# COMPOSITION OF MICROBIOLOGY, PHYTOPLANKTON AND BIO-TOXINS IN WATER AND MUSSEL ON FISH AND SHELLFISH FARMS IN BOKA KOTORSKA BAY (SE ADRIATIC SEA)

Paper received: 06.10.2018. Journal of Agronomy, Technology and Engineering Management
Paper accepted: 12.01.2019. ISSN: 2620-1755 Pestorić et al., 2019. Vol. 2(1): 207-217

*Original research paper*

# COMPOSITION OF MICROBIOLOGY, PHYTOPLANKTON AND BIO-TOXINS IN WATER AND MUSSEL ON FISH AND SHELLFISH FARMS IN BOKA KOTORSKA BAY (SE ADRIATIC SEA)

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**ABSTRACT:** This paper presents the results of sea water and mussels meat quality investigation on three sites in Boka Kotorska Bay. Two sites are situated in inner part on shelfish farms (IBMK and COGI) and one close to open sea, Žanjice, is chosen as referent one. The sanitary quality on shellfish farms was satisfactory according the "Regulation on the classification and categorization of surface and ground water 02/07" on referent location and COGI shellfish farm. Low abundances of fecal indicator bacteria in water were recorded, indicating that no direct human contamination had occurred. Results on IBMK shellfish farm were periodically unsatisfactory according the Regulation 02/07. Obtained results present that the IBMK showed the highist value of *E.coli* (800 cfu/100ml) and *Enterococi* (350 cfu/100ml) in September 2015. Recorded phytoplankton data showed that microplankton and nanoplankton abundances reached values up to $10^5$ cell/l. Diatoms was dominant group of phytoplankton and also reached values of $10^5$ cells/L. Abundances of diatoms were similar at two farm sites with some maximal abundances on COGI in September 2016. The same is for abundance of dinoflagellates, similar values were noticed with maximal abundances on COGI site in April 2016. Sometimes this group prevailed on diatoms during spring/summer. Quantitative and qualitative analysis of bio-toxins in mussels tissue showed that all obtained results were below detection limits (LOD) what indicate good quality of farmed mussels, safety for use in human nutrition.

**Key words:** *water quality, bio-toxins, mussels, farms, Boka Kotorska Bay*

## INTRODUCTION

The eastern coast of the Adriatic Sea consists mainly of numerous islands, bays, coastal embayments, and also some transitional areas can be found. Boka Kotorska Bay is a transitional system as it is a boundary environment between land and sea, characterized with specific biological communities which differ from other adjacent marine and continental biomes (Sarno et al., 1993).

Phytoplankton organisms are very sensitive to changes in its environment and the enrichment of water with nutrients (primarily nitrogen, silicon and phosphorus compounds) may result in its growth. As for that, they are useful indicator of water quality (Brettum and Andersen, 2005).

Under certain conditions, some phytoplankton species may produce toxins (bio-toxins). Their blooms cause illness and death in humans, fish, seabirds, marine mammals, and other oceanic life, often because of toxin transfer through the food web (Anderson et al., 2010). Sometimes toxins and harmful substances can be accumulated in the meat, i.e. in fish, in the larger quantities than in the water column (Kragulj et al., 2018).

Although the shellfish may not be visibly affected, any filtration of water of lower quality could lead to bioaccumulation of harmful substances in shell tissue and thus undesirable consequences for end users, i.e. consumers and the general population. Bearing that around 20 shellfish and fish farms are located in Boka Kotorska Bay (Gvozdenović et al., 2017) and that the bivalves are filter feeders, makes regular monitoring of water quality an even greater priority.

Bio-toxins are produced by phytoplankton in order to defense against predators (FAO, 2004), and can cause six syndromes in the humans: ASP, AZP, DSP, NSP, PSP, VSP. Toxic algae which produce bio-toxins mostly belong to dinoflagellates group (Taylor, 1976). Also diatoms such *Nitzschia navis-varingica*, *Pseudo-nitzschia australis*, *P. multiseries*, *P. seriata* can produce bio-toxins (Kotaki, 2008; Thessen, 2010). There is about 10 toxic and potentially toxic phytoplankton species which inhabits Boka Kotorska Bay (Drakulović, 2012; Drakulović et al., 2017). Monitoring of toxic and potential toxic phytoplankton species as well as monitoring of bio-toxins can benefit both human health and the shellfish industry, as costly harvesting can be avoided when those species are present.

