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OCENA MORSKEGA ONESNAŽENJA NA PODLAGI TOKSIČNEGA POTENCIALA VODE, SEDIMENTA IN BIOTE

(ESTIMATION OF MARINE CONTAMINATION BASED ON TOXIC POTENTIAL OF WATER, SEDIMENT AND BIOTA)

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Ocena morskega onesnaženja na podlagi toksičnega potenciala vode, sedimenta in biote

(Estimation of marine contamination based on toxic potential of water, sediment and biota)

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Izvleček: Za oceno tveganja za morske habitate je bistvenega pomena določiti usodo različnih onesnaževalcev, saj se različno razporejajo po vodnem stolpcu in se različno akumulirajo v bioti in usedlinah. Ta študija je pokazala toksični potencial morske vode, usedlin in biote (biološke tekočine klapavic *Mytilus galloprovincialis*) na obalnem območju Severnega Jadrana in odkrila možne povezave med temi tremi okolji. Poleg tega se je preučevalo toksični potencial glede na velikost zrn usedlin, kot tudi sposobnost potencialno strupenega okolja, da vpliva na fitnes klapavic. Strupenost je bila ocenjena z biološkim testom Microtox®. Velikost zrn usedlin je bila določena s pomočjo suhega sejanja, kondicija klapavic pa s pomočjo anoksičnega preživetja klapavic (SOS test). Med opazovanimi tremi okolji ni bila ugotovljena nikakršna povezava, zato je ključnega pomena ugotoviti toksični potencial vsakega posameznega okolja, da se pripravi celostno in natančno oceno stanja in potencialnega tveganja. Toksični potencial morske vode, usedlin in biološkega tkiva klapavic odraža nedavni vnos strupenih onesnaževal v morsko vodo, njihovo kronično kopičenje v usedlinah in dostopnost onesnaževal klapavicam. Med toksičnim potencialom in velikostjo zrn sedimentov, med potencialno strupenim okoljem in fitnesom klapavic niso bile ugotovljene povezave, kar kaže na posebne okoljske razmere na vsakem mestu vzorčenja v Severnem Jadranskem morju.

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Abstract: In order to estimate the risk for marine habitats it is essential to determine the different fates of contaminants as they are distributed differently throughout the water column and accumulated variously in the biota and sediment. This study established the toxic potential of seawater, sediment and biota (mussel *Mytilus galloprovincialis* biological fluids) in the coastal area of the Northern Adriatic Sea and revealed possible correlations between the three matrices. Furthermore, toxic potential relation to grain size of sediments was investigated, as well as the ability of the potentially toxic environment to affect mussel fitness. Toxicity assessment was estimated through Microtox® bioassay. Sediment grain size was determined through dry sieving while mussel fitness was assessed through mussel anoxic survival (SOS test). No correlations were found between the three matrices, therefore, it is crucial to establish the toxic potential of every single individual matrix to assess an exact environmental status and potential risk in an integrative way. The toxic potential of seawater, sediment and mussel biological tissue reflects the recent input of toxic contaminants in seawater, their chronic accumulation in sediments and the contaminants bioavailability in mussels respectively. No correlations were found between the toxic potential and grain size composition of sediments, neither between the potentially toxic environment and mussel fitness, indicating specific environmental conditions in each sampling site in the northern Adriatic Sea.

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LIST OF ABBREVIATIONS

RH - Rovinj harbour
RP - Rovinj pier
RM - Valdibora bay
RB - Valdibora bay, Institut for marine research Ruđer Bošković
LM - Lim Bay
PU - Pula harbor
BK - Bakar harbor
EC_{50} - Half maximal effective concentration
LT_{50} - Lethal time 50
SOS test - “Stress on stress” test
Tox - Toxicity
DDD - Dichlorodiphenyldichloroethane
DDT - Dichlorodiphenyltrichloroethane
DDE - Dichlorodiphenyldichloroethylene
PCB - Polychlorinated biphenyl
HCH - Hexachlorocyclohexane
DW - Dry weight
WW - Wet weight
W/V - Weight per volume

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1 INTRODUCTION

In the last decades traditional and emerging human activities, like renewable energy production, wastewater discharges, fishing, shipping and recreation, have highly expanded near coastal and open waters in order to support the increasing coastal worldwide population (OSPAR, 2009; Halpern et al., 2015; Borja et al., 2016). Although these activities are supplying valuable resources to humans, a lot of environmental pressures from overfishing, habitat destruction, noise pollution to chemical pollution are damaging marine ecosystems (Crain et al., 2008; Ban et al., 2010; Piggott et al., 2015). The regularity, amount, duration and type of disturbances on marine species and entire marine habitats have been modified by the accelerated increase in anthropogenic pressures (Nõges et al., 2016). Nowadays it is crucial to monitor and assess correctly marine environmental quality in order to preserve marine biodiversity and marine habitats all around the world.

1.1 Northern Adriatic Sea

The northern Adriatic Sea is bordered by the Balkans to the East and by the Italian peninsula to the West. It extends as far North between Venice and Trieste and far South towards a line connecting Zadar to Ancona (Figure 1). It is a shallow sub-basin of the Adriatic Sea characterised by sandy coasts and alluvial plain lands in the northern and western part (Italy). The eastern part (Croatia) is defined by a mountainous coast and large numbers of islands and bays (Poulain et al., 2001). According to Viličić (2014), temperature, salinity, amount of nutrients, light intensity, marine currents (horizontal, vertical and circular) and weather conditions are the most important oceanographic parameters influencing on the North Adriatic. The river Po has major influence on the amount of mineral nutrients found in the northern Adriatic Sea (Chiaudani et al., 1980; Degobbis and Gilmartin, 1990). Through the river Po, precipitations and snow melting in the Alps regulate the growth of phytoplankton (Viličić, 2014).

The main and dominant winds in the northern Adriatic are bura and jugo (Poulain and Raicich, 2001). Bura is a cold and dry wind blowing from north-east mixing the water column, decreasing the temperature, increasing evaporation and seawater density (Dorman et al., 2006). Bura has a great impact on the northern Adriatic, especially in Trieste Bay and Bakar Bay, from January to March (Viličić, 2014) lowering the seawater temperature at 12.3-12.5˚C and the salinity at 38.3-38.4 (Manca and Giorgetti, 1999; Vilibić et al., 2004; Vilibić and Supić 2005; Mihanović et al., 2013). The bura wind during the winter season in the Trieste Bay and Rijeka Bay is causing advection of the surface sea layer towards the western Adriatic (Italy). Jugo is a warmer wind blowing from south-east, transferring water masses from the southern Adriatic to the northern Adriatic mostly from November to April (Viličić, 2014).

Figure 1. Northern Adriatic Sea

The East Adriatic current transports warmer and saltier water masses from the eastern part of Mediterranean Sea and Ionic Sea towards the North Adriatic along the Croatian coast (Zore-Armanda, 1969). These currents rotate in Trieste Bay and continue their path West and then South, along the Italian coast. Forces, such as the rotation of the Earth and the bura wind, make vortexes in the northern Adriatic. A cyclonic vortex South of the Istrian coast is present during winter (Kuzmić et al., 2006) and can deliver rich north Adriatic waters to the Rijeka Bay (Viličić, 1991). This vortex possibly enables a significant growth of fish stock and phytoplankton (Kraus and Supić, 2011; Supić et al. 2012). A second cyclonic vortex is found near Trieste and an anticyclonic vortex in front of Rovinj (Lyons et al., 2007). Overall, the Northern Adriatic is described by a homogenous vertical water column during winter. Low temperatures and cyclonic system of currents, divided by the eastern high-salinity water flux and the western cold river's Po water flow, are contributing to the water column stability. During summer, the Northern Adriatic is characterised by a vertical stratification of the water column caused by higher temperatures. A cyclonic system above and an anticyclonic system below the river Po are present in those warmer months (Krajcar 2003; Supić et al., 2003; Frascari et al., 2006). Winter and summer circulation of surface waters are shown in Figure 2.

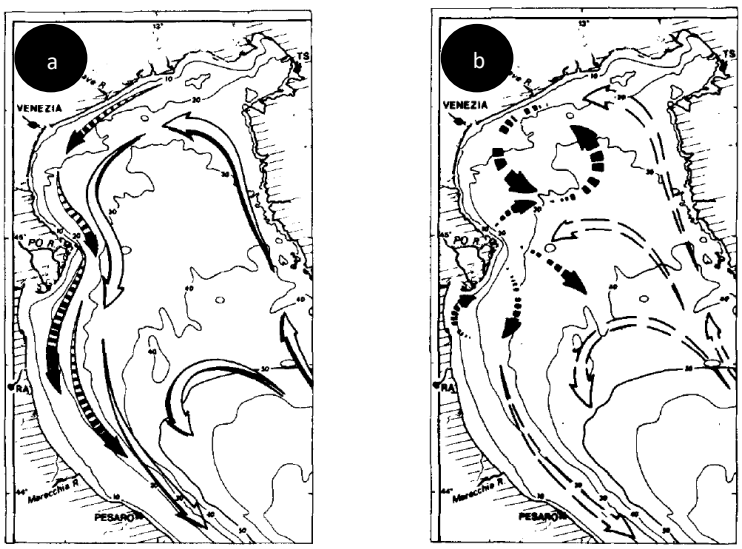

Figure 2. Winter (a) and summer (b) circulation of surface waters in the northern Adriatic Sea (Frascari et al., 2006).

High levels of mineral nutrients coming from the river Po support phytoplankton blooms measured by the accumulation of chlorophyll a (Smayda, 1997). During phytoplankton blooms they produce organic molecules, such as polisaharides which tend to assemble forming macro aggregates incorporating, among other anorganic particles (Azam et al., 1993; Faganeli et al., 2010; Heissenberger et al., 1996; Žutić and Svetličić, 2000). Macro aggregates can float towards the south Adriatic (Viličić, 1991) or remain in the northern Adriatic through the Istrian coastal counter current (Degobbis et al., 1999; Degobbis et al., 2000) and the southern Adriatic currents (Najdek et al., 2005). The issue with macro aggregates is their decomposition process which can lead to hypoxic and anoxic environmental conditions (Legović and Justić, 1997), endangering marine habitats. Usually in the northern Adriatic toxic species of phytoplankton bloom in the upper seawater layer during summer (France and Mozetič, 2006).

Other chemicals like toxic metals, artificial radionuclides, chlorinated pesticides and PCBs can be found in some concentrations in the Northern Adriatic. Such pollutants may disrupt the entire marine ecosystem. Like mineral nutrients their maximal concentration are found around the mouth of the river Po and in areas with low hydrodynamic energy (the northern part of Emilia Romagna coast and the Gulf of Trieste) (Frascari et al., 2006). Overall, the northern Adriatic is an oligotrophic area. From 1970 till today it has been recorded a decline in mineral nutrients inflow from the river Po, as Italy stopped using detergents containing phosphorus (Mozetič et al., 2009). For many years it looked like the pollution from the river Po could damage much more in short term the Northern Adriatic marine habitat. However,

the water renewal times are rather short, around one year. Cases of serious water pollution in the whole Northern Adriatic are quite rare with exceptions of particular local areas (Franco, 1983).

The Northern Adriatic is described by a homogenous distribution of toxic potential load with an overall fair water quality. Sites with very low human activities are relatable to the prevalence of excellent water quality (33,3%). Recreational areas and low density settlements display the highest frequency of good water quality (60%). Sites close to industrial areas or big cities display moderate frequency of fair water quality (39,4%). Sites directly in contact with industrial zones and urban cities show most of times fair water quality (64,3%). This kind of distribution may be defined by the higher number of big cities in the north, the semi-closed shape of the Adriatic Sea and its cyclonic current circulation (Fafanđel and Bihari, 2007).

1.2 Marine environmental quality assessment

Maintaining healthy oceans and seas leads to numerous beneficial ecosystem services which produce food, energy and raw materials (Costanza et al., 1997; Barbier et al., 2012; Turner et al., 2014; Turner and Schaafsma, 2015). Human activity can nevertheless compromise such ecosystem activities, stimulating conservationist, policy makers and scientist to respond. It is crucial to look at degradation of marine habitats by applying a science based integrated ecosystem approach where all parts from the marine ecosystem are linked (Agardy et al., 2011). The ecosystem approach is based on three objectives: conservation, sustainability and fair sharing of the benefits coming out from the resources (CBD ,2000). Maintaining and enhancing natural system structure leads to more ecosystem services and societal benefits needed by society (Elliott, 2011). According to Elliott (2014), the ecosystem approach to management requires the following elements: defining the source of the pressures deriving from activities, a risk assessment and risk management framework for each threat, a vertical integration of stakeholder from a local to a global level, a framework of stakeholder involvement and the delivery of ecosystem services and social benefits.

The levels of contaminants in water, sediment or biota and the changes in the physical or chemical properties of waters and sediments in relation to a natural state are some of the main parameters used in the marine environmental quality assessment (EPA, 2016). Marine habitats are characterised by their high complexity and large geographical extent. In order to assess correctly the quality of coastal and open sea systems, an extensive amount of data is needed. In favour of giving correct spatial and temporal data, precise monitoring methods are necessary (Borja and Elliott, 2013). It is crucial to take in consideration complex ecological parameters. They have an influence on: the distribution of contaminants in the marine system, the effect of contaminants or living organisms, the life cycle and organisms'

behaviour which influence the uptake of contaminants, the exposure time to contaminants, feeding and growth rate of organisms and biotranformation of contaminants in organisms (Bihari and Batel, 2018). Based on the ecosystem approach, marine monitoring should embrace quality and health assessment at all levels of biological organization (Elliott, 2011), from cells to tissue level, individuals of a population, populations and communities (Gray and Elliott, 2009; Borja et al., 2013). Overall, studies based on lower levels of biological organisations are easier to perform, evaluate and correlate with the cause of stress or toxicity. Studies on high levels of biological organisations have greater ecological importance showing long-term consequences. Nevertheless, it is more difficult to establish the exact correlation between lower biodiversity and concentration of potentially toxic compounds (Bihari and Batel, 2018).

