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Cardiac activity in the Mediterranean mussel (*Mytilus galloprovincialis* Lamarck, 1819) as a biomarker for assessing sea water quality in Boka Kotorska Bay, Southern Adriatic Sea

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Abstract

This paper presents an attempt to evaluate physiological response differences (cardiac activity) in the Mediterranean mussel *Mytilus galloprovincialis* and its use as a biomarker to assess water quality in Boka Kotorska Bay. Mussels were sampled in 2014 from three different sites in Boka Kotorska Bay, which faces various anthropogenic pressures. Both the heart rate recovery time (T_{rec}) and the coefficient of variation in the heart rate (CV_{HR}) were tested, in compliance with the standardized test procedure, using short-term reduction of water salinity as a stress factor to study the adaptive capacity of the tested mussels. The highest values of both T_{rec} and CV_{HR} in Port Bijela, T_{rec} = 77.3 ± 7.2 min; CV_{HR} = 10%, indicated that the water quality at this site was worse when compared to other locations (Stoliv and Dobrota). The values at Port Bijela were significantly higher compared to Stoliv and Dobrota. The lowest values of T_{rec} were found in the mussels from Stoliv (47.3 ± 3.8 min), which implies better water quality compared to the other locations. Although, higher values of T_{rec} were obtained for Dobrota (53.3 ± 4.8 min) compared to Stoliv, the differences were not statistically significant. The applied rapid method of environmental biomonitoring using cardiac activity in mussels could be a useful tool in water quality assessment. Thus, the relevant authorities could timely plan and carry out activities to maintain good ecological status of the marine coastal areas of Montenegro, in order to minimize the effect of emergency situations on both animal and human health in the region, as well as limiting wastewater discharge.

Keywords: Water quality assessment; mussels; cardiac activity biomarkers; Montenegro; Adriatic Sea.

Introduction

Most organisms are highly sensitive to environmental changes, so they are widely used as bioindicators (live bio-monitors) in the assessment of the quality of their dwelling (Saulović & Mujić, 2009). Bivalves have been recommended by several monitoring programs as bioindicators due to their cosmopolitan distribution and their ability to accumulate various pollutants, primarily heavy metals, and bio-toxins (Ninčević Gladan *et al*., 2010; Jović *et al*., 2011). Generally, results have been obtained by measuring the accumulation of pollutants in mussel tissues, as well as monitoring of pollutants effects on their growth, physiology, and reproduction (Lee *et al*., 1998; Miner *et al*., 2006; Jović *et al*., 2011; Turja *et al*., 2014; Luna-Acosta *et al*., 2015).

Biomonitoring programs are the most efficient and reliable in providing information about the natural state of aquatic ecosystems (Van der Oost *et al*., 2003). There are a variety of responses that can be used as tools to assess the health of animals exposed to certain chemicals, provide information on spatial and temporal changes in pollutant concentrations and indicate the occurrence of poor environmental quality or adverse ecological consequences (Turja *et al*., 2014). In fish biomonitoring programs, biotransformation enzymes, oxidative stress parameters, stress proteins, metallothionein induction and multi-xenobiotic resistance, haematological, immunological, reproductive and endocrine, neuromuscular, genotoxic, physiological and morphological parameters are widely used as biomarkers (Van der Oost *et al*., 2003). Most of them can also be applied to invertebrates including molluscs (Hoarau *et al*., 2006; Park *et al*., 2009; Kholodkevich *et al*., 2011; Despotović, 2013; Turja *et al*., 2014; Kuznetsova & Kholodkevich, 2015).

The Mediterranean mussel (*Mytilus galloprovincialis*) is widely used as an indicator species because of its well-known responsiveness to environmental contaminants, and it is included among the sentinel organisms in biomonitoring programs at both national and international level (Martin-Diaz *et al*., 2009).