The aim of this study is the presentation of the composition and distribution of microbiology and phytoplankton assemblages on mussel and fish farms and mussels meat quality in area of Boka Kotorska Bay.

## MATERIAL AND METHODS

In the period from June 2015 to September 2016, research was conducted at three sites in Boka Kotorska Bay. Two, situated in inner part on shelfish farms (IBMK and COGI) and one close to open sea chosen as referent site, Žanjice (Figure 1). Temperature, salinity and microbiology were taken by Niskin bottle on forth depth on each site (surface, 2m, 4m, and bottom), while phytoplankton was sampled at 2m and 4m at shellfish farms because these layers have the most impact on mussels farms. Mussel samples (*Mytilus galloprovincialis*) were taken to be tested for presence of bio-toxins.

Standard methods were used for detection and enumeration of *Escherichia coli* (MEST EN ISO 9308-1:2015) and for detection and enumeration of intestinal enterococci (MEST EN ISO 7899-2:2011).

For detection and enumeration of phytoplankton standard method was used (MEST EN 15204:2014). Phytoplankton samples were preserved in 3% formaldehyde. In laboratory samples were dropped in 25cm$^3$ chambers for sedimentation and after 24h of sedimentation it was done determination of samples. Phytoplankton cells were counted using Leica DMI4000 B inverted microscope in subsamples of 25 ml according Utermöhl (1958). For qualitative analysis appropriate keys for phytoplankton determination were used (Cupp, 1933; Hustedt, 1930a, 1930b; H. Peragallo & M. Peragallo, 1965; Dodge, 1985; Schiller, 1933, 1937; Sournia, 1989). Mussels for detection of bio-toxins were sampled and transported according to the rules of European laboratory for marine bio-toxins (http://www.aesan.msssi.gob.es/en/CRLMB/web/home.shtml). Toxins from ASP (domoic acid) and PSP group (saxitoxins) were analyzed. For domoic acid method based on HPLC-UV-DAD was used (Quilliam *et al.*, 1995), while for saxitoxins method on HPLC-FLD (AOAC, 2005) was used. Limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the blank.

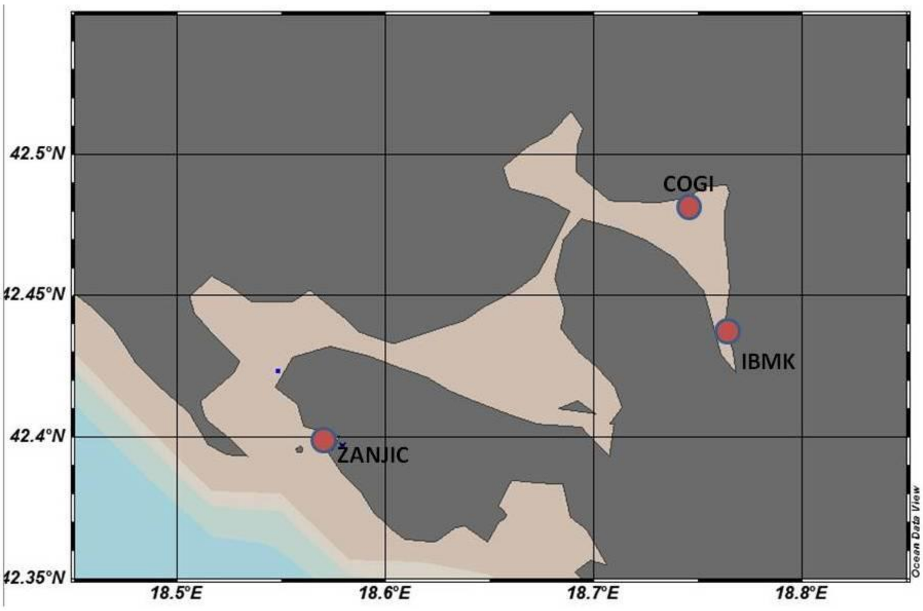



Figure 1. Map of sampling sites

Statistical method, Analysis of variance (ANOVA) was used to analyze differences among sites according to faecal indicators and phytoplankton groups.

