Stud. Mar. 26(1): 83–98 UDC 582.23(497.16 Boka Kotorska) (047.31)

IDENTIFICATION AND QUANTIFICATION OF MICROBIAL COMMUNITY BY CARD-FISH METHOD IN BOKA KOTORSKA BAY

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ABSTRACT

The aim of this study was to estimate number of bacterial community in Bokakotorska Bay by CARD FISH method that is one of the most useful culture-independent techniques for identification of organisms in the marine environment. Geographic position and specific ecological conditions make Boka Kotorska Bay a specific biotope where is very important to investigate bacterioplankton structure. The inner part of Boka Kotorska Bay represents a closed basin with specific hydrographic characteristics (freqent inflow of fresh water that is of seasonal character).Our preliminary data provide the first insight of bacteroplankton structure by this method. Samples were collected at 0m and 10m depth in June of 2011 and February 2012. Due to inflow of fresh water and organic load in Kotor and Risan Bay, an increase of all groups of bacteria was found.

Key words: Boka Kotorska Bay, Bacterial community, CARD FISH method

INTRODUCTION

The Boka Kotorska Bay is located in the southeastern part of Adriatic Sea comprising part of Montenegrin coast and represents specific semi-closed area created by the action of tectonic forces and fluvial erosion. Total water area is 87.3 km^2, coast length is 105.7 km. The Bay is composed of four smaller bays (Kotor, Risan, Tivat and Herceg Novi Bay

that are interconnected) and two straits. The first strait connects open sea with Herceg Novi Bay, and the second strait (Verige) connects Tivat Bay with Kotor and Risan Bay. Strong streams, springhead, rivers and wellsprings can be found on the many sides in the Bay especially its inner part (Kotor and Risan). All that streams and well springs receive water from Lovćen and Orjen massif and then from Tivat, Grbalj and Sutorina field. In that massifs are carst fields seasonally flooded and underground reservoir of water. Average depth of the Boka Kotorska Bay is 27m. (Stjepčević, 1974). Geographic position and specific ecological conditions make the Boka Kotorska Bay a specific biotope where is very important to investigate bacterioplancton structure. The knowledge of seawater microbial diversity is important to understand their community structure and pattern of distribution.

Important information about bacterioplankton structure by Catalyzed reporter deposition fluorescence in situ hybridization (CARD FISH) provided Glöckner et al. (1999) in Baltic Sea, Eilers et al. (2000), Pernthaler et al. (2002) in North Sea, Schattenhofer et al. (2009) along transect across the Atlantic Ocean from South Africa to United Kindgom, Cottrell & Kirchman (2000) in California, Simon et al. (1999) in Southern Ocean

Information on bacterioplankton structure in Adriatic Sea is scarce. Preliminary remarks of this topics was made by Manti et al. (2012) in central Adriatic region.

The aim of this study was to preliminary describe number of bacterial community in the Boka Kotorska Bay by CARD FISH method. Fluorescence in situ hybridization (FISH) for bacteria was described more than twenty years ago (Amann et al., 1990). Due to the low RNA content

of marine bacteria many attempts have been made to enhance the sensitivity of FISH. One example of application is the catalysed reporter deposition (CARD), introduced in 1989 by Bobrow et also known as tyramide signal amplification (TSA).(Manti et al., 2012)

MATERIAL AND METHODS

CARD FISH is one of the most useful culture-independent techniques for identification of organisms in the marine environment. This technique permits an investigator to "fish" for a specific nucleic acid sequence in a "pool" of unrelated sequences. (Pernthaler et al., 2002)

Samples were collected at 0m and 10m depth in June of 2011 and February 2012 in the following bays: Kotor, Risan, Tivat, Herceg Novi and Mamula (entry point of Bokakotorska Bay) (Fig.1, Table1). Samples were analysed by CARD FISH method (Pernthaler et al., 2002).