The cardiac activity and behavior of mussels have been proposed as indicators for assessing the biological effects of environmental pollution (Pirro *et al*., 1999; Curtis *et al*., 2000; Santini *et al*., 2000; Brown *et al*., 2004). Specifically, cardiac rhythm in mussels (and also in crabs) has been shown to be a sensitive responsive system (Kholodkevich *et al*., 2011; 2017; Kuznetsova *et al*., 2018). Thus, several physiological biomarkers based on the characteristics of cardiac activity, respiration rate and physical activity, reflecting an organism's integrated response to pollution, have been demonstrated to be effective tools for biomonitoring environment quality (Kholodkevich *et al*., 2011).

Boka Kotorska Bay is located in the south-eastern part of the Adriatic Sea and occupies an area of 87.3 km^2. The Bay is divided into four smaller bays, specifically Kotor, Risan, Tivat and Herceg Novi Bay, and has distinctive hydrographic and relief characteristics compared to the open sea. The Bay suffers from the intensive impact of anthropogenic pressure (Joksimović *et al*., 2001; Anonymous, 2012), especially due to population growth and the increased number of vessels sailing in its waters. Also, the Bay is characterized by freshwater input due to rainfall and freshwater from the shore and submerged sources. Maximum discharge of freshwater in the Bay occurs during winter, while minimum discharge is observed during summer (Mandić *et al*., 2016). In total, there are 13 coastal freshwater sources (four in Herceg Novi Bay, four in Tivat Bay, three in Risan Bay and two in Kotor Bay), while the highest number of submerged sources are located in Risan and Kotor Bay (Drakulović, 2012).

Since 2012, a biomonitoring program based on the heart rate of mussels is running in Boka Kotorska Bay (Martinović *et al*., 2013; Nikolić *et al*., 2014; 2015). However, regular environmental monitoring has also been conducted over the last decade (Mandić *et al*., 2012; Huter *et al*., 2017).

The aim of this study was to present the use of the cardiac activity of mussels as a biomarker for assessing the water quality in Boka Kotorska Bay. The three locations in the Bay were selected with regard to the fact that they have been subject to the impact of various anthropogenic pressures - Port Bijela as a former shipyard, Dobrota as a settlement with an unregulated waste water disposal system, and near Port Kotor, the settlement of Stoliv that has been considered as a referent location in previous studies (Martinović *et al*., 2013). We also focused on the physical, chemical and biological parameters of the sea water at selected locations for the assessment of water quality.

Material and Methods

Experimental animals and their maintenance

Specimens of *M. galloprovincialis* were collected during spring 2014 from three different locations in Boka Kotorska Bay (Fig. 1). Eighteen to twenty animals of similar shell length (from 50 to 55 mm) were collected from each location and transported to the laboratory. The coordinates of the study locations are given in Table 1.

In the laboratory, each individual was cleaned of fouling organisms. Thereafter, sensors were attached on the shells of the mussels over the heart arrangement area (eight mussels from each of three locations) in order to register their heartbeat. Then, each group of mussels orginating from each of the three sampling locations was placed in a separate aquarium submerged in sea water orginating from the same location, and kept there for about 24 hours in order to stabilize their heartbeat.

Physical and chemical analysis

The physical and chemical parameters of the seawater at the study locations were measured on the day of sampling. The temperature, salinity and O_2 saturation of the water were obtained *in situ* using a universal meter (Multiline P4; WTW), and water samples were taken for the analysis of NO_2, total coliforms and faecal coliforms. NO_2 concentrations were calculated by the standard colorimetric method (Strickland & Parsons, 1972) using a Perkin Elmer λ 2 spectrophotometer. Total coliforms and faecal coliforms were estimated by the membrane filtration method (ISO 9308-1: 2011).