## RESULTS AND DISCUSSION

Boka Kotorska Bay is a semi-enclosed region with strong water sources from streams or underground springs, during the precipitation season, which is higher in the late winter and spring then in autumn. In this period, streams and springs have an influence on the physical, chemical and biological dynamics of the seawater (Figure 2). Isothermic conditions were detected from March to mid May. In the summer period, temperature was variable because of weather conditions and intermittent precipitation, reaching a maximum in July 2015 (28.40°C) at referent site. Inverted stratification and a strong thermocline was detected from October 2015 to January 2016 when minimum was noticed (6.0°C, Figure 2) at IBMK site. Contrary to temperature, salinity showed inverted stratification during the entire research period with the presence of a strong halocline at shellfish farms from October 2015 to February 2016 with values range from 1.80 to 37.90 during investigated period. At referent site halocline was less pronounced and variability of salinity was lower, from 30 to 38.3 (Figure 2).

An insignificant number of faecal indicators were found at referent site and COGI shellfish farm during investigated period. The sanitary quality of seawater on these two sites was satisfactory according the "Regulation on the classification and categorization of surface and ground water 02/07". Faecal contamination indicators are significantly higher (including *E. coli*, intestinal enterococci) on IBMK shellfish farm and exceeded permissible limits prescribed by *Regulation* and reached highist value of *E.coli* (800 cfu/100ml) and intestinal *enterococi* (350 cfu/100ml) in September 2015 (Fig. 3). Over limited values were recorded in January 2016 (540 cfu/100ml) for *E. coli* and in February 2016 (220 cfu/100ml) for intestinal *enterococci* too. ANOVA test showed statistically significant difference among stations (p<0.001) for both indicators.