Based on the research of marine ecosystems quality in the last 30 years and the interactions with public, administrative and political bodies, the European Union issued in 2008 criteria and standards of Marine Strategy Framework Directive (MSFD). The aim was to achieve a coherent assessment of good environmental status (GES). The Baltic Sea, the North-East Atlantic Ocean, the Mediterranean Sea and the Black Sea are collaborating in this framework. The goal of the new EU Biodiversity Strategy for 2030 is to protect marine ecosystems and restore those which were damaged. By expanding protected areas, the EU wants to obtain a "good environmental status" (Marine Strategy Framework Directive, 2020). One of the main goals of the MSFD is to assess correctly contaminant concentrations and their toxic effects on the biota in the marine environment. In order to avoid any lethal effect of contaminants towards different biological levels of organisation (from an organism to a population, community or entire ecosystem), monitoring schemes should indicate critical pollution values as an early warning signal (Bihari and Batel, 2018). The goal of MSFD in contaminant assessment is summarized in the following three steps: 1. identify the concentration of contaminants stored in seawater, sediment and/or biota which is under the toxic threshold; 2. determine the toxic threshold of contaminants which endangers all living marine organisms of different biological levels of organisation; 3. avoid the rise of both contaminant concentration and toxic threshold (Bihari and Batel, 2018).

Sources of stress may include physical restructuring or other kind of perturbations, but mostly are related to marine pollution (Harding, 1992). Table 1., modified after review from Harding (1992)., shows the marine environmental quality assessment framework, with examples of stressors, characteristics of exposure and proper measure of stressor effect on marine habitats.

Table 1. Marine environmental quality assessment framework*: examples of stressors, characteristics of exposure and proper measure of stressor effect on marine habitats.

Stressors	Characteristics of exposure	Measure of effects
Reconstruction of the coastline or sea bottoms in harbours	Physical damage to the marine habitat	Less habitat for marine biota
Higher amount of nutrients	Algal blooms and hypoxic areas	Phytoplankton biomass and community analysis
Contaminants spills	Accumulation of toxic chemicals in water, sediments and biota	Total concentration of contaminants and their toxic effects on biota (bioaccumulatiton/biomagnification)
Bacterial contamination	Higher levels of pathogenic bacteria	Cases of human diseases in coastal area
Atmospheric contaminant loading	Residues on surface layer	Lower number of surface-dwelling biota

*Modified after review from Harding (1992).

Contaminant concentration assessment is very important but not enough from an integrative management point of view. Contaminants that enter the marine environment may vary. They consist of compounds and complex mixtures that may or may not be toxic. Furthermore, chemical synergistic and antagonistic interactions in the marine environment can generate new toxic compounds (Bihari et al., 2004a). For example, bromoform ($CHBr_3$) is formed by the interaction of chlorine, which is discharged from power plants cooling system and the normal constituents of the marine environment (WHO/UNEP, 1990). Ordinary chemical analysis alone can't display accurately information concerning potential synergism of chemical substances found in the marine environment. Moreover, chemical analysis are very expensive. Only few chemical tests could be performed, investigating the concentration and presence of only few potentially toxic substances (Kungolos et al., 2003). As a result, biological parameters such as mussel survival and toxicity bioassays were introduced. They establish the cumulative negative effects of all contaminants present, including those new complex chemical mixtures found in all marine matrices (water, sediment and biota). Biological parameters such as toxicity bioassays do not measure the cause of pollution. They measure the negative effect of toxic compounds present in seawater, sediment and biological fluids of mussels extracts on test organisms (Fafanđel et al., 2015; Kungolos et al., 2003). Mussels are often used as bioindicators and as representatives of the marine biota because

they are filter feeders. Through this process they are exposed to dissolved contaminants and to contaminants which are adsorbed on particles, making mussels the most researched marine organism when bioaccumulation is investigated (Bihari et al., 2007).

1.3 Contaminants in seawater

The water column is a highly diversified environment. Usually seawater samples are taken from the surface layer. They represent a spatial and temporal point where wind, tides, currents, waste waters and other factors are influencing the level of contaminants (Bihari et al., 2004a). Sampling water at a certain depth may give completely different levels of contaminants and toxic potential. Stratification based on temperature, salinity and level of oxygen can prevent the mixture of bottom and surface layers of seawater and therefore a mixture of contaminants. Although when bottom and surface layer mix it is very important to address that mixtures of chemicals and their synergetic-antagonistic relationship in water could lead to a new level of toxicity (Kungolos et al., 2006; Water Environmental Federation, 1997). Polycyclic Aromatic Hydrocarbons, or PAH, concentration in seawater can vary in different part of the world, from the Baltic Sea to the Mediterranean and South China Sea (Witt 1995; Kilikidis et al. 1994; Zhou and Maskoui, 2003). Different concentration of heavy metals like arsenic, copper, zinc, lead, mercury and cadmium were recorded as well around the world (Zhang et al., 2017; Suresh Kumar et al., 2013). Organochlorine pesticides and polychlorinated biphenyls are common marine contaminants found in the seawater column. PCB, DDE, DDT, DDD and HCH concentrations were recorded in the North Atlantic surface waters (Lammel et al., 2017).

1.4 Contaminants in sediment

Sediments have a very important role in nutrient recycling because. Their ability to absorb and release dissolved substances is fundamental in a marine ecosystem (Murray et al., 2006; Avramidis et al., 2014; Golterman, 2004). Sediment constitute a long term source of contamination as a sink area, affecting the food web since organisms that live in sediments consume and cycle organic matter (Burton, 2002). Organic matter, mud content and ammonia concentration may be the prevailing compounds of low to moderate toxicity when lower level of contaminants are registered (Montero et al., 2013). PAHs tend to bind to particulate matter because of their low solubility in water therefore accumulating in sediments. Concentration of PAHs in sediments depend on the proximity of urban areas to sampling sites and on the biodegradation of contaminants conditioned by biotic and abiotic factors (Bihari et al, 2007). High-molecular PAHs tend to accumulate in sediments (Bihari and Batel, 2018). Naes and Oug (1997) established that sediments carry a concentration of PAHs with a factor of 1000 or more than in the water column threatening the benthic biota.

Some PAHs are known to be toxic and their concentration is correlated to the toxic potential of sediment (Shor et al., 2004), like with PAHs from drill cuttings discharges from oil platforms in the North Sea (Grant and Briggs, 2002). On the other hand, in the Bay of Kavala Aegean Sea, Greece there is no significant correlation between PAHs concentration and toxic potential of sediment (Papadopoulou and Samara, 2002), suggesting that this relationship is not consistent in all marine environments. Concentrations of heavy metals like arsenic, copper, zinc, cadmium, lead and mercury were recorded in sediment in the northern Liaodong Bay of China (Zhang et al., 2017). In the Northern Adriatic traces of heavy metals like mercury, zinc, cadmium, lead, copper and organochlorine compounds like DDT, DDD, DDE, PCB and HCH were recorded in the last 20 years (EIONET, 2020).

1.5 Contaminants in bivalves

Bivalves (Mollusca) are one of the most researched organisms in bioaccumulation studies. As filter feeding organisms, they are exposed to dissolved contaminants and to contaminants which are adsorbed on particles. Bivalves are perfect bioindicators and representatives of the marine biota. The main factors that influence the bioaccumulation in bivalves include: species, diet, digestive physiology, season, physiological conditions, behaviour and biological regulation (reviewed in Bihari and Batel, 2018).

There are significant statistical differences in the amount of pollutants accumulation between different species of bivalves. Oysters tend to accumulate in higher concentrations zinc, copper and silver, mussels chrome and lead while scallops cadmium. Other bivalves, like *Dressena polymorpha*, which have a bigger amount of lipids in their tissue tend to accumulate high concentration of metals and organic compounds. Filter feeders and detritophagous selectively captive, sort and keep small particles of a specific size range which usually tend to have the highest concentration of contaminants. Bivalves concentrate and integrate chemicals in their tissue but their potential to metabolise those toxins is lower respect to other marine organisms. Digestive enzymes, intestinal amino acids and retention time in the intestine are specific factors which have an influence on the pollutants. They vary across all marine organisms. Bivalves can change their uptake depending on the quantity of dissolved pollutants and available food in the water environment. If there is enough food in the first place, bivalves absorb much less contaminants adsorbed on particles. If food is lacking in the environment, they have to rely on inorganic and organic matter adsorbed on particles, being more exposed to contaminants (reviewed in Bihari and Batel, 2018).

Bivalves are extremely flexible organisms because of their possibility to alter their physiological conditions in response to the actual state of the environment. They control the particle processing and the chemical stress inside their body. If the organism is unhealthy, it can accumulate less contaminants through time because the toxins that are already present

inside its body prevent normal filtration, physiology and growth. In order to evaluate the quantity of contaminants in the environment it is crucial to take into consideration the physiological condition of the mussel. It is not enough to base the conclusion on the toxin's chemical characteristics. It is important to address the importance of the behaviour of bivalves in the accumulation of toxins. They can detect when the environment has a higher concentration of toxins, closing their shells and stopping filtration as long as they can, in order to avoid being exposed to contaminants. Season has a major influence on the concentration of contaminants in bivalves. For example, in *Macoma balthica* there is a higher concentration of metals during fall and winter when their biomass is still small while it's lower in spring and summer because of their bigger biomass. Considering organic contaminants, *Mytilus edulis* are more exposed in summertime and spring when their amounts of lipids are higher during spawn (reviewed in Bihari and Batel, 2018). Accumulation though may vary in different species and geographical regions. PAHs were found in *Mytilus galloprovincialis* in South American Estuary (Oliva et al., 2017), in Northern Adriatic (Bihari et al., 2007), in Marmara Sea, Prince Islands (Balcioglu, 2016) and in Ionian Sea, Italy (Storelli and Marcotrigiano, 2000). Low-molecular PAHs tend to accumulate in mussels (Bihari and Batel, 2018). In the Northern Adriatic traces of toxic compounds like monobutylin, dibutylin and tributylin, heavy metals like copper, mercury, cadmium, zinc, lead, chromium and organochlorine compounds like DDT, DDD, DDE, PCB and HCH were recorded in mussels *Mytilus galloprovincialis* (EIONET, 2020).

1.6 Microtox® bioassay

Microtox® bioassay is a well-established technique used for assessing toxic potential of seawater, sediment and mussel biological fluids through indication of organic and metallic compounds. The bioassay is based on the measurement of the reduction in bioluminescence of the bacterium *Vibrio fischeri*, after it has been exposed to various concentration of the extracts of contaminated seawater, sediment or living tissue (Bulich, 1986). Usually bacteria emit a distinct level of bioluminescence of their own. When they are under stress they emit less light therefore indicating the presence of chemical contaminants in the Microtox® bioassay. Its use has been confirmed as successful in the determination of water and sediment toxicity in areas such as Venice lagoon, Italy (Passarini et al., 2000), including inner canals of the City of Venice (Pavoni et al., 1998), Albufera Natural Park, Valencia, Spain (Boluda et al., 2002), Bay of Brest, France (Quiniou et al., 1997), Lake Jamsanvesi, Finland (Hyotylainen and Oikari, 1999), Bay of Kavala Aegean Sea, Greece (Papadopoulou and Samara, 2002), North Sea (Grant and Biggs, 2002), Po River, Italy (Vigano et al., 2003), Adriatic coast of Croatia (Bihari et al., 2004a. Bihari et al., 2004b; Fafanđel and Bihari, 2007), Gulf of Rijeka, Croatia (Fafanđel et al., 2015; Bihari et al 2007), Rovinj area, Northern Adriatic, Croatia (Bihari et al., 2006) as well as in the determination of toxicity of

mussel biological extracts (Lau-Wong 1990; Cotou et al. 2002) in Rijeka Bay, Croatia (Bihari et al., 2007) and Rovinj area, Northern Adriatic, Croatia (Privileggio, 2017).

1.7 Mussel anoxic survival

In order to evaluate the physiological conditions ("fitness") of mussels exposed to certain pollutants, the measurement of anoxic survival was established. This biological parameter lays its basis on the natural ability of mussels to survive outside water in anoxic conditions, during periods of low tide. Taking in consideration that mussels located in areas with a higher amount of contaminants have an increased metabolic rate, they are expected to have a reduced anoxic survival time in comparison to mussels located in less pollutant areas (De Zwaan and DeKock 1988). Such theory is confirmed by studies on mussel's high levels of pollutants being positively correlated to a worse anoxic survival (Eertman et al. 1993; Viarengo et al. 1995; De Zwaan et al. 1995; Nesto et al. 2004). Anoxic survival rates were tested on *Mytilus trossulus,* after a long term exposure of spilled Exxon Valdez oil in Prince William Sound (Thomas et al. 1999), on *Mytilus edulis* in the Dutch coastal waters to confirm pollution induced environmental stress (Smaal et al. 1991), on *Mytilus galloprovincialis* in the Gulf of Rijeka, Croatia (Bihari et al., 2007) and in Rovinj area, Northern Adriatic, Croatia (Privileggio, 2017).

2 AIM AND HYPOTHESES

2.1 Aim

In this thesis three primary aims have been set:

- Determine the toxic potential of three different matrices (seawater, sediment and biota-*Mytilus galloprovincialis*) at each selected environments and test correlation among them.
- Determine if the toxic potential is related to the sediment type.
- Determine the ability of the potentially toxic environment to affect mussel anoxic survival.

2.2 Hypotheses

The hypothesis for this research are:

1. There is a correlation for toxic potential among three different matrices, as reflection of contamination load of selected sites.
2. Integration of toxic potential for three different matrices enable ranking of sampling sites according to contamination load.
3. The toxic potential of sediment depends on sediment type.
4. Potentially toxic environment affects mussel anoxic survival.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Chemicals used for the seawater extract preparation were: aceton, distilled dichloromethane, anhydrous sodium sulfate and dimethilsulfatoxide (DMSO). Chemicals used for the sediment extract preparation were: methanol, dichloromethane, distilled water, anhydrous sodium sulfate and dimethilsulfatoxide (DMSO). Chemicals used for the mussel biological fluids preparation were: 2% NaCl solution (Machery Nagel, Germany). Chemicals used for the toxicity assessment (Microtox® bioassay) were: 2% NaCl solution, bacterial reconstitution solution, bacterial medium (Macherey Nagel, Germany) and bioluminescence bacteria *Alivibrio fischeri* NRRL B-11177 (DIN EN ISO 11348-3, BioFix® Lumi, Machery-Nagel, Düren, Germany).