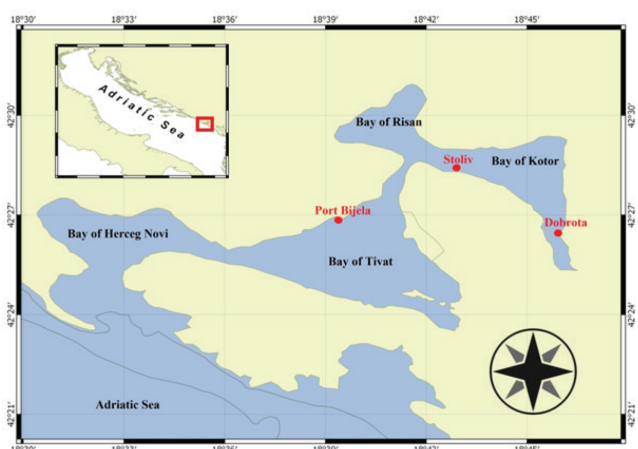

Fig. 1: Map of the study area showing the sampling locations in Boka Kotorska Bay (South Adriatic Sea).

Table 1. Coordinates and anthropogenic pressures at the study locations.

Location	N	W	Anthropogenic pressures
Stoliv	42.47216	18.70558	Reference location
Dobrota	42.45818	18.76505	Contaminated location / sewage wastewaters
Port Bijela	42.45140	18.65703	Contaminated location / former industrial activity zone

Biomarker analysis – Cardiac activity measurement in mussels

Heart rate measurements were performed using an original laser fibre-optic method for non-invasive recording of cardiac activity in selected invertebrates with exoskeletons (the shells of molluscs or the carapace of crustaceans). This method was developed at the laboratory of Experimental Ecology of the Scientific Research Centre for Ecological Safety (SRCES RAS Russia) (Fedotov *et al*., 2000; Kholodkevich *et al*., 2013). The infra-red light beam initially formed in the laser fibre-optic photoplethysmograph (LFOP) is transmitted to the animal by a thin fibre-optical cable with a small sensor (weighing <2g), which is attached to the shell, thus illuminating the heart area with scattered light. The optical signal modulated by the heart contractile activity of the animal contains information on cardiac activity. After appropriate amplification and filtration in the LFOP, the analogue signal is then transmitted to a 14-bit 16-channel analogue-to-digital converter and then via a USB port to a personal computer (Fig. 2). These measurements produce a photoplethysmogram, which can be further analyzed using mathematical and statistical methods (Kholodkevich *et al*., 2013).

After stabilization of the heart rhythm, the mussels from all three study locations were subjected to a salinity test. A rapid (1 min) decrease in water salinity (50% of the ambient level) at the sampling site was used as the functional load for evaluating the physiological state of the animals (Kuznetsova, 2013). Abrupt and unpredictable variations in salinity determine physiology in marine invertebrates such as molluscs and crustaceans (Henry *et al*., 2012). The salinity change in the tanks containing the mussels was performed by adding distilled water to the tanks (Turja *et al*., 2014). The mussels were exposed to low salinity for 1 hour and then salinity was restored rapidly (1 min) by channelling a flow of fresh sea water through the tank in which the mussels were tested.

During the entire period of the experiment (heart rate stabilization, salinity decrease and after returning to ambient salinity – a total of about 30 hours), the cardiac rhythm of the mussels was recorded and stored in the form of bin and text files for further analysis. Heart rate recovery time (T_{rec}) and the coefficient of heart rate variation (CV_{HR}) were used as physiological biomarkers of the alterations in the physiological state of the mussels from different contaminated sites (Kholodkevich *et al*., 2017).

Mean heart rate (beats per minute) was calculated automatically using 50 cardiointerval as a sample. T_{rec} was calculated as the time that mussels need to re-establish their initial heart rate after the salinity stress load. CV_{HR} is a quality characteristic of HR variability within a group of animals, measured 1 hour after removal of the stress treatment. CV_{HR} (expressed as a percentage, %) was calculated using the formula CV_{HR} = SD/HRm, where SD is the standard deviation and HRm is the mean HR for a group of mussels from a particular study site, measured after removal of the load and subsequent recovery of the background HR pattern (Kuznetsova *et al.*, 2018).