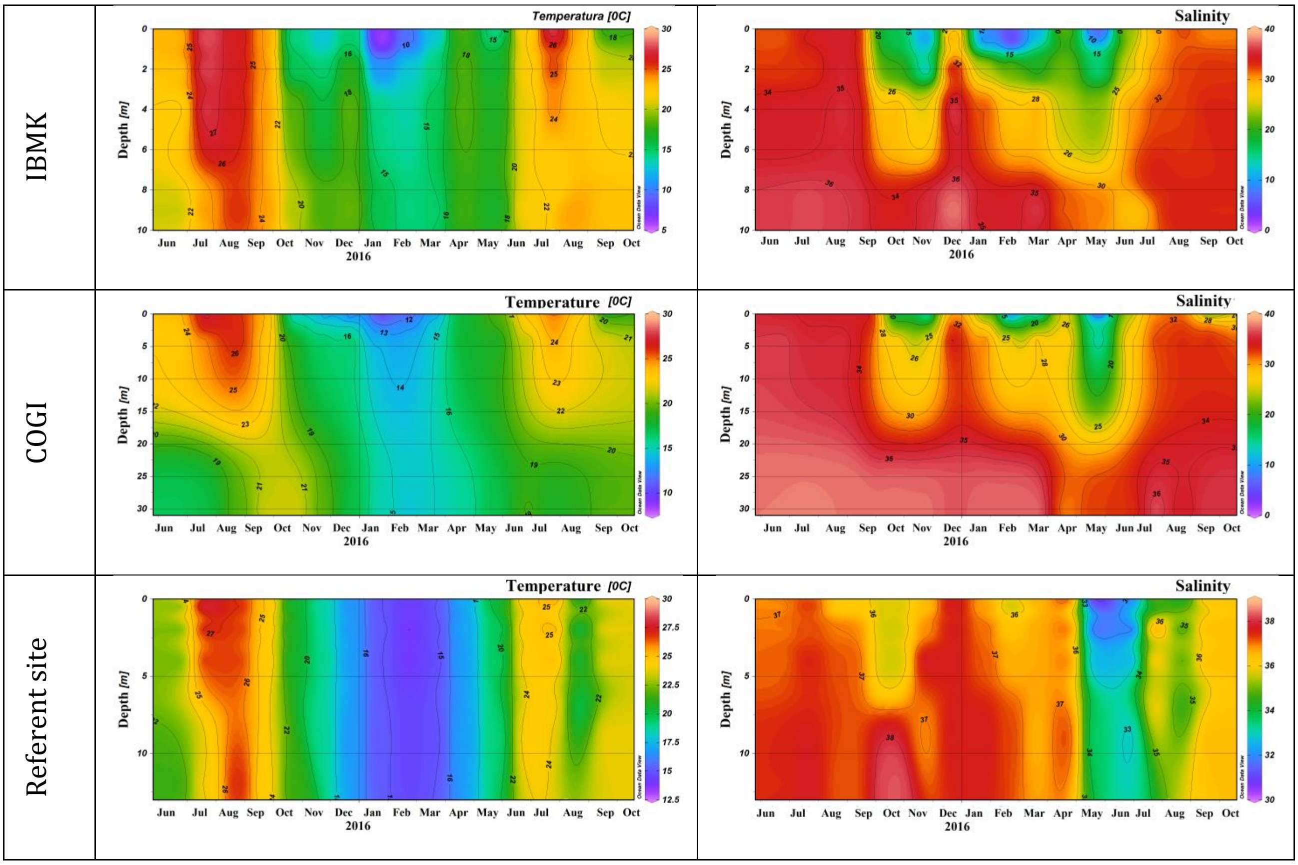
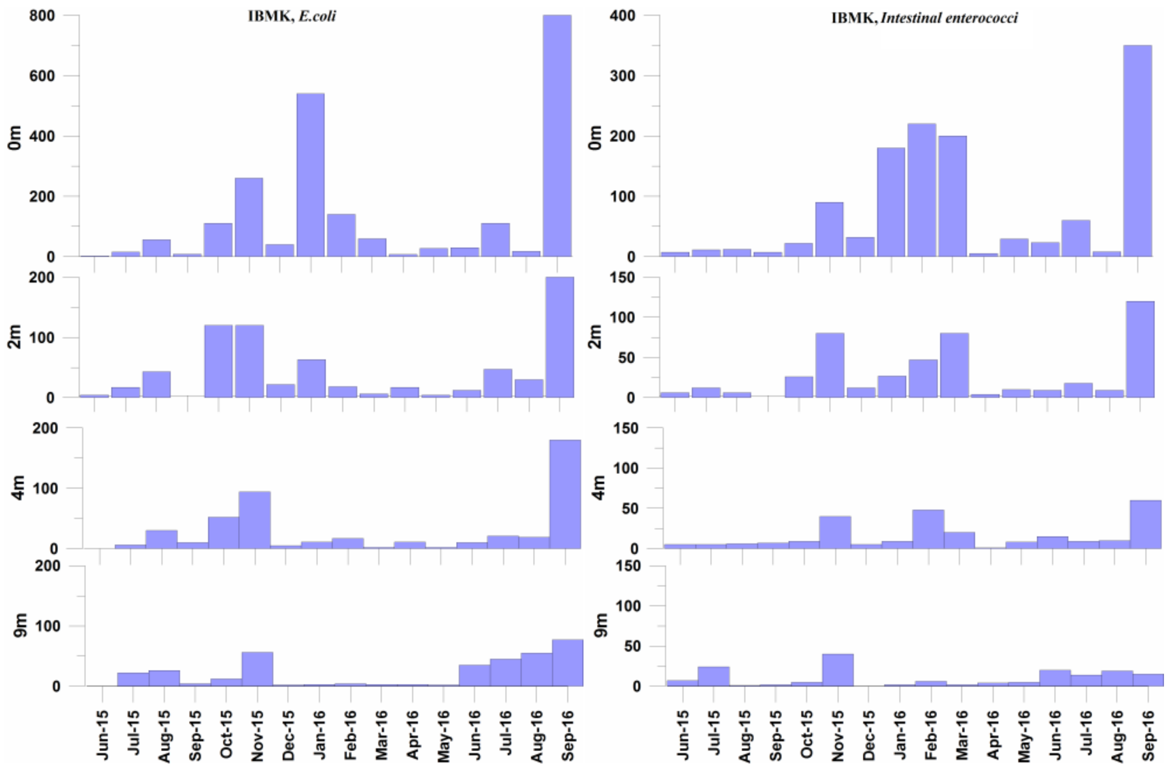