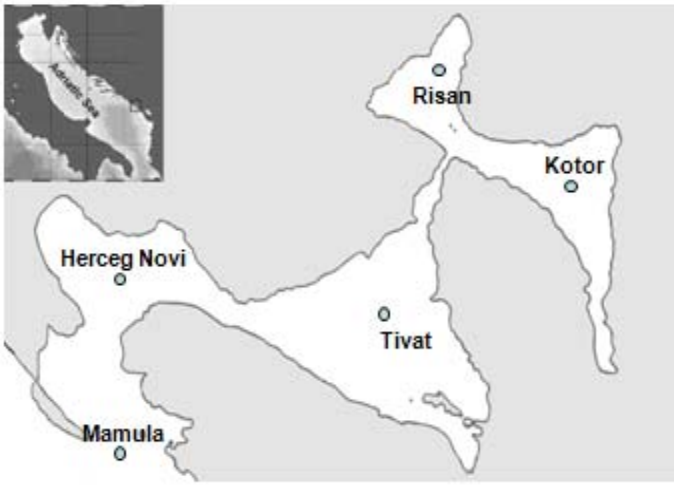

Fig.1 Investigated area

Table 1. Coordinates of investigated area

Station	Coordinates	
Kotor	N 42° 28’ 27.2”	E 18° 44’ 25.5”
Risan	N 42° 29’ 47.5”	E 18° 40’ 54.3”
Tivat	N 42° 25’ 54.5”	E 18° 39’ 24.5”
Herceg Novi	N 42° 26’ 14.5”	E 18° 32’ 41.4”
Mamula	N 42° 22’ 39.8”	E 18° 33’ 22.4”

Cells were immobilized on GTTP (0.2μm, 47mm, Millipore) filters and embeded in 0.2% agarose (Methapor Cambrex USA).

For permeabilization of bacterial cell walls, filters were incubated in 10mg/L lysozyme (in 50mM EDTA, 100mM Tris/HCl) for 1h at 37ºC.

Endogenous peroxidases were inactivated by using 0.15% H_2O_2 for 30min. Filters sections were cut and hybridized with HRP-labelled oligonucleotide probes (Biomers, Germany) - Table 2 for 3h at 46ºC.

Table 2. Overview of the probes and hybridization conditions applied for CARD FISH

Probe	Target organisms	Sequence (5'→ 3')	FA (%)	Reference
Eub 338	*Bacteria*	GCTGCCTCCCGTAGGAGT	35	Amann et al (1990)
Eub 338II	Supplement to Eub 338	GCAGCCACCCGTAGGTGT	35	Daims et al (1999)
Eub 338III	Supplement to Eub 338	GCTGCCACCCGTAGGTGT	35	Daims et al (1999)
GAM42a	*Gammaproteobacteria*	GCCTTCCCACATCGTTT	35	Manz et al. (1992)
CF 319a	*Bacterioidetes*	TGGTCCGTGTCTCAGTAC	35	Manz et al. (1996)
Alpha 968	*Alphaproteobacteria*	GGTAAGGTTCTGCGCGTT	35	Neef (1997)
Non338	Control	ACTCCTACGGGAGGCAGC	35	Wallner et al. (1993)

Formamide (FA) concentration (v/v) in CARD-FISH hybridization buffer

After washing buffer tyramid signal amplification (using fluorescein $Alexa_{448}$) was performed for 20 min at 46ºC. Then filter section were washed in 1xPBS, MQ water and 96% ethanol and arranged on microscope slides in a mixture of 1μg/mL DAPI. Particular probe was determined by epifluorescence microscopy (Axioplan2, Zeiss Jena,

Germany). At least 1000 DAPI (4',6-diamidino-2-phenylindole) stained cells were manually counted. Controls with antisense probe NON 338 were always negative. Total bacterioplankton abundance were analyzed by epiflurescence microscope with DAPI stained cells.(Porter & Faig, 1980). Samples for the determination of dissolved nutrients such as nitrate, nitrite, ammonium and phosphate were analyzed according to (Parsons et al., 1984). Temperature and salinity were measured *in situ* by WTW sonda Multi 350i.