T_{rec} was determined for each individual and then the average values of T_{rec} and its standard error (SE) were calculated for all the study locations. The T_{rec} values of mussels originating from all three locations were compared for

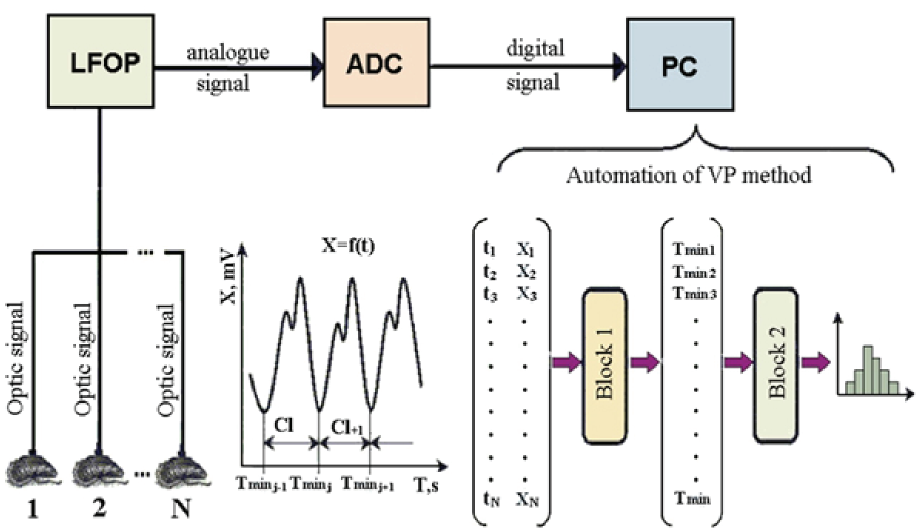

Fig. 2: Set up design for cardiac activity monitoring in mussels (from: Kholodkevich *et al.*, 2017).

indirect determination of water quality at the investigated locations. According to Kholodkevich *et al.* (2011), individuals collected from clean or less polluted sites have a shorter T_{rec} due to their better adaptive capacity, as well as lower CV_{HR} after the stress stimulus is removed.

Statistical Analysis

The normality and homogeneity of group variances were examined using Levene's test. Differences among the means were assessed by one-way analysis of variance – ANOVA (Fisher – F test), using the Data Analysis ToolPack in MS Excel 2003 for Windows; significant differences were found ($p < 0.05$.)

Results

Physical and chemical parameters

Table 2 presents the results of the physical and chemical parameters at the study locations. The temperature ranged from a minimum of 15.5°C at Dobrota to a maximum at Port Bijela of 19.9°C. Higher salinity (27.9‰) was observed at Port Bijela compared to other locations, 9‰ at Dobrota and 10.3‰ at Stoliv. On the other hand, oxygen saturation was quite similar at all the study locations and ranged from 102% at Dobrota to 109% at Stoliv. As with oxygen saturation, NO_2^- values did not differ significantly between locations; the lowest values were found at Stoliv (0.158 µmol/l) and the highest at Port Bijela (0.217 µmol/l.) The bacteriological results are given in Fig. 3. At Stoliv and Port Bijela, total coliforms were less than 100/100 ml of water, and faecal coliforms less than 300/100 ml of water. At Dobrota, the bacteriological values were significantly higher, with total coliforms being 2000/ 100 ml of water and faecal coliforms 500/100 ml of water.

Biomarker analysis

Mussels from all three locations showed a rapid heart rate reduction when water salinity was reduced by 50%, with exposure lasting one hour. Recovery of the heart rate was noted after stress removal. The T_{rec} and CV_{HR} values are given in Figures 4 and 5. After the salinity test and recovery, a prolonged T_{rec} was obtained in the mussels from Port Bijela (77.3 ± 7.2 min). Mussels from this location also presented a 10% change in the variation of the heart rate (CV_{HR}). The lowest T_{rec} was obtained from Stoliv (47.3 ± 3.8 min) and CV_{HR} (8.6 %.) At Dobrota, T_{rec} was 53.3 ± 4.8 min with a CV_{HR} of 8%.

The results of ANOVA showed that Port Bijela had statistically significantly higher values of T_{rec} compared to Stoliv and Dobrota. Although higher values of T_{rec} were obtained from Dobrota compared to Stoliv, the differences were not statistically significant (Table 3).