Figure 2. Temporal variability of temperature and salinity at investigated sites

Analysis of phytoplankton samples and recorded data showed that microplankton abundances reached values up to 105 cell/l. Diatoms as dominant group also reached values of 105 cells/L and higher abundances of diatoms mostly coincided with microplankton higher abundances (Figure 4). This group was dominant almost all investigation, except during some months in spring/summer when dinoflagellates prevailed. Abundances of diatoms were similar at two farm sites with some maximal abundances on COGI in September 2016. The same is for abundance of dinoflagellates, similar values were noticed with maximal abundances on COGI site in April 2016, with no difference by depths. ANOVA test showed statistically non-significant difference among stations (p>0.05).

The presence of diatom species from genus Pseudo-nitzschia is important due to its possibility of producing domoic acid as abundance of species from this genus was higher in comparison with other noticed toxic species. The composition, distribution and relation of domoic acid to physico-chemical parameters still needs to be clarified, even some domoic acid records have been recorded in the northern Adriatic (Marić et al., 2011). From dinoflagellates nine toxic species was noticed, from four genera: Dinophysis, Lingulodinium, Phalacroma and Prorocentrum. Recorded abundance of phytoplankton groups (dinoflagellates and coccolithophores-which were present in low abundance) are mostly typical for mesotrophic areas, with exception of microplankton and diatoms which abundances were increased and are more typical for eutrophic areas (Kitsiou and Karydis, 2002). Most of dominant and frequent species which are noticed in investigated area are characteristic for area enriched with nutrients (Bosak et al., 2009), means species prefer nutrients enriched waters. The inner part of Kotor Bay has several potent underwater springs and streams flowing into and providing almost constant influx of nutrients (Drakulović et al., 2017; Krivokapić et al., 2011). These findings might support the idea that the spatio-temporal distribution of the phytoplankton community is closely related to its environmental conditions (e.g. nutrients light and so on) as was reported by Buzančić et al., (2016).