RESULTS AND DISCUSSION

Bacteria, as detected by the probe mix of EUB338 I-III, dominated the microbial community (on average 80% of DAPI stained cell in June and 60% in February).EUB 338 I-III reached its highest value in Kotor about 88% and decreased in Mamula about 60%. Relative abundance of *Bacteria* was higher in surface waters in late spring because of increased phytoplankton growth. It is possible that the proportion of cells that can be detected with oligonucleotide probes may be linked to variations in the physiological conditions of the cells and ecosystem type. (Bouvier, 2002). Previous findings showed that EUB338 hybridizing cells ranged from 55% to 92% in Central Adriatic region (Manti et al.,2012), 31-71% in North Sea (Simon et al., 1998), 48-70% DAPI stained cell in Atlantic Ocean. (Schattenhofer et al., 2009), and 55%-88 % in California (Cottrell & Kirchman, 2000).

Fig.2 shows mean values of EUB I-III in investigated parts of Bay.

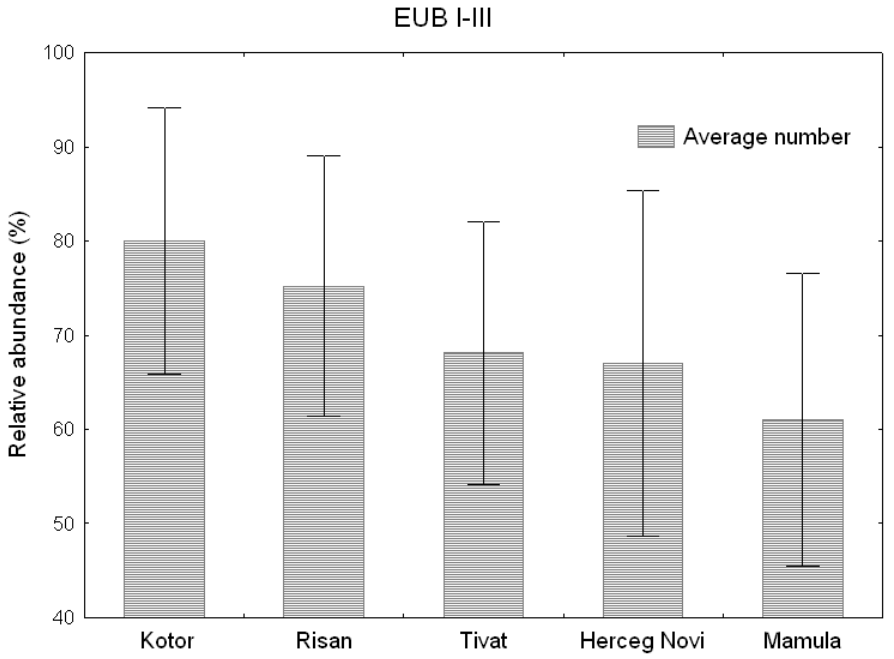

Figure 2: Mean values of EUB I-III in investigated area

The abundance of *Gammaproteobacteria* detected by the Gam 42a ranged from 5% in Mamula to 42% in Kotor Bay (on average 12.6% of DAPI stained cell in June and 15.5 % in February). It was noticed that relative abundance of *Gammaproteobacteria* was significantly higher in February in the Kotor Bay compared with other investigated sites. The Kotor Bay area is largely affected by river inputs that provide a basin with large flow of fresh water and land-derived nutrients.

Increased influx of fresh water in Kotor affects the occurrence of faecal bacteria from *Enterobacteriaceae* – large family of human pathogen species. Investigations showed that *Gammaproteobacteria* had average higher population densities outside the oligotrophic regions. In Northern Atlantic Drift *Gammaproteobacteria* showed maximum of 49% but at the other sites was about 3-9% (Schattenhofer et al., 2009).