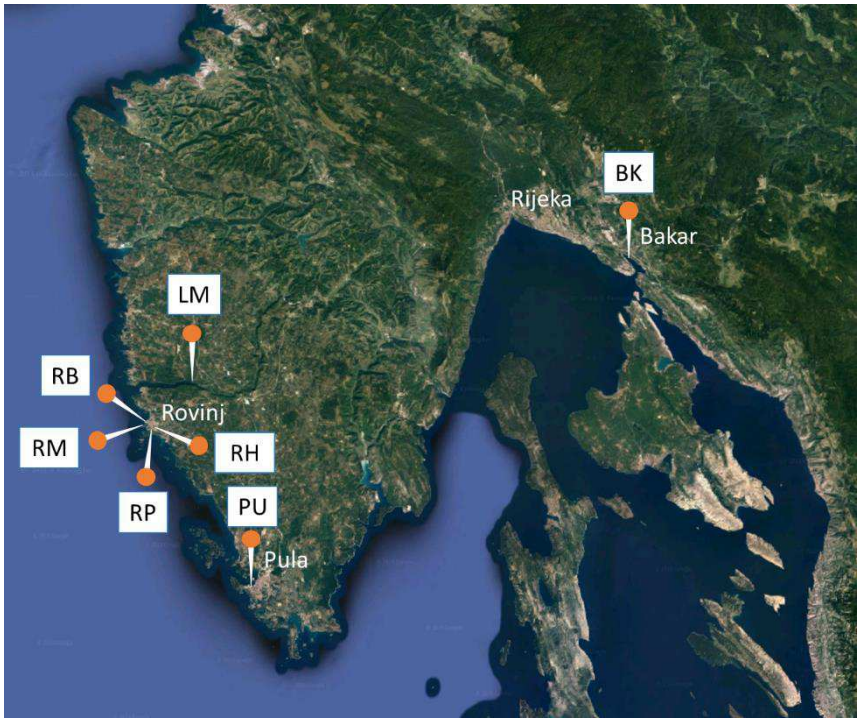

Figure 3A. Sampling sites: LM (Lim Bay), RH (Rovinj harbour), RP (Rovinj pier), RM (Valdibora bay), RB (Valdibora bay, Institut for marine research Ruđer Bošković), PU (Pula harbour), BK (Bakar harbour).

3.1.2 Sampling sites

Total of seven sampling sites across the Istrian western coast and Kvarner Bay were investigated (Figure 3A). Samples were collected from Lim Bay (LM), Rovinj harbour (RH), Rovinj pier (RP), Valdibora bay (RM), aquaculture basin for mussels at the Institute for marine research Ruđer Bošković (RB), Pula harbour (PU) and Bakar harbour (BK) (Figure 3B). LM is set in a long channel where mariculture, freshwater spills and its harbour have a great influence on water's biological, chemical and physical characteristics. Both RH and RP are closed areas characterised by low marine circulation and under great anthropogenic influence because of the high boat traffic. RM is an area situated near a sewage discharge from the old part of the city with a high abundance of boats coming to the shore. Marine water samples taken from RB were collected from pools which are used for various experiments. The water that comes into the pools is pumped from the sea in front of the Institute, but purified with various filter systems. The area in front of the Institute is exposed to boat traffic and spillages from the Mirna fish industry. PU is set in the city centre in a very close area between many small piers under a constant influence of boats. BK is the only sampling site outside the Istrian region, found in Kvarner area, a semi-closed part of the Adriatic Sea, characterised by big anthropogenic influence in the past. Through its narrow mouth the bay communicates with the Rijeka Bay. It is under rather high influence of local activities but also relatively well protected from disturbances that come from the open sea. Bakar Bay is well known for its industrial zone having a shipyard, oil refinery and a bulk cargo terminal for handling bauxite ore, iron ore, coal and bulk cargo (Fafanđel et al., 2015).

Figure 3B. Sampling sites: LM (Lim Bay), RH (Rovinj harbour), RP (Rovinj pier), RM (Valdibora bay), RB (Valdibora bay, Institut for marine research Ruđer Bošković), PU (Pula harbour), BK (Bakar harbour).

3.2 Methods

3.2.1 Sampling

Samples of sea water, sea bottom sediment and mussel *Mytilus galloprovincialis* were collected at each of 7 sampling sites. For the seawater toxicity assay, one sampling of 30 L of sea water was collected with a bucket from the surface layer of 0.5 m at each sampling site. For the sediment toxicity assay and granulometric composition determination, one sampling of 200 g of sea bottom sediment was collected at each sampling site. The sediment surface layer, not deeper than 2 cm, was obtained manually by diving at a depth of 2-3 m near the shore. For mussel anoxic survival and mussel toxicity assay, one sampling of 50 specimens of mussels *Mytilus galloprovincialis* was collected from the shore or from ropes hanging from the boats at each sampling site.

3.2.2 Grain size composition determination

The samples of sediments that would have been analysed for the granulometric composition were first dried at room temperature. Later they were weighed on an electronic scale with an accuracy in the range of 0.001 g to 1 g (Mettler Toledo) and treated with the wet and dry sieving method (Buchanan & Kain, 1971). Wet sieving through a 63 μm mesh sieve was performed to separate fine-grained (silt and clay) from the coarse-grained sediment fraction (sand and gravel). The coarse-grained part of the sediment was dried, weighed and sieved through a 2 mm and 63 μm sieve (Figure 4). From the difference between the initial sediment weight and the weight after wet sowing, the proportions of two basic sediment fractions (fine-grained and coarse-grained) were determined. Gravel and sand fractions were separated on the basis of weighting off the sediment from the 2 mm and 63 μm sieve. The sediment composition analysis was performed based on Shepard (1951) and Folk and Ward (1957).

Figure 4. Sieves used to separate fine-grained from the coarse-grained sediment fraction.

3.2.3 Seawater extract preparation

From the seawater samples (30 L) collected at each sampling site, all nonpolar compounds were concentrated on Amberlite XAD-7 resin columns (20 mL) according to Bihari et al. (2004a) (Figure 5), letting the water pass by gravity at a flow rate of 200 mL/min. After the completed elution of sea water, the resin column was dried with a vacuum pump. The column was eluted one more time with 150 mL of aceton in a 250 mL chemical flask and evaporated near dryness at 40 °C to reduce the volume to 30 mL. 100 mL of distilled dichloromethane was added with a few tea spoons of anhydrous sodium sulfate to bond with the remaining molecules of water. The solution was filtered, mixed with 300 µL of dimethilsulfatoxide (DMSO) and evaporated to dryness one more time. Samples of dimethilsulfatoxide (DMSO) containing the concentrated organic substances derived from the 30 L seawater was stored at 4ºC prior to toxicity testing.

Figure 5. Amberlite XAD-7 resin columns (20 ml) used to concentrate all nonpolar compounds from the seawater.

3.2.4 Sediment extract preparation

The sediment extraction was performed from 50 g samples (wet weight), according to Schiewe et al. (1985) and Bihari et al. (1989), for each sediment sample collected at respective sampling sites. The sample was put in glass bottles with 50 mL of methanol for half an hour. Afterwards the methanol was carefully poured out leaving the entire sediment sample still inside the glass bottle. 100 mL of dichloromethane and 50 mL of methanol were additionally poured in the glass bottle containing the sediment sample. Later the glass bottle

was put on an electric powered roller for 3 hours (Figure 6). The mixed methanol and dichloromethane were carefully poured out of the glass bottle without any sediment resident in a separation funnel. 100 mL of distilled water was added to the separation funnel in order to isolate the dichloromethane at the bottom of the funnel and letting it flow into a baker. Additional few tea spoons of anhydrous sodium sulfate bonded with the remaining molecules of water. The solution was filtered, mixed with 300 µL of dimethilsulfatoxide (DMSO) and evaporated to dryness at 100 mbar. Samples of dimethilsulfatoxide (DMSO), 300 µL, containing the concentrated organic substances derived from the sediment samples, was stored at 4ºC prior to toxicity testing.

Figure 6. Electric powered roller used for sediment extract preparation.

3.2.5 Mussel biological fluids preparation

In order to assess the toxicity of the mussel, the mussel tissue homogenate was prepared accordingly to Bihari et al. (2007). For every sampling station the soft tissue of three mussel specimens *Mytilus galloprovincialis* (Figure 7) was removed from the shells, sliced and mixed together. 1 gram of mixed tissue was sampled and mixed with a 2% NaCl solution (Machery Nagel, Germany) in a 1:3 ratio weight per volume (w/v). The sample was then homogenized in Potter-Teflon homogenizer and centrifuged at 4º C, 1160 rpm for 15 minutes. Mussel biological fluids extracts were stored at 4º C prior to toxicity testing.

Figure 7. Mussel *Mytilus galloprovincialis*, with open shells showing soft tissue.

3.2.6 Toxicity assessment (Microtox® bioassay)

For the detection of toxic potential in seawater, sediment and mussel biological fluids extracts, Microtox® bioassay was performed accordingly to Schiewe et al. (1985) and Bihari et al. (2004a). The marine bacterium *Alivibrio fischeri* NRRL B-11177 (DIN EN ISO 11348-3, BioFix® Lumi, Machery-Nagel, Düren, Germany) was exposed to eight dilutions of the corresponding seawater, sediment and mussel biological fluids extract. The luminescence of bacteria was measured in Microtox® model 500 Analyser (Azur Environmental, Carlsbad, USA) as shown in Figure 8.

For the toxicity assessment of seawater, 25 µL of the total organic seawater extract (300 µL), corresponding to 2500 mL of seawater, were added to the 1 mL 2% saline suspension. This initial solution of 1 mL containing the seawater extract in a 2% saline suspension was used as a starting point for additional seven 1:2 serial dilutions of each sample. The bioassay consisted of adding 0.5 mL, of the diluted seawater extract in a 2% saline suspension, to 0.5 ml of 2% saline suspension containing 10^6 exponentially growing luminescent bacteria. The bioluminescence of bacteria was measured after a 15-min incubation, compared with blank samples and corrected for spontaneous photoactivity decline. The results are expressed as millilitres (mL) of seawater causing a 50% reduction in bioluminescence after 15 min (EC_{50}) (Bihari et al., 2004a).

For the toxicity assessment of sediment, 10 µL of the total sediment extract (300 µL), corresponding to 83 mg of sediment, were added to the 1 mL 2% saline suspension. This initial solution of 1 mL containing the sediment extract in a 2% saline suspension was used as a starting point for additional seven 1:2 serial dilutions of each sample. The bioassay

consisted of adding 0.5 mL, of the diluted sediment extract in a 2% saline suspension, to 0.5 ml of 2% saline suspension containing 10^6 exponentially growing luminescent bacteria. The bioluminescence of bacteria was measured after a 15-min incubation, compared with blank samples and corrected for spontaneous photoactivity decline. The results are expressed as milligrams (mg) of sediment causing a 50% reduction in bioluminescence after 15 min (EC_{50}) (Bihari et al., 2004a).

For the toxicity assessment of mussel biological fluids, 1 mL of mussel biological fluids extract was used as a starting point for additional seven 1:2 serial dilutions of each sample. The bioassay consisted of adding 0.5 mL, of the diluted mussel biological fluids extract in a 2% saline suspension, to 0.5 ml of 2% saline suspension containing 10^6 exponentially growing luminescent bacteria. The bioluminescence of bacteria was measured after a 15-min incubation, compared with blank samples and corrected for spontaneous photoactivity decline. The results are expressed as microliters (μL) of mussel biological fluids causing a 50% reduction in bioluminescence after 15 min (EC_{50}) (Bihari et al., 2004a).

Figure 8. Microtox model 500 Analyser (Azur Environmental, Carlsbad, USA) used for the toxicity assessment of sea water, sediment and mussel biological tissue samples.

3.2.7 Anoxic mussel survival determination (SOS test)

The anoxic survival test (SOS test) was performed on 30 specimens of *Mytilus galloprovincialis* which were sampled from seven stations. The mussels were placed in plastic containers on wet filter paper and covered with aluminium foil (Figure 9). The goal was to maintain a constant humidity and temperature of 22° C following protocol by Bihari et al. (2007). Mussel survival was assessed once a day until all mussels die. Mussels are considered dead when they have a specific smell, their shells are open and don't close after

physical contact (Kaplan and Meier, 1958). The result of the anoxic mussel survival is represented as LT_{50} (Lethal time 50) expressed in days, otherwise known as the time required to kill 50% of all individuals.

Figure 9. Anoxic survival determination: 30 specimens of mussels *Mytilus galloprovincialis* were kept in plastic containers at constant humidity and temperature.

3.2.8 Data analyses

Estimates of EC_{50} with corresponding confidence intervals for the toxic potential of seawater, sediment and mussel biological fluids were obtained using linear regression analysis through MicrotoxOmni^{TM} Software package.

Spearman's rank analysis (Statistica 8.0), linear regression and correlation analysis (Excell 2016) were used to evaluate possible correlations between the toxic potential of seawater, sediment and mussel biological fluids, mussel anoxic survival and grain size of sediment between sampling sites.

PROBIT analysis (Excell 2016) was used to calculate time, expressed in days, needed for 50% of the mussel specimens to die in anoxic conditions, without water, at constant humidity and temperature. Results are expressed as LT_{50} (Lethal time, CI 95%). PROBIT analysis converts the logistic function into a linear function, then a regression is performed over their relationship.

Principal Component Analysis (PCA) (Statistica 8.0) was used for correlating the toxic potential of seawater, sediment and mussel biological fluids with mussel anoxic survival between sampling sites and toxic potential of sediment with grain size of sediment between sampling sites. PCA lies its origin in multivariate data analysis but it can be used in a wide range of application, mostly for identifying patterns in a set of data and then presenting such data in a way to highlight their differences and similarities. PCA is a technique which uses sophisticated underlying mathematical principles to transform a number of possibly correlated variables into a smaller number of variables called principal components.