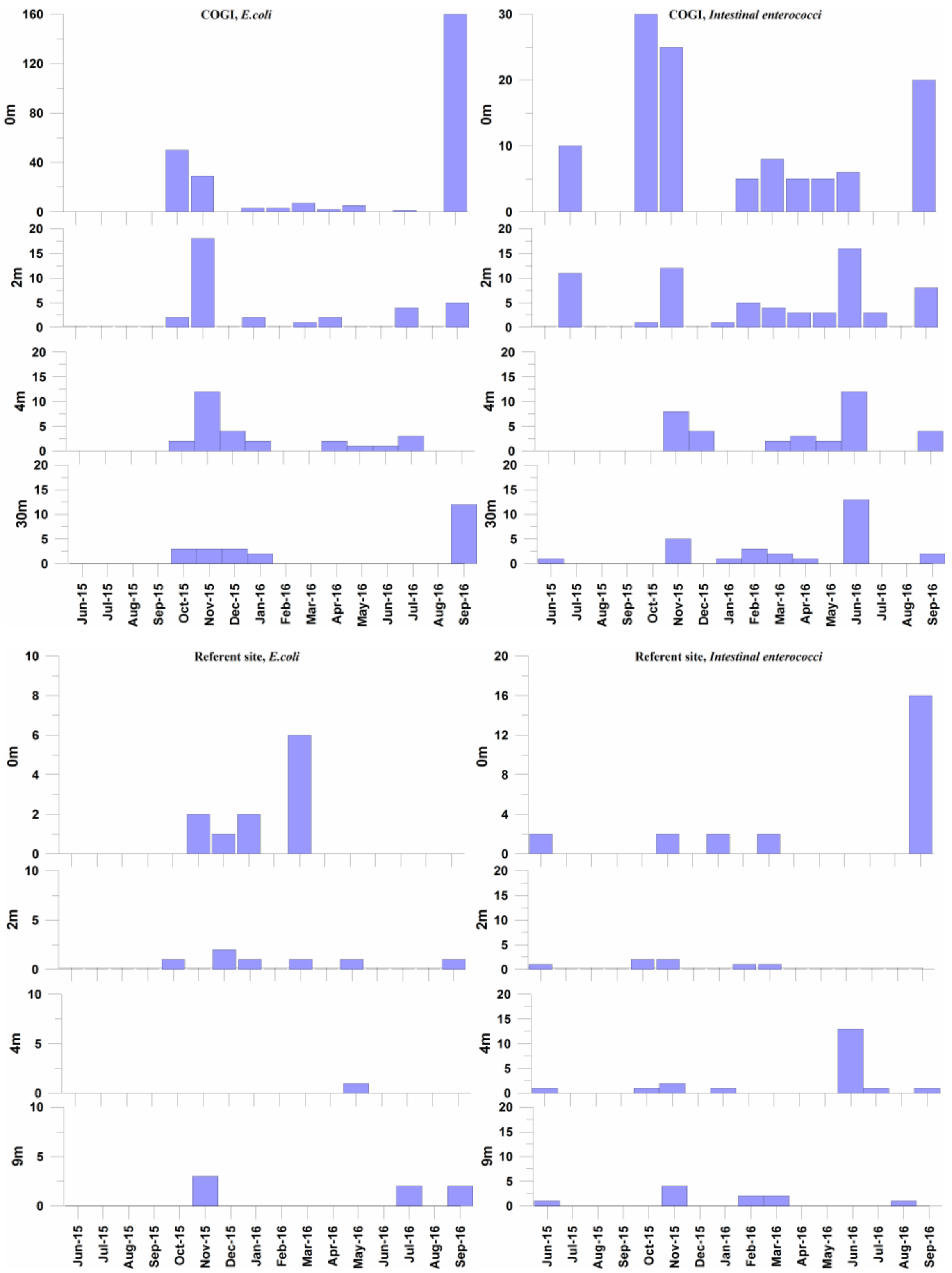



Figure 3. Temporal distribution of *E.coli* and intestinal *enterococci* at three investigated sites (units cfu/100ml).