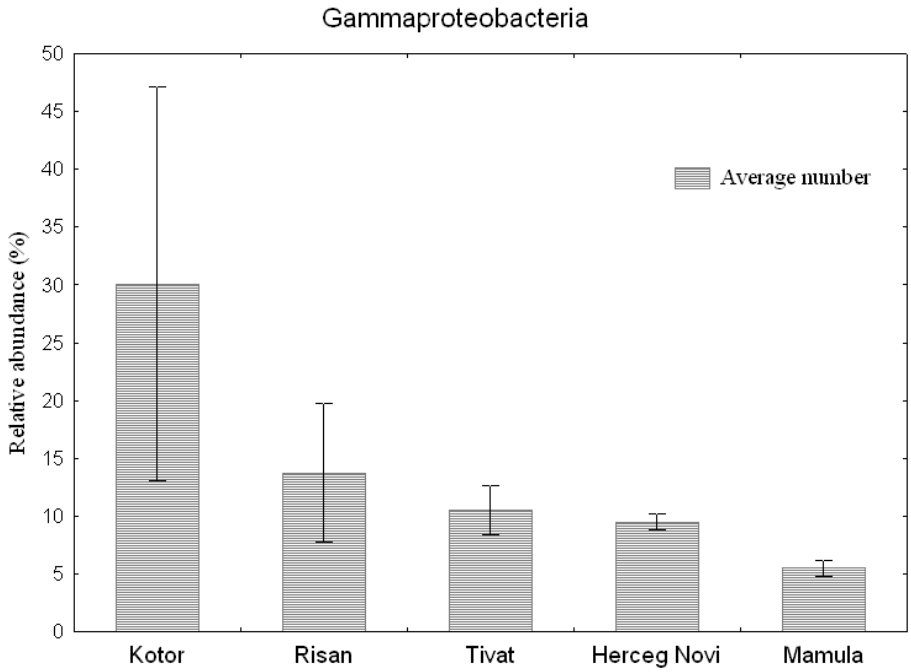

Figure 3: Mean values of *Gammaproteobacteria* in investigated area

In the samples from the North Sea and the surface layers of the Antartic Ocean abundances of *Gammaproteobacteria* ranged from 6% to 9% (Glöckner, 1999). Findings (Manti et al., 2012) indicate that contribution of *Gammaproteobacteria* was 13% of total bacteria.

Investigations of Cottrell & Kirchman (2002) in California showed that *Gammaproteobacteria* varied between from 7% to 42%. Fig.3 shows mean values of *Gammaproteobacteria* in ivestigated parts of the Bay.

Similar situation was obtained with other groups of bacteria. The genus *Cytophaga-Flavobacterium*, detected by the CF319a probe is known to constitute a substantial fraction of coastal marine communities (Kirchman, 2000). Its mean values varied between 3% in Mamula to 15% and 16% in Kotor and Risan Bay.(Fig.4). Relative abundance of Bacterioidetes in all investigated stations was higher in June and can be

linked with algal bloom. Higher percentage of free-living *Cytophaga-Flavobacterium* may have been caused by high phytoplankton exudation rates related to nutrient depletion (Lancelot, 1983).Most notably Cytophaga-Flavobacterium are chemoorganotrophic and are especially proficient in degrading various biopolymers such as cellulose, chitin and pectin (Kirchman, 2002). In June in Central Adriatic region, samples probe CF319a detected about one-fourth of the total bacterioplankton compared to the EUB 338 I-III.(Manti et al., 2012), in Atlantic surface waters was 3.5-9% (Schattenhofer et al., 2009). The relative abundance of *Cytophaga-Flavobacterium* ranged from 2% in Baltic Sea (depth, 78m) to 72% in the Antarctic Ocean (depth 0m)(Glöckner et al., 1999).

Cottrell&Kirchman (2000) indicated that in California the abundance of this group varied from 18 %-33%.