4 RESULTS

4.1 Granulometric sediment composition

Granulometric sediment composition analysis was performed for five sampling sites across Rovinj, Pula and Lim Bay area. Gravel was composed by sediment particles >2 mm, sand by sediment particles between 0.63 μg-2 mm and silt-clay by sediment particles <0.63 μg. RH and RM had nearly the same sediment composition with almost 90% of sand, 3-4% of silt-clay and 7-8% of gravel (Figure 10). LM had the highest percentage of silt-clay with 22%, the sand component was slightly smaller with 70% while the gravel component retained the same percentage of 8% as RH and RM. RP had the highest percentage of gravel of all sampling sites with almost 50% while PU was close with a 40%. The two sites had both identical percentages of sand with a 48% but PU had a higher component of silt-clay, almost 10%, while RP a low 4%. BK and RB were not analysed.

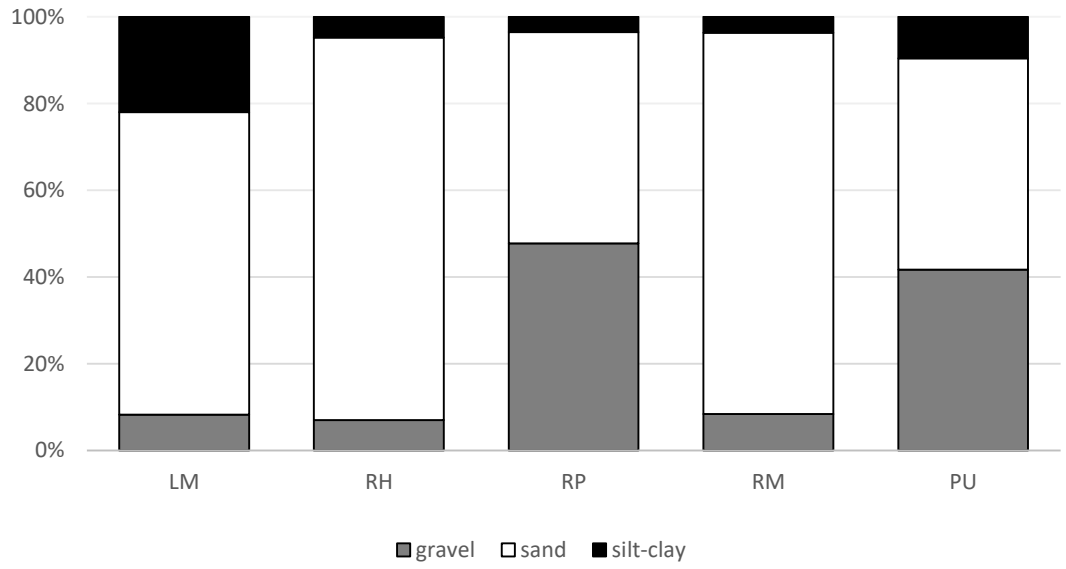

Figure 10. Grain size composition (Gravel >2 mm; Sand 0.63 μm-2 mm; Silt-clay <0.63 μm) for sediment from five sampling sites (LM - Lim Bay; RH - Rovinj harbour; RP - Rovinj pier; RM - Valdibora bay; PU - Pula harbour).

With all the exact percentages of the sediment composition (sand, gravel and silt-clay) all five sampling sites could be classified through Shepard's diagram shown in Figure 11. RM and RH were clearly sandy areas with nearly identical composition percentages. LM was composed of silty sand while RP and PU, although having similar composition, were characterised by sandy gravel and silty gravely sand respectively.

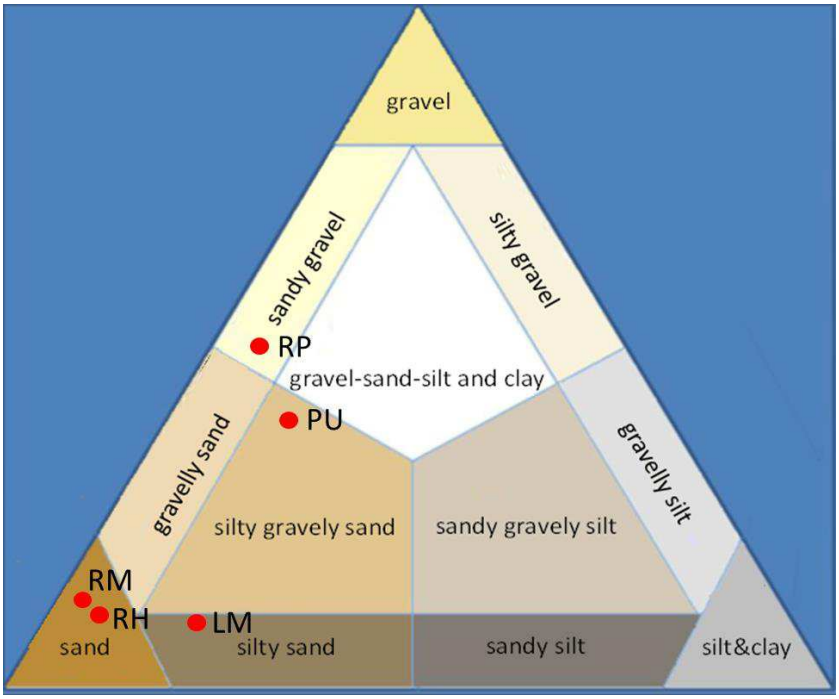

Figure 11. Shepard's diagram showing sediment classification for five sampling sites (LM - Lim Bay; RH - Rovinj harbour; RP - Rovinj pier; RM - Valdibora bay; PU - Pula harbour).

4.2 Toxic potential of seawater, sediment and mussel biological fluids

Toxic potential values for samples of seawater, sediment and mussel biological fluids expressed as EC_{50} (estimated concentration to reduce the bacterial luminescence for 50%) and Tox (converted toxicity values) are shown in Table 2. EC_{50} values had an ascending order as the lowest EC_{50} score was the most toxic while the highest EC_{50} score was the least toxic. Tox values had a descending order as lowest Tox value was the least toxic while the highest Tox value was the most toxic.

Table 2. Toxic potential of seawater (mL), sediment (mg WW) and mussel (µL of soft tissue homogenate) expressed as toxicity (Tox) and half maximal effective concentration (EC_{50}) for seven sampling sites (LM - Lim Bay; RH - Rovinj harbour; RP - Rovinj pier; RM - Valdibora bay; RB - Valdibora bay, Institut for marine research Ruđer Bošković; PU - Pula harbour; BK - Bakar harbour), CI confidence interval (95%).

	Seawater[a]		**Sediment**[b]		**Mussel**[b]	
Station	EC_{50} (CI)	Tox (CI)	EC_{50} (CI)	Tox (CI)	EC_{50} (CI)	Tox (CI)
LM	46 (30-72)	21.6 (13.9-33.3)	11 (8-16)	9.1 (6.3-12.5)	7 (2-17)	14.3 (5.9-50)
RH	29 (18-47)	35.1 (21.3-55.6)	4 (3-7)	25 (14.3-33.3)	14 (10-20)	7.1 (5-10)
RP	25 (11-57)	39.9 (17.5-90.9)	4 (3-6)	25 (16.7-33.3)	18 (12-28)	5.6 (3.6-8.3)
RM	33 (23-72)	30.7 (13.9-43.5)	3 (2-4)	33.3 (25-50)	42 (31-59)	2.4 (1.7-3.2)
RB	37 (20-65)	27.2 (15.4-50)	/	/	66 (36-115)	1.5 (0.9-2.8)
PU	31 (26-35)	32.7 (28.6-38.5)	1 (1-2)	100 (50-100)	30 (22-40)	3.3 (2.5-4.5)
BK	28 (21-38)	35.7 (26.3-47.6)	100 (62-159)	1 (0.6-1.6)	92 (58-146)	1.1 (0.7-1.7)

[a] Calculated as $1/EC_{50} * 1000$.

[b] Calculated as $1/EC_{50} * 100$.

Toxic potential values of seawater extracts were in a range of 25-46 mL through all sampling sites. The most toxic site was RP with a EC_{50} of 25 mL, followed by BK (28 mL), RH (29 mL), PU (31 mL), RM (33 mL) and RB (37 mL). LM was the least toxic site of all tested (46 mL). Toxic potential values of sediment extracts were in the range of 1-100 mg. PU was the most toxic site (1 mg) with RM being close second (3 mg) while RH and RP sharing the third place (4 mg). LM site had a EC_{50} value of 11 mg and BK site was the least toxic of all with 100 mg. Toxic potential values of mussel biological fluids were ranging from 7-92 µL. LM was the most toxic site with a EC_{50} of 7 µL, followed by RH (14 µL), RP (18 µL), PU

(30 µL), RM (42 µL) and RB (66 µL). BK was the least toxic site of all tested with 92 µL needed to reduce the bacterial bioluminescence for 50%.

Table 3. Ranked sampling sites (LM - Lim Bay; RH - Rovinj harbour; RP - Rovinj pier; RM - Valdibora bay; PU - Pula harbour; BK - Bakar harbour) from highest toxic potential (1) to lowest toxic potential (6) based on the toxic potential of seawater, sediment, mussel biological fluids.

Overall Toxicity	
Ranking	Sampling sites
1	RP
2	RH
3	PU
4	LM, RM
5	
6	BK

Taking in consideration the individual ranking of toxic potential of seawater, sediment and mussel biological fluids, it was possible to calculate the overall toxicity ranking, including all three matrices, for all sampling sites (Table 3). RP was the most contaminated area followed by RH and PU. LM and RM were sharing the second last spot while BK resulted to have the lowest toxic potential. RB was not taken in consideration because the lack of sediment toxicity results.

4.3 Mussel anoxic survival

Mussel anoxic survival for mussels *Mytilus galloprovincialis* sampled at six different sampling sites is presented in Figure 12. In all sampling sites all mussels survived the first three-four days. The shortest survival time was recorded for mussels from BK (10 days) while the longest were for RM, RP and LM (14 days). Mussels specimens from RH and PU died after 11 and 12 days respectively.

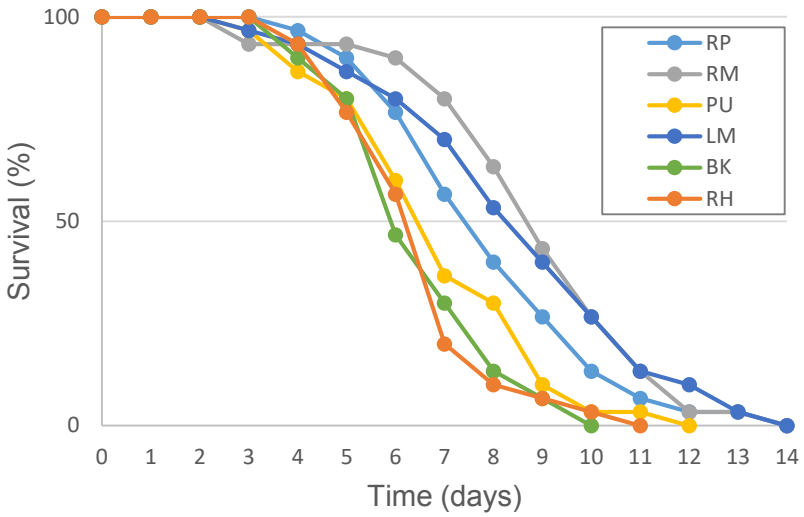

Figure 12. Mussel survival curves for mussel specimens from six different sampling sites (LM - Lim Bay; RH - Rovinj harbour; RP - Rovinj pier; RM - Valdibora bay; RB - Valdibora bay, Institut for marine research Ruđer Bošković; PU - Pula harbour; BK - Bakar harbour).

Lethal time (LT_{50}), when 50% of the total 30 mussel specimens per sampling site were dead, is presented in Table 4. All the LT_{50} results were based on the toxicologist regression analysis PROBIT. The shortest LT_{50} was recorded at RH with 6 days with a close second at BK with 6.1 days. PU followed with 6.5 days, RP with 7.4 days and LM with 8.2 days. The mussels from RM had the highest LT_{50} of all with 8.6 days.

Table 4. Lethal time (days), LT_{50} (Confidence Interval - CI 95%) (PROBIT analysis), for mussel specimens from six different sampling sites (LM - Lim Bay; RH - Rovinj harbour; RP - Rovinj pier; RM - Valdibora bay; PU - Pula harbour; BK - Bakar harbour).

Sampling sites	LM	RH	RP	RM	PU	BK
LT_{50}	8.2	6	7.4	8.6	6.5	6.1
CI (95%)	(6.9 - 9.8)	(5.4 - 6.7)	(6.4 - 8.7)	(7.6 - 9.8)	(5.5 - 7.7)	(5.4 - 6.9)

4.4 Correlation among matrices

Values of Spearman's rank correlation coefficients among toxic potential of seawater (EC_{50}), toxic potential of sediment (EC_{50}), toxic potential of mussel biological fluids (EC_{50}) and mussel survival time (SOS test) are found in Table 5. All sampling sites (LM, RH, RP, RM, PU, BK) were taken in consideration in the correlation except for RB because of its lack of toxic potential of sediment and mussel anoxic survival time. Negative correlations were found between the toxic potential of mussel and seawater (-0.314), toxic potential of sediment and mussel (-0.057) and toxic potential of seawater and sediment (-0.202). SOS test showed a negative correlation towards the toxic potential of mussel (-0.028) and toxic potential of sediment (-0.231), while a positive correlation was found in relation to the toxic potential of seawater (0.542). All the correlations were not though statistically significant.

Table 5. Values of Spearman's rank correlation coefficients among toxic potential of mussel biological fluids (Mussel), toxic potential of seawater (Seawater), toxic potential of sediment (Sediment) and mussel anoxic survival (SOS test) (All p-values > 0.05).

	Seawater	Sediment	SOS test
Mussel	-0.314286	-0.057977	-0.028571
Seawater		-0.202920	0.542857
Sediment			-0.231908

Values of linear correlation coefficients among the toxic potential of sediment samples and gravel, sand and silt-clay content are shown in Table 6. All sampling sites (LM, RH, RP, RM, PU) were taken in consideration in the correlation except for RB and BK. Gravel was positively correlated to the toxic potential of sediment (0.535) compared to silt-clay (-0.167) and sand (-0.485) content which were negatively correlated, but none of the results was statistically significant.

Table 6. Values of linear correlation coefficients among gravel, sand and silt-clay grain size composition and toxic potential of sediment (All p-values > 0.05).