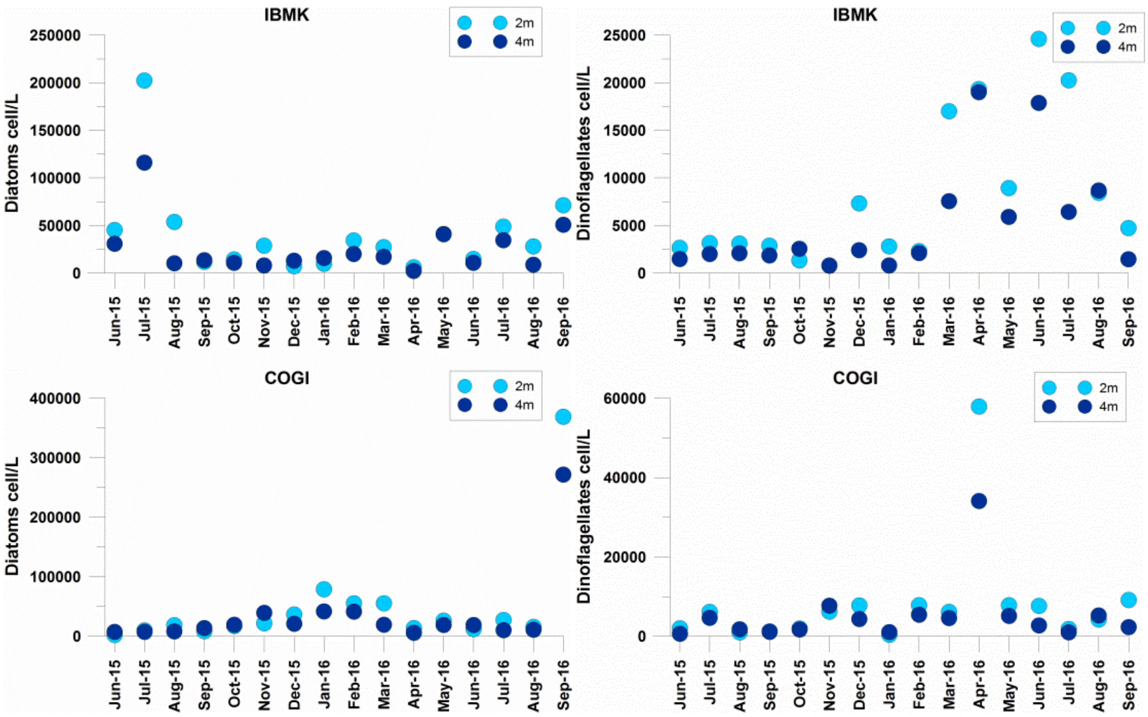



Figure 4. Temporal variability of most abudant groups diatoms and dinoflagelates at shellfish farms (2 and 4 m) (cells/L)

Analysis of bio-toxins in mussels meat showed negative results, all processed samples had values lower than LOD. In Table 1 LOD's for each toxin is presented. According to the EU Regulation (EC, 2004) maximum allowable concentration for STX and it derivates in mussels tissue is 800 µg/kg, and for DA is 20 mg/kg.

Table 1. LOD for ASP and PSP toxins obtained based on the standard deviation of the blank

| Bio-toxin | STX | dcSTX | GTX 1,4 | GTX 2,3 | NEO | C 1,2 | DA |
|---|---|---|---|---|---|---|---|
| LOD | 51.42 (µg/kg) | 1.168 (µg/kg) | 55.5 (µg/kg) | 8.368 (µg/kg) | 43.9 (µg/kg) | 7.368 (µg/kg) | 0.326 (mg/kg) |

Obtained results regarding bio-toxins analysis indicate that mussel in area of Boka Bay has good meat quality and it is safe for use in humans. Although high abundance of diatom *Pseudo-nitzschia* spp. was mentioned, levels of domoic acid were below LOD, what can indicate that maybe toxic species from this genus were not so abundant, or not even been present. Ujević et al. (2010) indicate that toxic species do not always express their toxicity. According to the same authors if abundance does not reach $1 \times 10^5$ cells/L then the area can be considered safe with respect to ASP. If the abundance exceeds $1 \times 10^6$ cells/L it might indicate the possible occurrence of ASP contamination. Bosak et al. (2010) indicate one toxic species, *Pseudo-nitzschia calliantha* in Boka Bay, with low abundace. Authors consider that bloom of this species appears in the Bay. Also Krivokapić (2005) indicate toxic *P. delicatissima* for iner part of Boka Kotorska Bay.

Arapov et al. (2017) found five toxic species from genus Pseudo-nitzschia in area of central Adriatic: *P. calliantha, P. delicatissima, P. fraudulenta, P. pseudodelicatissima/P. cuspidata* i *P. subfraudulenta*. In last twenty years domoic acid has been found at low levels, below regulatory limit of 20 mg/kg, for entire Adriatic Sea (Arapov, 2013). Domoic acid was found for the first time in Croatian water in 2006 in shellfish samples from Šibenik Bay and northern part of the coast (Ujević et al., 2010). Contamination samples were from winter season during the high abundance of *Pseudo-nitzschia* spp. in surrounding water. The highest concentration of domoic acid was 6.5486 mg/kg during February.