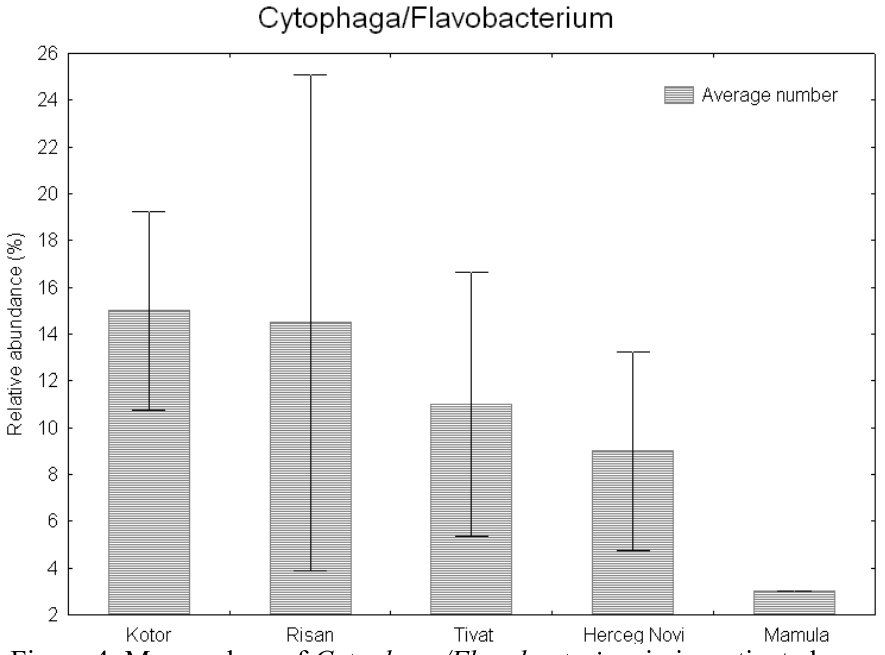

Figure 4: Mean values of *Cytophaga/Flavobacterium* in investigated area

Average number of *Alphaproteobacteria* detected by probe Alpha968 was the most abundant in June in Kotor Bay (23%). It showed the lowest values in Mamula (6%) Fig.5. The *Roseobacter* clade subgroup constitute a significant fraction (40%) of α-proteobacteria (Eilers et al., 2000) and especially abundant and widely distribute in plankton as well as in sediment. SAR 11 is the most abundant group for pelagic bacteria. Relative abundance of SAR11 varied from 28%-43% in central Adriatic area (Manti et al., 2012). Findings from Glöckner et al. (1999) showed various range of *Alphaproteobacteria* from 1% in North Sea to 14% in Baltic Sea. On average *Alphaproteobacteria* comprised 10% of the bacteria detected by DAPI staining in California's investigations (Cottrell &Kirchman, 2000).

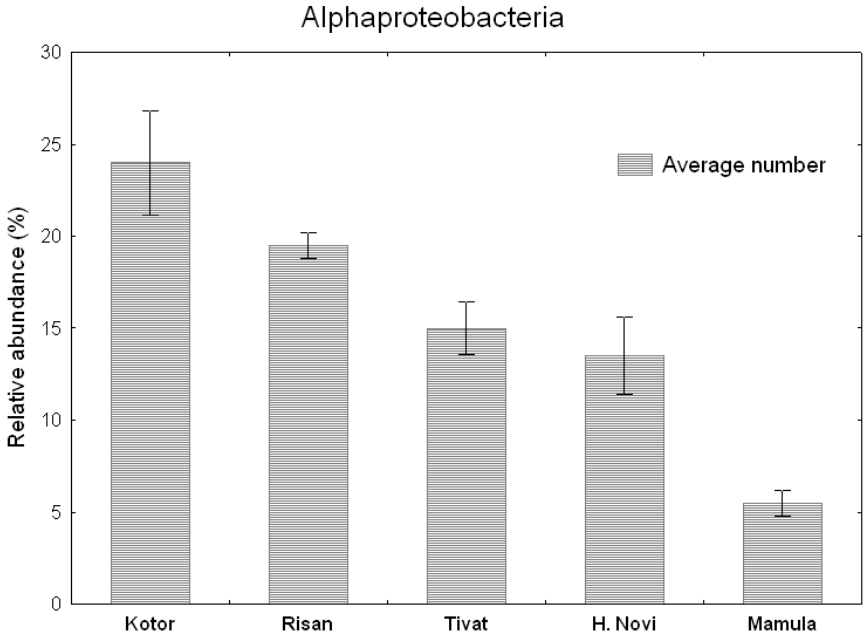

Figure 5: Mean values of *Alphaproteobacteria* in investigated area

It is obviously that abundance of all groups of bacteria decreased going from inner part in Kotor and Risan Bay to opener part so values in Mamula were lower during entire investigated period. The oligotrophic characteristics of this area and low freshwater influence are correlated with low abundance of investigated bacterioplankton especially *Bacterioidetes* and *Gammaproteobacteria*.