	Gravel	Sand	Silt-clay
Sediment toxicity	0.5354528	-0.48555	-0.16703

Principal component analysis (PCA) of toxic potential of seawater, sediment and mussel biological fluids, for six sampling sites (LM, PU, RH, RM, RP, BK), was performed as a base. On top of it, respective mussel anoxic survival results were superimposed (Figure 13) in order to find possible correlations. The first two PCA components (PC1, PC2) described 90.08% of the variances. The first principal component (PC1) explained 58.69% of the total variance. The second principal component (PC2) accounted for 31.39% of the total variance.

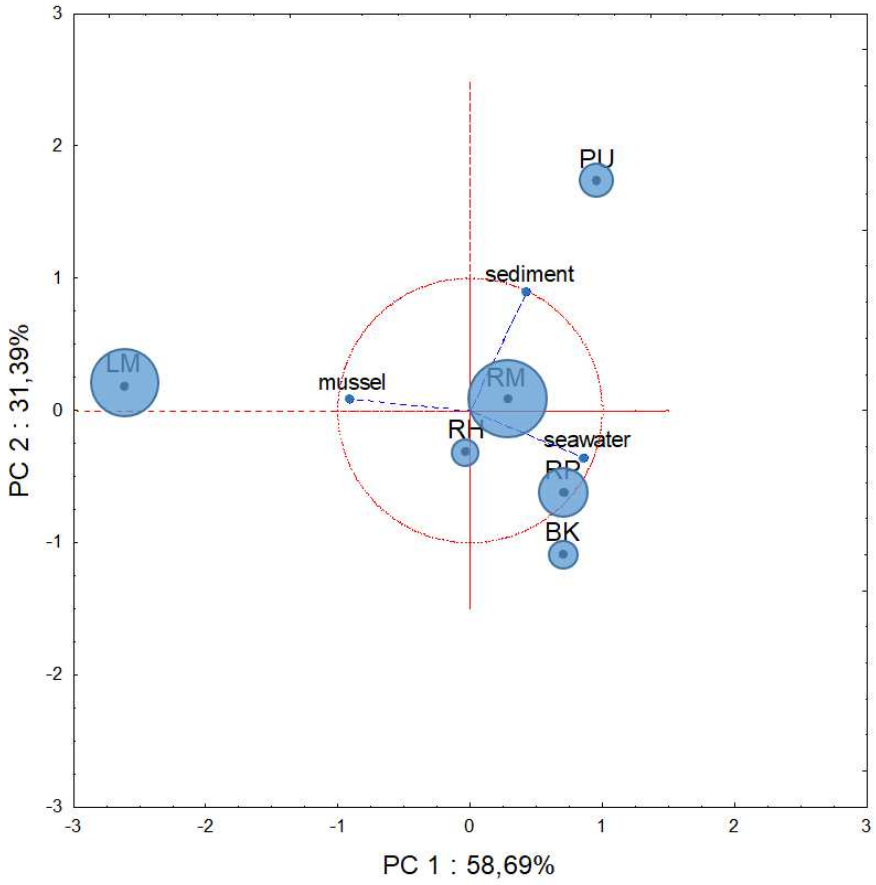

Figure 13. Principal component analysis (PCA) for sampling sites (LM - Lim Bay; RH - Rovinj harbour; RP - Rovinj pier; RM - Valdibora bay; PU - Pula harbour; BK - Bakar harbour) based on the toxic potential of seawater, sediment and mussel biological fluids, with superimposed mussel anoxic survival.

Values of PCA coordinates (PC1, PC2) for seawater, sediment and mussel biological fluids are shown in Table 7. From the PCA, LM was the only site in the top left quadrant as it was dominated by the toxic potential of mussel. RH was the only site in the bottom left part characterised by the toxic potential of mussel and seawater. PU and RM were situated in the top right quadrant while RP and BK in the bottom right part sharing the similar tendency towards the toxic potential of seawater. RM was set almost in between of all three matrices but it was still between the toxic potential of sediment and seawater while PU was situated

far above, indicating a characterisation towards the toxic potential of sediment. Based on the first principal component PC1, which had the most variation, LM and PU site were the most distant apart. On the other hand, PU and BK were the most separated in the second principal component PC2, which had the second most variation of the hole analysis. From Figure 12., no statistically significant correlation could be observed among toxic potentials of sampling sites or between toxic potentials of sampling sites and mussel anoxic survival.

Table 7. Values of PCA coordinates (PC1, PC2) for toxic potential of seawater, sediment and mussel biological fluids in principal component analysis (PCA).

	PC 1	PC 2
Seawater	0.861459	-0.354146
Sediment	0.422941	0.899770
Mussel	-0.916395	0.082352

Principal component analysis (PCA) of grain size composition of five sampling sites (LM, RM, PU, RH, RP) was performed as a base, on which respective toxic potential values of sediment were superimposed (Figure 13), in order to find possible correlations. The first two principal components (PC1, PC2) described 100% of the variances. The first principal component (PC1) explained 64.54% of the total variance. The second principal component (PC2) accounted for 35.46% of the total variance. The PCA was based on three parameters, sand, gravel and silt-clay grain size composition. Values of PCA coordinates (PC1, PC2) for gravel, sand and silt-clay grain size composition are shown in Table 8.

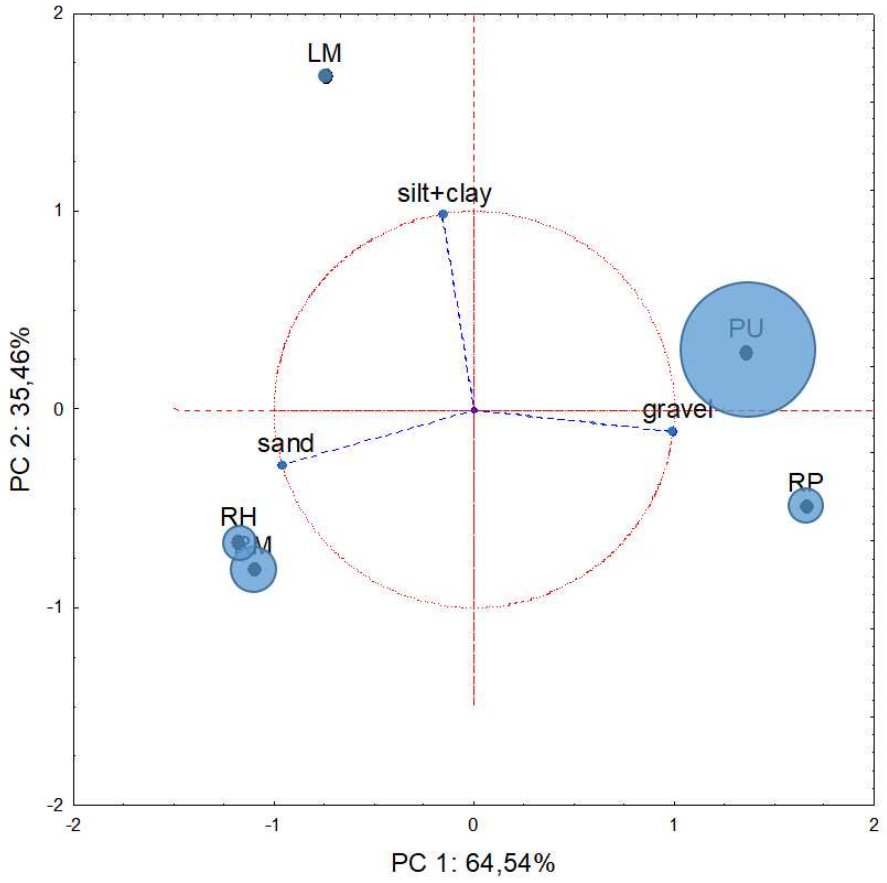

Figure 14. Principal component analysis (PCA) for sampling sites (LM - Lim Bay; RH - Rovinj harbour; RP - Rovinj pier; RM - Valdibora bay; PU - Pula harbour;), based on sand, gravel and silt-clay grain size composition with superimposed toxic potential of sediment.

LM was set in the top left quadrant gravitating mostly towards silt-clay grain size composition. PU was located in the top right quadrant being characterised mostly by gravel grain size composition, like RP, although RP was situated in the bottom right quadrant. RM and RH were both in the bottom left quadrant defined by sand grain size composition. Based on the first principal component PC1, which had the most variation, RH and RP were the most far-off sampling sites. Based on the second principal component PC2, which had the second most variation of the hole analysis, LM and RM were the most distant apart. No statistically significant correlation between grain size compositions of sampling sites was found. Neither between grain size composition of sampling sites and toxic potential values of sediment which were superimposed (Figure 14).

Table 8. Values of PCA coordinates (PC1, PC2) for gravel, sand and silt-clay grain size composition in principal component analysis (PCA).

	PC 1	PC 2
Gravel	0.994047	-0.108956
Sand	-0.960067	-0.279771
Silt-clay	-0.162449	0.986717

A linear correlation between PC1 values of sampling sites (RH, RM, LM, RP, PU) based on grain size composition and toxic potential of sediment was done finding a slightly positive correlation (0.523) although not statistically significant ($p > 0.05$).

5 DISCUSSION

The marine ecosystem is constantly under the influence of natural and anthropogenic pressures. It is very important to be aware of such environmental disturbances within the frame of integrative environmental management (Elliot, 2014). Contaminants presence alone can't give enough information about the condition of a marine system. It is crucial to take in consideration contaminants behavior, as it is the result of the interplay of contaminants sources, as well as physical-chemical properties of individual compounds, sediment and water movement, biotic and abiotic factors and specific conditions found in marine areas such as anoxia, eutrofication, freshwater springs and sewage spills. In order to estimate the risk for the marine habitats it is essential to determine the different fates of contaminants. They are distributed differently throughout the water column and accumulated variously in the biota and sediment. Chemical analyses of specific contaminants provide insight in contaminant input and possibly contaminant source. However, relationship between the presence of specific contaminants, their distribution through different matrices and their ability to affect marine organisms is very complex (Bihari et al., 2007). Contaminants in the marine environment include a number of compounds and form complex mixtures that may or may not be toxic (Bihari et al., 2004a). Toxicity assessment is a well-established tool to provide insight on contaminant accumulation, behavior and their potentially negative effects on the marine environment, specifically in seawater, sediment and biota. Toxicity assessment takes in consideration all environmental factors that are possibly modifying contaminants in the environment (Bihari et al., 2007).

The purpose of this study was to estimate marine contamination based on the toxic potential of seawater, sediment and biota. Mussels (*Mytilus galloprovincialis*) were used as representatives of the marine biota. Mussels are generally used as bioindicators. Being filter feeding organisms, they are exposed to dissolved contaminants and to contaminants which are adsorbed on particles. The toxic potential of seawater, sediment and mussel biological tissue would probably reflect the recent input of toxic contaminants in seawater, their chronic accumulation in sediments and the contaminants bioavailability in mussels respectively. Furthermore, toxic potential relation to sediment grain size composition was investigated, as well as the ability of the potentially toxic environment to affect mussel fitness.

5.1 Toxic potential of seawater

The toxic potential of seawater (EC_{50}) was obtained through Microtox® bioassay analysing seawater samples collected during summer in 2019. The Microtox® bioassay was performed on organic extracts of seawater samples, therefore only organic toxic compounds may had induced toxicity. RH, RM, RB, PU and BK show a similar toxic potential between 28-37

mL, while RP has the highest toxic potential (25 mL) and LM the lowest toxic potential (46 mL).

From Bihari et al. (2004b), in 1998 at LM, the toxic potential of seawater was >1250 mL while in 1999 it was 208 mL. From 1999-2005 the most frequent toxic potential of seawater samples collected at LM were both <138 mL and 138-613 mL with an overall increase in toxicity from 2000-2001 and later with a steady toxic potential values from 2001-2005 (Fafanđel and Bihari, 2007). Comparing this data with the toxic potential values of seawater from this research (46 mL), it seems that seawater at LM may got more toxic through the years due to the boat traffic in the harbour and the specific water circulation of the Lim Bay. RH (29 mL), RP (25 mL), RM (33 mL) and RB (37 mL) have similar toxic potential values of seawater probably due to their proximity in the Rovinj aquatory and having the same role as harbour. At PU in 1998 the toxic potential value of seawater was 625 mL, while in 1999 it was 76 mL (Bihari et al., 2004b). From 1999-2005 the most frequent toxic potential of seawater samples collected at PU were both <138 mL and 138-613 mL. An overall significant increase in toxicity from 2000-2002 and then a decrease in toxicity from 2002-2005 was recorded (Fafanđel and Bihari, 2007). Taking into consideration previous data and the toxic potential of seawater from this research (31 mL), seawater at PU may have increased its toxicity. It may be a response to a higher level of toxic chemicals due to the poor water circulation and increased boat traffic in the harbour. At BK in 1999 the toxic potential of seawater was >1250 mL (Bihari et al., 2004b). From 1999-2005 the most frequent toxic potential samples of seawater collected at BK were both <138 mL and 138-613 mL with an overall increase in seawater toxicity (Fafanđel and Bihari, 2007). In 2006, Bihari et al. (2007), measured a toxic potential of seawater of 12 mL. In this research seawater sample from BK resulted toxic with a EC_{50} of 28 mL, indicating a possible slight improvement of the environmental conditions.

Bihari et al. (2007) found a statistically significant correlation between total PAH concentration (291 ng/L) and toxic potential of seawater at BK, in the Gulf of Rijeka. This suggests that PAHs are the main toxic compounds present in organic extracts of seawater. Based on the toxic potential results of seawater from this research and the correlation determined by Bihari et al. (2007), PAHs are probably present at BK inducing seawater toxicity. No studies were done to establish the concentration of PAHs and their possible correlation with the toxic potential of seawater at RH, RP, RM, RB and PU. Nevertheless, Bihari et al. (2006) established the presence of PAHs in sediment samples in those sampling sites. PAHs must have been present in the water column in some point of time before reaching the sediment, therefore PAHs may be the toxic compounds inducing seawater toxicity at RH, RP, RM, RB and PU in this research. However, seawater samples represent a temporal and spatial point which may have variations even in the same sampling areas (Bihari et al., 2004a). Based on 50 sea water samples in the Arabian Gulf, collected in a

period of 6 months from an industrial discharge zone, Al-Muzaini et al. (1995) concluded that variations in toxic potential of seawater wouldn't get smaller with a higher sampling frequency. Such variations of toxic potential values of seawater should be taken in consideration with cluster analysis, discriminating areas based on their seawater toxicity (Bihari et al., 2004a).