Species from the genus Alexandrium, as well as *Gymnodinium catenatum* and *Pyrodinium bahamense* are known to produce saxitoxins (Arapov, 2013). Those species were not found in the water samples from investigated stations during this research. Also those species have not been detected in Boka Kotorska Bay in earlier research (Drakulović, 2012; Drakulović et al., 2017). As analized mussel samples were negative on presence of saxitoxins, this indicates that there is almost no likelihood that paralytic shellfish poisoning (PSP) appears. Ciminiello et al. (1995) found PSP toxins for the first time in Adriatic Sea in shellfish during 1993. Low concentrations of GTX2 and GTX3 were detected in shellfish from the Emilia-Romagna coast (west Adriatic). In 1994 PSP toxins in concentrations above regulatory limits appeared in this part of Adriatic Sea. Toxins appeared after bloom of *Alexandrium minutum* (Honsell et al., 1995). In area of north-west Adriatic (Medulin Bay) levels of PSP toxins above regulation limits were found in shellfish during winter 2009 (Ujević et al., 2012).

*Dinophysis* spp., *Phalacroma* spp., *Prorocentrum* spp., as well as *Gonyaulax spinifera*, *Lingulodinium polyedrum* and *Protoceratium reticulatum* can produce DSP toxins (ocadaic acid, dinophysistoxins, pectenotoxins and yessotoxin) (Arapov, 2013). Some of those species were identified in water samples during our investigation but did not have high abundances, exept *Prorocentrum cordatum* which had a little higher abundance. Toxic species which produce DSP toxins were mentioned in earlier research for Boka Kotorska Bay (Bosak et al., 2011; Drakulović, 2012; Drakulović et al., 2014; Drakulović et al., 2017) but abundances were not high except for the *Prorocentrum* spp. Bosak et al. (2011) found *Prorocentrum cordatum* in abundance of 4 × $10^4$ cells/L in area of Boka Kotorska Bay, while Drakulović et al. (2017) found the same species in abundance of 4.7 × $10^3$ cells/L. Drakulović et al. (2014) also found high abundance of potentially toxic species *Prorocentrum micans* in the Bay ($10^6$ cells/L). Due to technical disadvantages we could not perform analysis of DSP toxins in mussels, so it is possible (higher abundance of some toxic species) that some DSP toxins were present in mussels during investigated. According to Gvozdenović et al. (2015) official data about poisoning in humans by bio-toxins in Montenegro does not exist, but it is possible that some kinds of poisoning (especially diarrhetic shellfish poisoning, DSP) were mistaken with bacterial and viral infections due to the fact that the clinical picture of these disease are quite similar. Arapov et al. (2015) found yessotoxins in almost all mussel samples from Croatian coast but below regulation limits. Ocadaic acid and dinophysistoxins in levels above regulation limits have been determined in mussels from Lim Bay. Levels were 1222 μg/kg for ocadaic acid and 1041 μg/kg for dinophysistoxins, respectively (Ninčević-Gladan et al., 2010; Ninčević-Gladan et al., 2011).

## CONCLUSIONS

Phytoplankton species ability to produce toxins can lead to accumulation of toxins in mussels tissue and throughout the food chain to the final consumers, humans, causing serious shellfish poisoning that can seriously endanger human life. In current research, toxic and potentially toxic species were noticed. The presence of diatom *Pseudo-nitzschia* spp. is also important due to its possibility of producing domoic acid as abundance of this diatom was higher in comparison with other noticed toxic species but as in mussels tissue bio-toxins were not found, the presence of these taxa did not affect the quality of mussels as potentially sea food. Monitoring of bio-toxins including also toxic and potential toxic phytoplankton monitoring should perform in future what give us possibility to react on time so possible alarming situations can be avoided for prediction and prevention of its harmful effects and potential toxicity in one of the most sensitive areas of Adriatic.

## ACKNOWLEDGEMENTS

This study has been supported by the Ministry of Science of Montenegro and the HERIC project through the BIO-ICT Centre of Excellence (Contract No. 01-1001).

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