Heterotrophic bacteria abundance dominated the picoplancton community as determined by DAPI counting varied from $0.2x10^6$ cells mL^{-1} to $1.3x10^6$ cells mL^{-1} (in June) and $0.4x10^6$ mL^{-1} cells to $1.5x10^6$ cells mL^{-1} (in February). Highest cell numbers were measured in Kotor in June and Risan in February. Also at 0m was noticed higher bacterioplanctone abundance compared with 10m. (Fig.6).

Bacterial abundance is associated with enrichment of nutrients in particular aquatic area. According to Cottner & Biddanda (2002) abundances of heterotrophic bacteria of less than $1x10^6$ cells mL^{-1} cells considered to be typical for oligotrophic seas. That indicated that Kotor and Risan Bay represents eutrophic area during investigated period,Tivat slightly eutrophic area, Herceg Novi and Mamula oligotrophic area. Bacterial abundance in Kaštela Bay - Adriatic Sea (Šolic & Krstulović, 1994) ranged from 0.40 cells mL^{-1} to 2.06 $x10^6$ cells mL^{-1} .The average monthly abundance of heterotrophic bacteria along the eastern coast of the Central and Southern Adriatic Sea ranged from 0.21 $x10^6$cells mL^{-1} to 2.39 $x10^6$ cells mL^{-1} and in the open sea areas ranged from 0.23 $x10^6$ cells mL^{-1} to 0.63 $x10^6$ cells mL^{-1}.(Šantic et al., 2012).In the Northern Adriatic maximal values (124 $x10^7$ cells mL^{-1}) were found at the surface water because of large influence of Po River (Fuks et al., 2012).