5.2 Toxic potential of sediment and grain size composition

The toxic potential of sediment (EC_{50}) was obtained through Microtox® bioassay analysing sediment samples collected during summer in 2019. The Microtox® bioassay was performed on organic extracts of sediment samples, therefore only organic toxic compounds may had induced toxicity. Moreover, granulometric sediment composition was performed on the same samples. LM, RH, RP, RM and PU sampling sites show a very high toxic potential of sediment (<11 mg), while BK (100 mg) doesn't result to be toxic. Based on the toxic potential values of sediment, all sampling sites, except for BK, show very low variance and high homogeneity (1-11 mg).

At LM the sediment EC_{50} was 11 mg probably due to the high anthropogenic influence on the area. The granulometric composition reveals a higher percentage of silt and clay compared to the other sites. LM is situated in an estuary with specific geomorphological characteristics giving the area specific sediment grain size. Bihari et al. (2006), in 2005 at RH, recorded a toxic potential of sediment of 509 mg while in this research it was 4 mg. The toxic potential of sediment in 2005 at RP was 45 mg (Bihari et al., 2006) while in this research it was 4 mg. At RM in 2005, the toxic potential of sediment was 22,6 mg (Bihari et al., 2006) while in this research it was 3 mg. At RH, RP and RM the toxic potentials of sediment have increased from 2005, indicating a possible accumulation of toxic chemicals in the sediment through the years. High amount of total PAHs were recorded at RH (1988 μg/kg DW), RP (13681 μg/kg DW) and RM (347.93 μg/kg DW) in 2005, although no statistical significant correlation between PAH content and toxic potential of sediment at RH, RP and RM was found by Bihari et al. (2006). Toxicity of samples from polluted environments could be caused by hundreds of different compounds other than PAHs (Papadopoulou and Samara, 2002; Olajire et al., 2005), which could be the case for sampling sites RH, RP and RM. RH and RM show a very similar granulometric composition, characterized mostly by sand, possibly being explained by their proximity, being situated in the same city and having similar role as harbors, although RH has a lower water circulation compared to RM. RP on the other hand, has a big percentage of both gravel and sand, just like PU, despite being in Rovinj and Pula aquatory respectively. Although being a harbour like other sampling sites PU is one of the oldest and considering it has the highest boat traffic and lowest water circulation enhancing accumulation of contaminants in the sediment. Furthermore, PU has the worst toxic potential of sediment recorded in this research (1 mg).

BK from 1998-2006, despite of occasional peaks, showed a declining trend in PAHs, most likely linked to the improved environmental protection (Alebic-Juretic, 2011). One of the peaks in PAHs concentration was measured in 2006 by Bihari et al. (2007) concluding that BK was characterized by heavier PAHs from petrogenic and pyrogenic origin and listed it as highly contaminated area with 624 μg/kg DW of total PAHs. Toxic potential of sediment recorded in 2006 at BK was very high (113 ng), in fact a statistical significant correlation between PAH content and toxic potential of sediment was found (Bihari et al., 2007). In 2015, low or no toxic potential of sediment (EC_{50} from 23 mg to >197 mg) was measured by Fafanđel et al. (2015) and no correlation between concentrations of nutrients and toxic potential of sediment was found. In this research, the toxic potential of sediment at BK was 100 mg, concluding that the toxic potential of sediment and therefore environmental conditions haven't changed since 2015. PAHs may be still be the major source of toxicity at BK.

From statistical and PCA analysis in this research, no statistically significant correlations were found between gravel, sand, silt-clay grain size composition and toxic potential of sediment at LM, RM, RP, RH and PU. The higher positive correlation, although not statistically significant, between the toxic potential of sediment and gravel grain size composition could be associated to the fact that wet frozen sediment was used for the toxicity assay. The water may have increased the toxicity of the sample. It would have been better to dry the sediment before the toxicity assay as they did in a study in the Gulf of Gdansk (Baltic Sea) (Lukawska-Matuszewska et al., 2009) and in analogue studies (Ringwood et al., 1997; Wall et al., 2001) where a statistically significant correlation was found between the sediment granulometry and the toxic potential of sediment. Bihari et al. (2006) concluded as well that the toxic potential of sediment is bonded to sediment particle size. In their research LM and RM were characterized by the highest toxic potential of sediment with a sandy silt composition. The lowest toxic potential of sediment found at RH and RP, showed a silty gravely sand type of sediment which were characterized by the lowest amount of silty clay and the highest amount of gravel (Bihari et al., 2006). From the PCA analysis in this research it may be appropriate to indicate that every sampling site has specific toxic contaminant input. There is no statistically significant correlation between sediment grain size composition and toxic potential of sediment. Toxic contaminants are stored in sediments through accumulation depending on the specific conditions found in every sampling site.

5.3 Toxic potential of mussel biological fluids

The toxic potential of mussel biological fluids (EC_{50}) was obtained through Microtox® bioassay analyzing mussel soft tissue samples collected during summer season in 2019. Toxic potential values of mussel biological fluids at RH, RP, RM, RB and PU range from

14-66 µL. Nevertheless, LM has the highest toxic potential (7 µL) while BK has the lowest toxic potential (92 µL).

At LM in 2016, Privileggio (2017) recorded a toxic potential of mussel biological fluids of 174 µL, while in this research a far higher toxic potential of 7 µL was recorded. At PU in 2016, Privileggio (2017) recorded a toxic potential of mussel biological tissue of 111 µL, while in this research a much higher toxic potential of 30 µL was recorded. From 2000-2009 at LM it has been recorded an increasing accumulation of DDE, chromium, zinc and mercury while at PU an increasing accumulation of copper and mercury (EIONET, 2020). There are no records of their accumulation from the last ten years but following the trend, those toxic contaminants may have got increased in concentration in LM and PU, inducing a higher toxic potential response from mussel samples. At RP the toxic potential of mussel biological fluids in this research was 18 µL, probably due to the anthropogenic influence, increased boat traffic and low water circulation. At RH in 2016, Privileggio (2017) recorded a toxic potential of mussel biological fluids of 32 µL, while in this research it was 14 µL. At RM in 2016, Privileggio (2017) recorded a toxic potential of mussel biological fluids of 72 µL, while in this research a higher toxic potential of 42 µL was recorded. The toxic potential of mussel biological fluids, in both RH and RM, got higher probably because of the increased anthropogenic influence in those two harbour areas. The toxic potential at RH is higher compared to RM probably due to the lower water circulation at RH.

At BK, in 2006, Bihari et al. (2007) recorded a toxic potential of mussel biological fluids levels of 74.3 µL and Glad et al. (2017) confirmed similar levels of toxic potential in 2017. This research seems to follow this trend as 92 µL were recorded. Bihari et al. (2007) established that at BK mussel biological fluids had a low total amount of PAHs of 56.7 ng/g WW. They couldn't correlate it to the toxic potential of mussel biological fluids. The same lack of correlation was confirmed by Glad et al. (2017). Since the 1980s there is a shortage of data about the presence of specific toxic contaminants in marine organisms in the Gulf of Rijeka (Picer et al. 1982; Branica and Raspor 1982). Bihari et al. (2007) speculated that phenols, heavy metals and polychlorinated biphenyls could be present in mussel tissues, being common marine toxicants found in 1982. From 2000-2007 there are records of an increase in accumulation of chromium, mercury and zinc (EIONET, 2020), which may explain the toxic potential results of Bihari et al. (2007). Chromium, mercury and zinc concentration are probably still present at BK because of the limited water exchange in the closed bay of Bakar (Degobbis, 1981) and therefore may be causing a toxicity response in mussel samples from this research.

5.4 Mussel anoxic survival

In order to evaluate the mussel fitness response to anoxic conditions, which simulates a stressful environment, mussel anoxic survival (LT_{50}) was performed. Mussel samples were collected in summer season in 2019. At LM, RP, PU and BK LT_{50} values ranged from 6.1-8.2 days. RM shows the best (8.6 days) while RH shows the worst (6 days) mussel anoxic survival time.

At LM in 2016, Privileggio (2017) reported a mussel anoxic survival time of 3.1 days while in this research a much better fitness was recorded (8.2 days). At RH in 2016, the mussel anoxic survival time was 3.5 days (Privileggio, 2017) while in this research 6 days. At RM in 2016, Privileggio (2017) reported a mussel anoxic survival time of 3.1 days while in this research a much better fitness was recorded (8.6 days). At PU, in 2016, the mussel anoxic survival time recorded was 4.7 days (Privileggio, 2017), while in this research 6.5 days. At BK, in 2017, Glad et al. (2017) recorded a mussel anoxic survival time of 4.4 days, a lower result compared to these research's 6.1 days.

Although the toxic potential of mussel biological fluids has risen through the years at LM, RH, RM, PU and BK the mussel anoxic survival time has improved. Such difference could be explained by the fact that present mussel population are now adapted to the concentrations of toxic contaminants present in the marine environment. A lot of abiotic and biotic factors like temperature, oxygen levels, salinity, food availability and nutrients may affect the anoxic mussel survival. Studies in the Adriatic Sea (Petrović et al., 2004) and in the Northern Sea (Eertman et al., 1993) established that mussels should survive less in anaerobic condition during the summer period when the sea water temperature is higher, because they develop their gonads before the spawning season in fall. On the other hand, Pampanin et al. (2005) and Nesto et al. (2004), based on their research made in the Venetian Bay, have determined that high sea water temperature and low oxygen levels may have caused a preadaptation in the mussels, increasing their chance of surviving in anaerobic conditions.

A positive correlation was expected between the toxic potential of mussel biological fluids and a reduction of the mussel anoxic survival such as in Bihari et al. (2007) research in the Gulf of Rijeka. From the statistical analysis and PCA analysis no statistically significant correlation were found in this research between the mussel anoxic survival time and the toxic potential of seawater, sediment mussel and biological tissue. Mussels with lower toxic potential levels and a lower anoxic survival time, like BK, could be affected by mussels' oversensitive organisms as they struggle to overcome even the slighter level of contaminants. On the other hand, LM recorded the highest toxic potential of mussel biological fluids as well as the second best anoxic survival time which could be associated by the specific mussel ability to tolerate such high levels of contaminants or other environmental conditions affecting mussel fitness. The lack of correlation between mussel anoxic survival time and

toxic potential of seawater, sediment mussel and biological tissue imply that many other environmental factors as well as physiological properties of the mussels should be taken in consideration. The only positive correlation, although not statistically significant, was found between mussel anoxic survival time and toxic potential of seawater. Mussels are filter feeders organisms and in order to survive they have to filter great amounts of seawater. Inevitably they intake a lot of toxic compounds inside their bodies which could affect their fitness.

5.5 Characterization of sampling sites

From the PCA analysis LM was distinguished by the toxic potential of mussel biological fluids. LM is situated in Lim Bay, a 11 km long estuary of the underground river Pazinčica, which plunges into the bay, overall having very specific environmental characteristics. Samples were collected at the beginning of the bay in a small harbor where boat traffic is regular especially in summer season. A few km away from the harbor, in the middle of the bay, fish and mussel farms are situated as well as a protected area at the end of the bay. Nonetheless many freshwater springs can be found all around the Lim Bay. All this factors may condition the contaminant load and toxic potential. Toxic potential of sediment and mussel biological fluids varies a lot depending on the specific area of the bay where samples were collected as confirmed by Bihari et al. (2007), Bihari et al. (2006) and (EIONET, 2020). Interestingly mussels from LM showed the highest toxic potential but one of the best anoxic mussel survival results. This may indicate that a special adaptation has developed in mussels, under the influence of boat traffic and fish/mussel farms mineral nutrients released in the area.

RM and RH are situated in the same aquatory, but RH is characterized more by both toxic potential of mussel biological fluids and seawater while RM by toxic potential of sediment and seawater. RH was under a great anthropogenic influence because of the reconstruction of the area in the last few years. Such conditions may have influenced more mussels than the sediment considering the shorter period of high antropogenic influence, since sediment represent a contaminant load accumulated through many years. RM is more of an open area where a greater water mass circulation is found compared to RH. That could be the reason of the best anoxic mussel anoxic survival.

RP is the overall most toxic sampling site in this research considering all three matrices, distinguished by the toxic potential of seawater. It is situated in a harbor in Rovinj with a low water circulation, heavy boat traffic and anthropogenic influence which might have influenced such toxic potential response in all three matrices. PU was distinguished by the toxic potential of sediment rather than by seawater or mussel biological fluids. Such result may be explained by the fact that samples were collected in an old city harbor in Pula. The

low seawater circulation and great sedimentation rate could have made the sediment full of pollutants and therefore toxic.

At BK the estimated annual load of industrial and urban wastewater is about 23 million m^3 (Cvitković, 2005) although BK is the least toxic sampling site of this research based on the three matrices. The low toxic potential of sediment and mussel biological fluids which could be attributed to a good environmental status in those two matrices. From the PCA analysis BK was distinguished by the toxic potential of seawater. Being in a sheltered bay, BK is characterised by a high water column stability (Degobbis, 1981), limited water exchange (Legović and Vučak, 1981) and abundant underwater springs which could have influenced the higher toxic potential of seawater detected in this research.

5.6 Correlation among matrices

Through Spearman's rank analysis and PCA analysis in this research, no statistically significant correlations were found between the toxic potential of seawater, sediment and mussel biological fluids, just like Bihari et al. (2007) in the Gulf of Rijeka. The lack of correlation proves that the marine environment is very structured and complex. Contaminants distribution in different matrices reflects their different type of storage and availability.