Discussion

Environmental pollution is recognized as a major threat to the aquatic environment all over the world. Heavy metals, organic compounds, microplastics and other pollutants directly and indirectly affect the functioning of the aquatic benthic community in natural habitats (Gallo *et al*., 2018; Kahlon *et al*., 2018). From this perspective, permanent biomonitoring of the ecological health of aquatic ecosystems is a global issue in international scientific programs (MEDPOL, HELCOM and OSPAR). Mussels are widely used as bioindicators, mainly because of their cosmopolitan distribution and the fact that populations are constant, they lead a sessile way of life and have the ability to accumulate high levels of contaminants in their tissues (Dailianis, 2010). The most widely used biomarkers in mussels for monitoring environmental health status are (Dailianis, 2010):

1. General stress biomarkers (lysosomal membrane stability, lysosomal lipofuscin content, lysosomal neutral lipid, peroxisomes proliferation, total oxidant scavenging capacity and lipid peroxidation content);
2. Specific stress biomarkers (acetylcholinesterase activity and metallothionein content);
3. Genotoxicity biomarkers (DNA damage).

In this study, we focused not only on the physical and chemical parameters of the sea water, but also on water

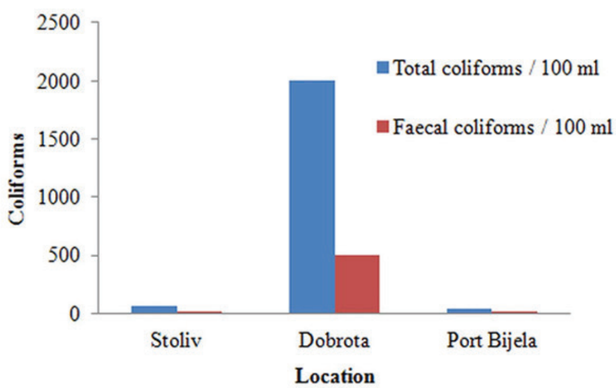

Fig. 3: Total and faecal coliforms at sampling locations.

Table 2. Physical and chemical parameters of the water at the sampling locations.

Location/Parameters	Temperature (˚C)	Salinity (‰)	O_2 saturation (%)	NO_2^-(µmol/l)
Stoliv	19.1	10.3	109	0.158
Dobrota	15.5	9	102	0.178
Port Bijela	19.9	27.9	108	0.217

Table 3. Fisher F-test ccomparative analysis of T_{rec} for the mussels from the study locations in Boka Kotorska Bay.

ANOVA	df	MS	F	p
Stoliv x Dobrota	1	144	1.571	0.2305[ns]
Dobrota x Port Bijela	1	2304	14.484	0.0019*
Stoliv x Port Bijela	1	3600	23.518	0.0002*

df - degrees of freedom; MS - mean squares; F - Fisher test;

ns - non significant differences; * - significant differences at $p < 0.05$

quality at the habitat sites of the mussels by evaluating their physiological state. The measured physical and chemical parameters of the water at all three locations indicate good ecological quality, which makes the water a suitable habitat for mussels (Table 2). All the parameters, i.e. salinity, O_2 saturation and NO_2^- concentration, were within the permitted limits (salinity < 40‰; O_2 saturation > 70%; $NO_2^- < 0.652$ μmol/l) required by the Regulation on the Classification and Categorization of Surface and Ground Waters (Official Gazette of Montenegro, 2007). Based on the same Regulation, the bacteriological results from Stoliv and Port Bijela were within permitted limits (total coliforms less than 100/100 ml of water; faecal coliforms less than 300/100 ml of water). The results obtained from Dobrota, with total coliforms of 2000/100 ml of water, indicated that those values were 20 times higher than permitted. Faecal coliforms, at 500/100 ml of water, were almost double the permitted value (Official Gazette of Montenegro, 2007). The results can be explained by the low currents in this area, high population pressure and as yet untreated sewage discharges (Stjepčević, 1974; Joksimović *et al*., 2001; Drakulović, 2012).