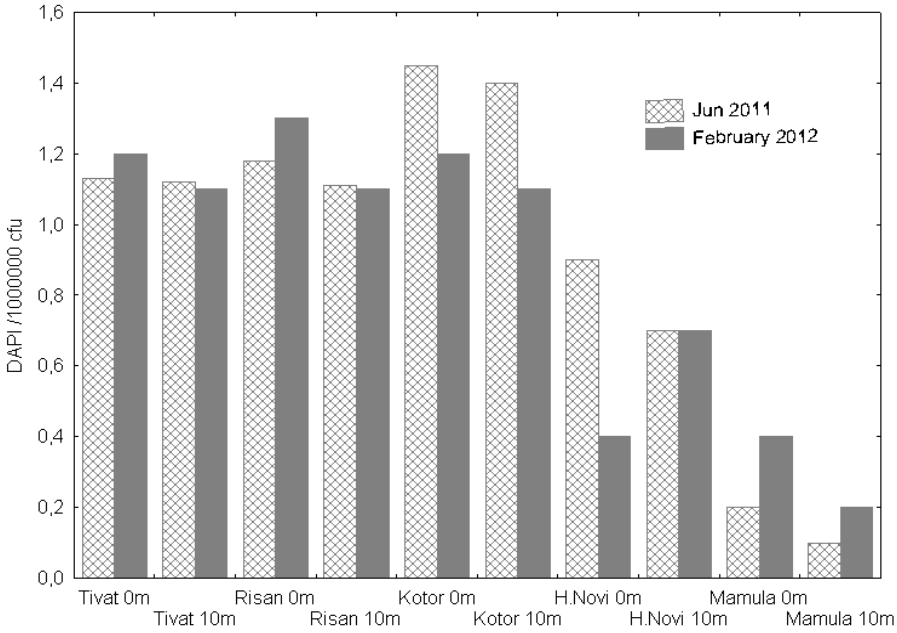

Figure 6 Total bacterioplankton abundance in investigated area in 1mL seawater

Nutrient concentration showed the lowest values in Mamula in June (nitrate 0.789µmol/L; nitrite 0.099 µmol/L,ammonia 0.044 µmol/L, phosphates 0.044 µmol/L) and highest in Kotor Bay in February (nitrate 5.955 µmol/L, nitrite 0.405 µmol/L, ammonia 0.345 µmol/L, phosphates 0.289 µmol/L). Kotor area showed maximum of measured nutrients because of large impact of coastal region. Rapid urbanization in this area also contributes higher pollution pressure.

Temperature varied from 11.1°C (February) in Risan to 25°C (June) in Tivat. Salinity varied from 16 PSU (February at 0m) in Kotor to 37.1 PSU (June) in Mamula. In thermal sense Bay is not homogenous area and the oscillations are most prominent in surface layers.

Salinity is influenced by variety of factors: the influx of fresh water, precipitation and currents. Gradual increasing of seawater temperature and salinity can be observed going from Kotor to Mamula. When the infux of

fresh water from the land is intesive surface water (0-2m) in Kotor and Risan Bay is characterized by very low salinity. Fig.7. Water flows are irregular. In summer the input current is strong, it has north-western direction. After heavy and long-term precipitation powerfull output current appears and moves along west coast and get out from the Bay in South-Eastern direction (Stjepčević, 1976).

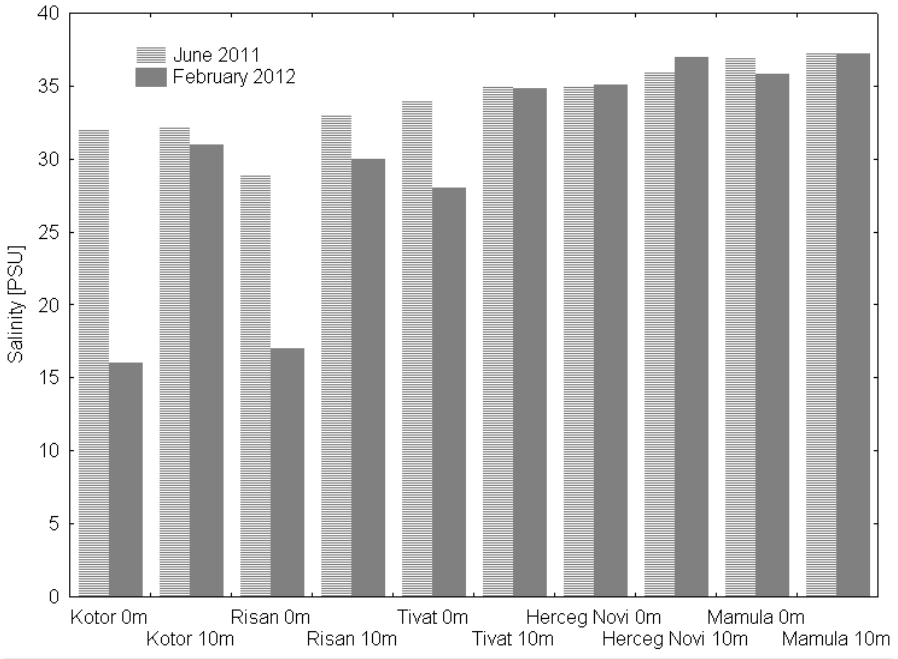

Figure7 Mean values of salinity in investigated area

CONCLUSION

Due to inflow of fresh water and organic load in Kotor and Risan Bay, an increase of all groups of bacteria was found. Further investigation will be based on more frequent sampling and using other oligonucleotide probes.

ACKNOWLEDGMENTS

This work is supported by Montenegro-Croatian project: "Molecular and physiological bacterial diversity of the trophic gradients in the Adriatic sea" and by the BABAS project (Croatian Science Foundation)

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