Seawater represents a specific spatial and temporal point under the influence of wind, tides, currents, waste waters and other factors (Bihari et al., 2004a). Sediments act as a sink area for many chemical compounds which may result toxic threatening benthic organisms (Lukawska-Matuszewska et al., 2009). Sedimentation rate is one of the main factors which influence the toxic potential of sediment and it is specific to every area in the sea. In some marine areas, fast sedimentation is forming thicker layers of sea bottom layers, while elsewhere sedimentation is very slow (Lukawska-Matuszewska et al., 2009). Generally collecting the first few cm of marine sediments would represent the novel contaminants present in the environment from most recent period of time. Deeper layers of sediments could show much older contaminants which were present in the environment decades or even centuries before. Accumulation of contaminants in mussels is under the influence of many factors such as diet, digestive physiology, season, physiological conditions, behaviour and biological regulation (Bihari and Batel, 2018). Because of their ability to accumulate large number of contaminants through filter feeding, the most important variable in mussels is their metabolic rate which consents them to neutralize contaminants. Such metabolic rate varies in mussels from different habitat around the world so it is difficult to correlate it with the toxic potential of seawater or sediment. Moreover, some contaminants may interact exclusively in mussel tissues and become toxic while in seawater those molecules would never interact (Bihari and Batel, 2018). Size is an important factor as well as Privileggio

(2017) concluded that between the winter and summer season there is a positive statistically significant correlation between mussel morphologic characteristics and toxic potential.

LM and PU showed an increasing trend through the years in the toxic potential of seawater and mussel biological fluid while RH and RM were characterized by an increasing trend in the toxic potential of sediment and mussel biological fluid. RP was characterized by an increasing trend through the years in toxic potential of sediment. LM, PU, RH and RM with an increased trend in at least two matrices and RP with an increased trend in one matrix indicate a possible deterioration of the marine environment through the years, probably associated to a higher anthropogenic influence. BK was characterized by a decreasing trend in toxic potential of seawater and by a stable trend through the years in the toxic potential of sediment and mussel biological fluids, probably related to a possible improvement of the marine environment and to a lower negative anthropogenic influence.

Considering all data present in this research, the relationship among the toxic potential of seawater, sediment and biota is very complex. It is crucial to establish the toxic potential of every single individual matrix to assess an exact environmental status and potential risk in an integrative way (Fafanđel et al., 2015). In the same way it is important to establish mussel survival as a standalone biological parameter because of the lack of correlation towards the potentially toxic environment. Microtox® bioassay is very convenient as a primary test by which sites can be easily discriminated according to their toxic potential load. Additional chemical analysis could be performed supplementary to those sites which may demand further investigation trying to find the sources and lower the toxic potential load (Bihari et al., 2004a; Kungolos et al., 2003). Just as in Fafanđel and Bihari (2007), the Microtox® bioassay in this research demonstrated to have the advantage of a fast acquisition of results while still getting a good overall insight of the environmental conditions of the area (Lukawska-Matuszewska et al., 2009). All sampling sites in this research, except for the toxic potential of sediment and mussel biological fluids in BK, show a moderate to high toxic potential for all three matrices. RP is the most toxic site while BK is the least toxic considering the toxic potential of seawater, sediment and mussel biological fluids. Chemical analysis should follow to reveal the major toxic contaminants present in the sampling areas.

6 CONCLUSION

The following conclusions can be drawn as a result from this research:

- The bacterial bioluminescence assay (Microtox® bioassay) is suggested as a screening tool in order to identify potentially toxic conditions in marine environments. Such assay is defined by a fast acquisition of results and it gives a very good general insight of the polluted area considering seawater, sediment and biota.
- Because of the lack of correlation among the toxic potential of seawater, sediment and mussel biological fluids and mussel anoxic survival, and the specific conditions which lead to a toxic potential load, all matrices should be taken in consideration to get a true picture when trying to evaluate marine environmental quality assessment.
- RP is the most toxic site while BK is the least toxic considering the toxic potential of seawater, sediment and mussel biological fluids.

7 POVZETEK V SLOVENSKEM JEZIKU

Morski ekosistem je pod nenehnim vplivom naravnih in antropogenih pritiskov. V okviru celostnega upravljanja okolja se je zelo pomembno zavedati prisotnosti takšnih okoljskih motenj. Sam podatek o prisotnosti onesnaževal nam ne more dati dovolj informacij o stanju morskega sistema. Ključnega pomena je upoštevati vedenje onesnaževal, saj je le-to posledica medsebojnega vpliva virov onesnaževal, pa tudi fizikalno-kemijskih lastnosti posameznih spojin, gibanja sedimenta in vode, biotskih in abiotskih dejavnikov ter specifičnih pogojev, ki jih najdemo na morskih območjih, kot so anoksija, evtrofikacija, sladkovodni izviri in izlivi kanalizacije. Za oceno tveganja za morske habitate je bistvenega pomena določiti usodo različnih onesnaževal, saj so ta različno razporejena po vodnem stolpcu in se različno kopičijo v bioti in sedimentu. Kemične analize specifičnih onesnaževal omogočajo vpogled v vnos onesnaževal in potencialno tudi vir onesnaževal. Vendar pa je odnos med prisotnostjo specifičnih onesnaževal, njihovo porazdelitvijo po različnih okoljih in njihovo zmožnostjo vpliva na morske organizme zelo zapleten. Onesnaževala v morskem okolju vključujejo številne spojine in tvorijo kompleksne mešanice, ki lahko so ali niso strupene. Ocena strupenosti (»Toxicity assessment«) je uveljavljena metoda za vpogled v kopičenje onesnaževal, njihovo vedenje in potencialno negativne vplive na morsko okolje, zlasti v morski vodi, sediment in bioti. V oceni toksičnosti so upoštevani vsi okoljski dejavniki, ki lahko spreminjajo onesnaževala v okolju, zato je bil namen te študije ugotoviti toksični potencial morske vode, usedlin in biote ter razkriti možne povezave med temi tremi okolji. Klapavice (*Mytilus galloprovincialis*) so bile uporabljene kot predstavniki morske biote. Klapavice se, zaradi zmožnosti filtracije morske vode (za prehranjevanje in dihanje), običajno uporabljajo kot bioindikatorji. S to dejavnostjo so izpostavljene raztopljenim onesnaževalom in onesnaževalom, ki se absorbirajo na delcih. Toksični potencial morske vode, sedimenta in tkiva klapavic bi najverjetneje odražala nedavni vnos toksičnih onesnaževal v morsko vodo, njihovo kronično kopičenje pa bi se kazalo v sedimentu in (bio)dostopnosti onesnaževal v školjkah. Dodatno je bil preučevan tudi toksični potencial glede na sestavo zrn v sedimentu ter vpliv potencialno toksičnega okolja na fitnes školjk.

Hipoteze:

1. Med tremi različnimi okolji obstaja povezava med toksičnim potencialom kot odraz onesnaženosti izbranih vzorčnih mest.

2. Integracija toksičnega potenciala v vseh treh različnih okoljih omogoča razvrstitev vzorčnih mest glede na obremenjenost z onesnažili.

3. Toksični potencial sedimenta je odvisna od vrste sedimenta.

4. Potencialno toksično okolje vpliva na anoksično preživetje klapavic.

Metodologija:

Vzorci morske vode (30 L), sedimenta (500 g w.w.) in klapavic *Mytilus galloprovincialis* (40 osebkov) so bili zbrani na 7 vzorčnih mestih ob istrski obali (Jadransko morje, Hrvaška), ki predstavljajo težje obremenjena industrijska območja, urbana območja in referenčna mesta. Vzorčna mesta so: LM (Limski zaliv), RB (zaliv Valdibora, Institut za morske raziskave Ruđer Bošković), RM (Rovinjski severni zaliv Valdibora), RP (pomol Rovinj), RH (pristanišče Rovinj), PU (pristanišče Pula), BK (pristanišče Bakar).

Organske snovi iz 30 L morske vode so bile koncentrirane po gravitaciji takoj po odvzemu na stolpcih Amberlite XAD-7 (20 mL) s pretokom 200 mL/min (Fafanđel in Bihari, 2007). Absorbirane organske snovi so bile raztopljene s 150 mL acetona in uparjene, da je bila prostornina zmanjšana na 30 mL. Dodan je bil prečiščen diklorometan (70 mL), ki zelo učinkovito ekstrahira širok razpon od nepolarnih do polarnih spojin. Prosojnemu diklorometanu je bilo dodanih 300 µL dimetilsulfoksida (DMSO) in skupaj uparjeno pod vakumom pri 38°C. Preostalih 300 µL DMSO, ki vsebuje koncentrirane organske snovi, pridobljene iz 30 L vzorcev morske vode, se je pred preskušanjem strupenosti shranilo pri 4°C.

Teža suhega sedimenta je bila določena s segrevanjem 10 g pri 105°C za 24 h. Prisotnost skupnega organskega materiala je bila ocenjena z izgubo teže po segrevanju pri 450°C 5 ur. Vzorci za določitev velikosti zrn so bili ločeni z mokrim sejanjem. Frakcija N65 µm je bila nato 24 ur sušena v pečici pri 90°C in ločena s šestimi sejanji (specifikacija ASTM E11). Nato je bila frakcija še na suho sejana v intervalih 1 phi do phi +4 (65 µm) in razvrščena s trikotnim grafom (Buchanan in Kain, 1971).

Sediment (100 g mokre mase) je bil ekstrahiran s ponovno destiliranim diklorometan-metanolom (2:1), kot je opisano zgoraj (Schiewe in sod., 1985). Po več pranjih za odstranitev metanola se je diklorometanske ekstrakte uparilo do suhega in raztopilo v dimetilsulfoksidu (DMSO). Ekstrakcijski ostanki so bili pripravljeni po istem postopku, a brez usedlin. Ekstrakti sedimenta so bili pred preskušanjem strupenosti shranjeni pri 4°C.

Klapavice so bile postavljene v škatle (po 30 osebkov na škatlo) v vlažno okolje pri 16°C (Bihari in sod., 2007). Preživetje se je preverjalo vsak dan. Pokazatelji smrti so bili specifičen vonj, odsotnost mišične aktivnosti in sproščeni zaklopki. Rezultati so predstavljeni kot povprečni čas preživetja (LT50).

Toksični potencial organskih ekstraktov morske vode in sedimenta ter biološkega ekstrakta klapavic je bila merjena z biološkim testom Microtox® (Bihari et al., 2007), s katerim se meri zmanjšanje bakterijske (*Alivibrio fisheri*) luminescence po nizu razredčitev vzorcev po postopku BioFix® Lumi, ki ga je predpisal proizvajalec Macherey-Nagel, Nemčija, v

luminometru Microtox® Model 500 (AZUR, Environmental, ZDA). Ocene EC_{50} so bile pridobljene s programskim paketom MicrotoxOmni™.

Z analizo Probit je bil določen LT_{50} za vsako skupino klapavic. Za oceno stopnje strupenosti iz treh okolij (morska voda, sediment in biota) in preučevanje razmerja med podatki o strupenosti na raziskanih mestih je bila izvedena PCA analiza (Principal Component Analysis) z uporabo STATISTICA 8.0 (StatSoft Inc, ZDA).

Rezultati in diskusija:

Vzorčna mesta RH, RM, RB, PU in BK so pokazala podoben toksični potencial morske vode (med 28-37 mL), RP je imel najvišji toksični potencial (25 mL), LM pa najnižji (46 mL). Vzorčna mesta LM, RH, RP, RM in PU so pokazala zelo visoki toksični potencial sedimenta (<11 mg), izjema med vzorci pa je bilo mesto BK (100 mg), ki ni bilo strupeno. Vrednosti toksičnega potenciala bioloških tekočin klapavic pri RH, RP, RM, RB in PU so se gibale med 14 - 66 µL, mesto LM je imelo najvišji toksični potencial (7 µL), BK pa najnižji (92 µL). Z regresijsko analizo in analizo PCA v tej raziskavi niso bile najdene statistično pomembne korelacije med toksičnim potencialom morske vode, sedimentov in bioloških tekočin klapavic, prav tako kot je ugotovil Bihari in sod. (2007) v Reškem zalivu. Pomanjkanje korelacije dokazuje, da je morsko okolje zelo strukturirano in kompleksno. Porazdelitev onesnaževal v različnih okoljih odraža različno naravo skladiščenja in razpoložljivosti za privzem. Morska voda predstavlja specifično prostorsko in časovno točko pod vplivom vetra, plimovanja, tokov, odpadnih voda in drugih dejavnikov (Bihari et al., 2004). Sediment predstavlja številne usedle kemične spojine, kar lahko pomeni strupenost za bentoške organizme (Lukawska-Matuszewska et al., 2009). Hitrost usedanja je eden glavnih dejavnikov, ki vpliva na toksični potencial sedimenta in je značilen za posamezna območja. V nekaterih morskih območjih je usedanje zelo hitro, saj usedline tvorijo debelejše plasti morskega dna, drugje pa je usedanje zelo počasno (Lukawska-Matuszewska et al., 2009). Kopičenje onesnaževal v klapavicah je pod vplivom številnih dejavnikov, kot so prehrana, prebavna fiziologija, letni čas, fiziološke razmere, vedenje in biološka regulacija (Bihari in Batel, 2018). Zaradi sposobnosti kopičenja velikega števila onesnaževal preko filtracije (preko dihanja in hranjenja) je najpomembnejša spremenljivka, ko govorimo o klapavicah, hitrost njihovega metabolizma, ki omogoča, da takšna onesnaževala privzemajo. Hitrost presnove se razlikuje med klapavicami različnih habitatov, zato jih je težko povezati s toksičnim potencialom morske vode ali sedimentov. Poleg tega lahko nekatera onesnaževala medsebojno delujejo izključno v tkivih klapavic, kjer postanejo strupena, medtem ko v morski vodi te molekule ne bi nikoli medsebojno vplivale (Bihari in Batel, 2018).

Na podlagi statističnih in PCA analiz v tej raziskavi niso bile ugotovljene statistično pomembne korelacije med peskom, mivko, glinenimi sedimenti in toksičnim potencialom

usedlin na vzorčnih mestih LM, RM, RP, RH in PU. Iz analize PCA je morda smiselno nakazovati na dejstvo, da je vsako mesto vzorčenja specifično glede na način vnosa in tip onesnaževal ter da statistično pomembne korelacije med sestavo velikosti zrn sedimenta in toksičnega potenciala usedlin niso opažene. Strupena onesnaževala so shranjena v usedlinah, kjer se kopičijo - odvisno od specifičnih pogojev, vezanih na vsako posamezno mesto vzorčenja.