According to the results, the microbiological status of the water from Dobrota can be classified as poor but, according to the World Health Organization (WHO, 2010), thermo-tolerant (faecal) coliforms are not a comprehensive indicator of health risk because their presence in water does not show a positive correlation with bacterial and viral pathogens. The WHO recommendation is that *E. coli* should be taken as a credible indicator of the presence of pathogens instead of thermo-tolerant coliforms (Mandić *et al*., 2012).

Despite the fact that water from Dobrota exhibits the highest total and faecal coliforms, mussels from Port Bijela exhibit the most prolonged T_{rec}, thus indicating poor water quality at this location compared to Dobrota. Mussels are probably less sensitive to "microbial pollution", since faecal coliforms represent a food supply for mussels, but not a primary food source. Šolić *et al.* (1999) indicated that the concentration of faecal coliforms in seawater had no effect on their concentration in mussels. Mussels are selective filter feeders, having the ability to sort particles, ingest food and reject pseudofaeces particles that are non-nutritive or of an unfavourable size (Jorgensen, 1966).

Although the salinity values were within the permitted limits at all three locations, it is evident that salinity at Stoliv and Dobrota was much lower than at Port Bijela during the investigated period. It is known that salinity values in Boka Kotorska Bay vary throughout the year, and depend on rainfall and the inflow of freshwater from the shore and submerged sources (Mandić *et al*., 2016). When compared to Port Bijela, Dobrota and Stoliv that are located in the inner part of the Bay, are characterized by greater salinity fluctuations, especially during winter and early spring (Drakulović, 2012; Mandić *et al*., 2016). However, the local biota representatives are characterised by evolutionary based pre-adaptation to unstable salinity in their dwelling. Species within the genus *Mytilus* are considered as very tolerant to a wide range of physical and chemical conditions (Mainwaring *et al*., 2014). Therefore, differences in salinity are not significantly reflected in the cardiac response of mussels, especially as we reduced salinity by 50% at each sampling location.

The lowest values of T_{rec} from Stoliv (Fig. 4) imply good physiological condition of the mussels inhabiting this location. These results are consistent with the observations of Martinović *et al.* (2013) and Nikolić *et al.* (2014) at the same sampling locality, attributed as a reference site. There are strong currents in this area of the Bay (Stjepčević, 1974), which might mean that pollutants (such as heavy metals, biotoxins, PAHs, pesticides, organometallic materials, and waste waters) are carried away.

The comparative analysis of the data obtained for the three locations showed statistically significant differences in T_{rec} obtained from Port Bijela compared to the oth-

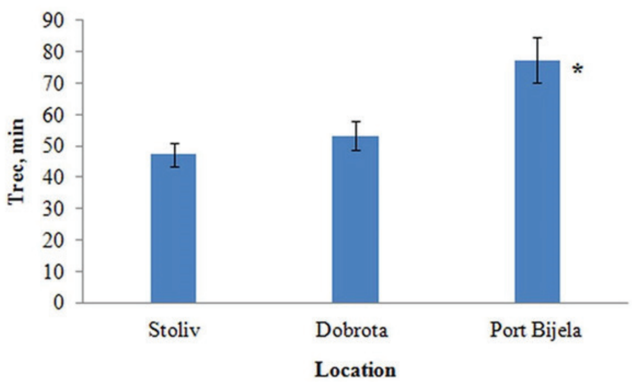

Fig. 4: Trec in mussels from the study locations, including standard deviation, * statistically significant difference from the other two locations (ANOVA, $p < 0.05$).

