrations of organic and inorganic nutrients change in a relatively short time after fish feeding, which affect the bacterial production. Changes in bacterial activity and community composition can lead to changes in biogeochemical cycling and ecological status of the environment. Sedimentation of organic matter has a strong impact on sediment quality and benthic community, but changes in the concentration of organic and inorganic nutrients in the water column, as well as microbial abundance, production, and community composition in a relatively short time are not negligible. Preliminary fluorescence *in situ* hybridization results indicated changes in abundance of certain bacterial groups in response to pollution, as well as the presence of bacteria pathogenic to humans and fish. In the future, seasonal changes and long-term consequences should be investigated in the water column as well as sediment, to better understand changes in microbial community structure, and biogeochemical processes.

Povzetek

Dokumentirani številni negativni vplivi marikulture na okolje so povezani predvsem s kopičenjem ne-zaužite hrane in ekskrecijskih produktov v morskih vodi in na/v sedimentu. Rezultati naših meritev prostorskih razlik števila in hitrosti rasti populacije heterotrofnih planktonskih bakterij, kot tudi analize vsebnosti hranilnih snovi, so pokazali povišane vrednosti merjenih parametrov v bližini ribogojnice, ki pa se z oddaljenostjo od vira onesnaženja zmanjšujejo. Rezultati analize sestave bakterijskih združb z metodo fluorescentne *in situ* hibridizacije in izbranih bakterijskih oligonukleotidnih sond so pokazali prisotnost amonij-oksidirajočih bakterij, β-*Proteobacteria*, γ-*Proteobacteria* and *Cytophaga – Flavobacterium* kot tudi potencionalno patogenega seva *Vibrio.* Kopičenje organsko obogatene ribje hrane in odpadnih produktov, ki se sproščajo v morsko vodo znotraj ribje kletke in okolici v relativno kratkem času po hranjenju, je povzročilo znatno povečanje koncentracij partikulatne organske snovi, ortofosfata in amonija. Kot odgovor na povečanje anorganskih hranil smo izmerili znatno povečano bakterijsko produkcijo, medtem ko razlik v številu bakterij ni bilo opaziti.

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