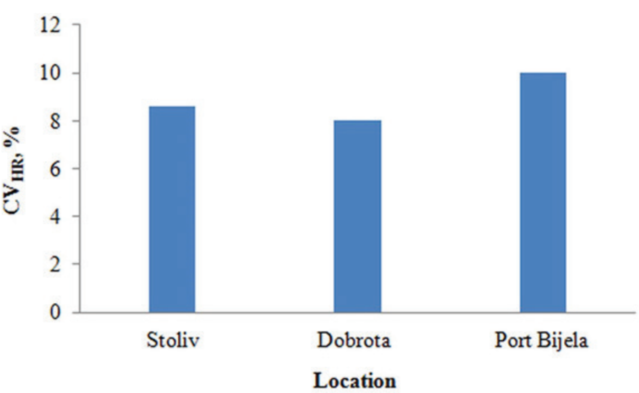

Fig. 5: CV_{HR} in mussels from the study locations.

er two locations, while differences in T_{rec} between Stoliv and Dobrota were not significant (Table 3). This could be explained by the fact that this area was a highly active industrial zone (a shipyard area). It is generally known that shipyards produce large amounts of waste grit that adversely impacts the environment. In the area of Port Bijela, waste grit is dumped in the sea with potential sources of contamination being: heavy metals – As, Ba, Cu, Zn, F, Cr, Sn, Co, Ni, Pb, Hg and organic compounds such as TBT and PAHs (EPA, 2016). All these pollutants are toxic for molluscs. This leads us to the conclusion that mussels are highly sensitive to pollutants such as heavy metals and organic compounds and that significantly prolonged T_{rec} in Port Bijela mussels was probably observed because of the presence and accumulation of those pollutants. According to the EPA (2016), in the area of Port Bijela, TBT levels in mussels in 2010 and 2011 were 20-30 times higher than permitted.

As the T_{rec} obtained from Port Bijela exceeded the T_{rec} obtained from Stoliv (considered a reference site for Boka Kotorska Bay in long-term biomonitoring studies), and as those differences are statistically significant, this finding confirmed the presence of stress in the mussels at Port Bijela.

Our results confirm that the applied method allows discrimination of the polluted site from the reference one by measuring recovery time in marine invertebrates. Earlier, Kholodkevich *et al.* (2011) found that rapid recovery of *Mytilus* (50-60 min) may signify good health and high adaptive potential of mussels and, therefore, good environmental status of the study site. Turja *et al.* (2014) also found higher T_{rec} values in mussels from contaminated sites compared to their reference location.

Only one similar study has been conducted in the area of Boka Kotorska Bay. Martinović *et al.* (2013) performed a salinity test in mussels from three different localities, including Stoliv and Dobrota. Their research coincides with ours and indicates best water quality in Stoliv, with a T_{rec} of less than 60 min.

The results of our study indicate that the non-invasive laser optic fibre method for cardiac activity monitoring can be a useful tool for finding early signs of deterioration in the physiological condition of marine bioindicator organisms. The latter was evaluated by detecting their adaptive potential in order to restore their heart rate after a short-term experimental stress load. A prolonged T_{rec} in organisms reflects the tendency of the community to be affected by environmental challenges of various origins. The data obtained shows that the proposed physiological assessment method for selected species of local biota can serve as a basis to discriminate between sites subject to different anthropogenic pressures. The good state of the biota proves the good ecological status of the environment and ensures long-term sustainability of ecosystem functioning.

The method proposed in this research has the following limitations as regards implementation:

Only certain species with a hard exoskeleton, suitable for attaching cardiac registration sensors, can be used (other types of sensors have to be developed);

Regarding the physiology of the experimental species, special short-term physiological periods (i.e. spawning), which are characterized by natural oxidative stress, could mask the biological effects of pollution (Soldatov *et al.*, 2007) as it indicates a disturbed organism state.

However, one of the advantages of the method worth mentioning is its rapid performance, low cost, the fact that it does not require expert staff to conduct the experimental procedure, and automatic analysis of the data. In addition, the fibre-optic cables and sensors system does not limit the usual locomotor activity in the animals and does not cause stress to the animals, thus offering the opportunity to use this method for permanent long-term environmental biomonitoring studies. The method used is complementary and should be combined with various other methods used to assess environmental quality (Turja *et al.*, 2014).

Future studies on the peculiarities of adaptive reorganization in the functioning of aquatic organisms under environmental challenges are required, as well as studies on seasonal variations in the responses of bioindicator organisms to pollution. The need for such studies is obvious and some of these areas will be explored by the authors in the future.

Acknowledgements

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