Vrednosti LT_{50} (čas anoksičnega preživetja klapavic) vzorčnih mest LM, RP, PU in BK so se gibale od 6,1 do 8,2 dni. Vrednost vzorčnega mesta RM je pokazala najboljši (8,6 dni), RH pa najslabši (6 dni) čas preživetja školjk. Med statistično analizo in analizo PCA v tej raziskavi ni bila opažena statistično pomembna korelacija med časom anoksičnega preživetja klapavic in toksičnega potenciala morske vode, sedimentih in bioloških tekočin klapavic. Zaradi pomanjkanja korelacije med časom anoksičnega preživetja klapavic in toksičnega potenciala morske vode, sedimentih in bioloških tekočin klapavic je potrebno upoštevati številne druge okoljske dejavnike in fiziološke lastnosti klapavic.

Glede na vse pridobljene podatke v tej raziskavi je odnos med toksičnim potencialom morske vode, sedimenta in biote zelo zapleteno, zato je ključnega pomena ugotoviti toksični potencial vsakega posameznega okolja, da se s celostnim pristopom natančno oceni okoljsko stanje in potencialno tveganje (Fafanđel in sod., 2015). Na enak način je zaradi pomanjkanja korelacije s potencialno strupenostjo okolja pomembno vzpostaviti samostojni biološki parameter preživetja klapavic. Biološki test Microtox® je zelo primeren kot primarni test, s katerim je mogoče izpostaviti vzorčna mesta, ki jih je mogoče zlahka razločiti glede na toksični potencial, nadaljnja kemijska analiza pa bi lahko služila kot dodatna analiza za mesta, ki bodo zahtevala nadaljnjo preiskavo, da bi našli vir onesnaženja in zmanjšali obremenitev onesnaževal (Bihari in sod., 2004a; Kungolos in sod., 2003). Tako kot sta ugotovila že Fafanđel in Biharij (2007), je biološki test Microtox® v tej raziskavi pokazal prednost hitrega pridobivanja rezultatov, hkrati pa daje še vedno dober splošen vpogled v okoljske razmere na območju vzorčenja (Lukawska-Matuszewska idr., 2009). Vsa mesta vzorčenja v tej raziskavi (razen toksičnega potenciala mesta BK za sediment in klapavic), kažejo zmerno do visoki toksični potencial za vsa tri okolja. Vzorčno mesto RP je najbolj strupeno vzorčno mesto, medtem ko je BK najmanj strupeno glede na toksični potencial morske vode, sedimentov in bioloških tekočin klapavic. Raziskavi bi morala slediti še kemijska analiza, ki bi razkrila glavna strupena onesnaževala na območjih vzorčenja.

8 REFERENCES

Agardy T., Davis J., Sherwood K., Vestergaard O. 2011. "Taking steps toward marine and coastal ecosystem-based management - an introductory guide," in UNEP Regional Seas Reports and Studies, Volume 189 (Nairobi), 68.

Al-Muzaini S., Beg M.U., Al-Mutairi M., Al-Mullalhah A. 1995. Seawater quality at industrial effluents discharge zone. Water Science Technology 32: 21-26.

Alebic-Juretic A. 2011. Polycyclic aromatic hydrocarbons in marine sediments from the Rijeka Bay area, Northern Adriatic, Croatia, 1998-2006. Marine Pollution Bulletin 62: 863-869.

Avramidis P., Bekiari V., Christodoulou D., Papatheodorou G. 2014. Sedimentology and water column stratification in a permanent anoxic Mediterranean lagoon environment, Aetoliko Lagoon, western Greece. Environmental Earth Sciences 73(9): 5687-5701.

Azam F., Smith D.C., Stewart G.F., Hagström A. 1993. Bacteria - organic matter coupling and its significance for oceanic carbon cycling. Microbial ecology 28: 167-179.

Balcioglu E.B. 2016. Assessment of polycyclic aromatic hydrocarbons (PAHs) in mussels (Mytilus galloprovincialis) of Prince Islands, Marmara Sea. Istanbul University, Faculty of Fisheries, Marine Biology Department, Laleli 34130, Istanbul, Turkey. Marine Pollution Bulletin 109(1): 640-642.

Ban N.C., Alidina H.M., Ardron,J.A. 2010. Cumulative impact mapping: advances, relevance and limitations to marine management and conservation, using Canada's Pacific waters as a case study. Marine Policy 34: 876-886.

Barbier E.B., Hacker S.D., Koch E.W., Stier A., Silliman B. 2012. "Estuarine and coastal ecosystems and their services: Ecological economics of estuaries and coasts," in Treatise on Estuarine and Coastal Science Series by E. Wolanski and D. McLusky, Vol. 12, eds M. van den Belt and R.Costanza (Waltham, MA: Academic Press), 109-127.

Bihari N., Najdek M., Floris R., Batel R., Zahn RK. 1989. Sediment toxicity assessment using bacterial bioluminescence: effect of an annual phytoplankton bloom. Marine Ecology Progress Series 37: 307-310.

Bihari N., Mičić M., Fafanđel M. 2004a. Seawater Quality Along the Adriatic Coast, Croatia, Based on Toxicity Data. Environmental Toxicology 19: 109-114.

Bihari N., Mičić M., Fafanđel M., Hamer B., Jakšić Ž., Batel R. 2004b. Testing quality of seawater from the Adriatic coast of Croatia with toxicity, genotoxicity and DNA integrity tests. Acta Adriatica 45(1): 75-81.

Bihari N., Fafanđel M., Hamer B., Kralj-Bilen B. 2006. PAH content toxicity and genotocixity of coastal marine sediments from the Rovinj area, Northern Adriatic, Croatia. Science of the Total Environment 366: 602-611.

Bihari N., Fafanđel M., Piškur V. 2007. Polycyclic aromatic hydrocarbons and ecotoxicological characterization of seawater, sediment, and mussel *Mytilus galloprovincialis* from the Gulf of Rijeka, the Adriatic Sea, Croatia. Archives of Environmental Contamination Toxicology 52: 379-387.

Bihari N., Batel I. 2018. Ekotoksikologija mora i oceana. Institut za oceanografiju i ribarstvo Split. pp. 11-69.

Boluda R., Quintanilla JF., Bonilla JA., Saez E., Gamon M. 2002. Application of the Microtox test and pollution indices to the study of toxicity in the Albufera Natural Park (Valencia, Spain). Chemosphere 46: 355-369.

Borja A. and Elliott M. 2013. Marine monitoring during an economic crisis: the cure is worse than the disease. Marine Pollution Bulletin 68: 1-3.

Borja A., Elliott M., Andersen J.H., Cardoso A.C., Carstensen J., Ferreira J.G., et al. 2013. Good Environmental Status of marine ecosystems: what is it and how do we know when we have attained it? Marine Pollution Bulletin 76: 16-27.

Borja A., Elliott M., Andersen J.H., Berg T., Carstensen J., Halpern B. S., Heiskanen A., Korpinen S., Stewart Lowndes J.S., Martin G., Rodriguez-Ezpeleta N. 2016. Overview of Integrative Assessment of Marine Ecosystems: The Ecosystem Approach in Practice. Frontiers in Marine Science 3: 20.

Branica M., Raspor B. 1982. Heavy metals. In: Jeftić M (ed) Ecological study of Rijeka Bay. Institut "Ruđer Bošković", Zagreb, Rovinj, pp. 241-255.

Buchanan J.B., Kain J.S. 1971. Measurement of the physical and chemical environment. In Methods for the Study of Marine Benthos (ed. N. A. Holme and A. D. McIntyre), 30-53. Blackwell Scientific Publications.

Bulich AA. 1986. Bioluminescence assay. In: Bitton G, Dutka BJ, editors. Toxicity testing using microorganisms. Volume 1. Cleveland, OH: Press. pp. 57-64.

Burton Jr GA. 2002. Sediment quality criteria in use around the world. Limnology 3: 65-76.

CBD 2000. Convention on Biological Diversity United Nations. Available online at: www.cbdint/doc/legal/cbd-en.pdf (date of access: 28.5.2020.).

Chiaudani G., Marchetti R., Vighi M. 1980. Eutrophication in Emilia - Romagna coastal waters (North Adriatic Sea, Italy): A case hystory. Progress in water technology 12: 185-192.

Costanza R., d'Arge R., de Groot R., Farber S., Grasso M., Hannon B., et al. 1997. The value of the world's ecosystem services and natural capital. Nature 387: 253-260.

Crain C.M., Kroeker K., Halpern,B.S. 2008. Interactive and cumulative effects of multiple human stressors in marine systems. Ecology Letters 11: 1304-1315.

Cvitković E. 2005. Statistical chronicle of the Primorsko-goranska County. pp. 456.

Degobbis D. 1981. Hydrographic characteristics of the Rijeka Bay (the Northern Adriatic). Thalassia Yugoslavica 17: 141-154.

Degobbis D., Gilmartin M. 1990. Nitrogen, phosphorus and biogenic silicon budgets for the northern Adriatic Sea. Oceanologica acta, 13: 31-45.

Degobbis D., Malej A., Fonda–Umani S. 1999. The mucilage phenomenon in the northern Adriatic Sea. A critical review of the present scientific hypotheses. Annali dell Istituto superiore di sanita 35: 373-381.

Degobbis D., Precali R., Ivančić I., Smodlaka N., Fuks D., Kveder S. 2000. Long-term changes in thenorthern Adriatic ecosystem related to anthropogenic eutrophication. International Journal of Environment and Pollution 13: 495-533.

De Zwaan A., DeKock WC. 1988. The development of a general biochemical stress index. Marine Environmental Research 24: 254-255.

De Zwaan A., Cortesi P., Cattani O. 1995. Resistance of bivalves to anoxia as a response to pollution-induced environmental stress. Science of the Total Environment 171: 121-125.

Dorman C.E., Carniel S., Cavaleri L., Sclavo M., Chiggiato J., Doyle J., Haack T., Pullen, J., Grbec B., Vilibić I., Janeković I., Lee C., Malačić V., Orlić M., Paschini E., Russo A., Signell R.P. 2006. February 2003 marine atmospheric conditions and the bora over the northern Adriatic. Journal of Geophysical Research: Oceans 111.

Cotou E., Papathanassiou E., Tsangaris C. 2002. Assesing the quality of marine coastal environments: comparison of scope for growth and Microtox bioassay results of pollution gradient areas ion eastern Mediterranean (Greece). Environmental Pollution 119: 141-149.

Eertman RHM., Wagenvoort AJ., Hummel H., Smaal AC. 1993. "Survival in air" of the blue mussel *Mytilus edulis* L. as a sensitive response to pollution induced-environmental stress. Journal of Experimental Marine Biology and Ecology 170: 179-195.

EIONET - European Environment Information and Observation Network. http://baltazar.izor.hr/azopub/beionet_tab (date of access: 01.06.2020.)

Elliott M. 2011. Marine science and management means tackling exogenic unmanaged pressures and endogenic managed pressures - A numbered guide. Marine Pollution Bulletin 62: 651-655.

Elliott M. 2014. Integrated marine science and management: wading through the morass. Marine Pollution Bulletin 86: 1-4.

EPA - Environmental Protection Authority 2016. Environmental Factor Guideline: Marine Environmental Quality. https://www.epa.wa.gov.au/policies-guidance/environmental-factor-guideline-marine-environmental-quality (date of access: 30.05.2020.)

Fafanđel M., Bihari N. 2007. Temporal and spatial variations of seawater toxicity along Adriatic coast, Croatia: a long-term study. Fresenius Environmental Bulletin 16(11): 1457-1463.

Fafanđel M., Piljagić J., Smodlaka Tanković M., Travizi A., Bihari N. 2015. Nutrients vs toxicity in sediments: a case study of two samy-closed basins in Rijeka Bay, Croatia. Fresenius Environmental Bulletin 24(9a): 2888-2897.

Faganeli J., Mohar B., Kofol R., Pavlica V., Marinšek T., Rozman A., Kovač N., Šurca Vuk A. 2010. Nature and lability of northern Adriatic macroaggregates. Marine Drugs 8: 2480-2492.

Folk R.L., Ward W C. 1957. Brazos River bar: a study in the significance of grain size parameters. Journal of Sedimentary Petrology 27: 3-26.

France J., Mozetič P. 2006. Ecological characterization of toxic phytoplankton species (*Dinophysis spp., Dinophyceae*) in Slovenian mariculture areas (Gulf of Trieste, Adriatic Sea) and the implications for monitoring. Marine Pollution Bulletin 52: 1504-1516.

Franco P. 1983. In Proceedings of the Vth Congress of the Italian Association of Oceanology and Limnology (AIOL) 1-27.

Frascari F., Frignani M., Guerzoni S., Ravaioli M. 2006. Sediments and Pollution in the Northern Adriatic Sea. Istituto di Geologia Marina, Consiglio Nazionale delle Ricerche 40127 Bologna, Italy. Annals of the New York Academy of Sciences 534(1): 1000-1020.

Glad M., Bihari N., Jakšić Ž., Fafanđel M. 2017. Comparison between resident and caged mussels: Polycyclic aromatic hydrocarbons accumulation and biological response. Marine Environmental Research 129.

Golterman HL. 2004. The chemistry of Phosphate and Nitrogen Compounds in Sediments: Springer Netherlands.

Grant A., Briggs AD. 2002. Toxicity of sediments from around a North Sea oil platform: are materials or hydrocarbons responsible for ecological impacts? Marine Environmental Research 53: 95-116.

Gray J.S. and Elliott M. 2009. Ecology of Marine Sediments. From Science to Management, 2nd Edn. New York, NY: Oxford University Press.

Halpern B.S., Frazier M., Potapenko J., Casey K.S., Koenig K., Longo C., et al. 2015. Spatial and temporal changes in cumulative human impacts on the world's ocean. Nature Communications 6: 7615.

Harding L.E. 1992. Measures of Marine Environmental Quality. Environment Canada, 224 West Esplanade, North Vancouver, B.C., Canada V7M 3H7. Marine Pollution Bulletin, Volume 25, 1-4, pp. 23-27.

Heissenberger A., Leppard G.G., Herndl G.J. 1996. Ultrastructure of marine snow. II. Microbiological considerations. Marine Ecology Progress Series 135: 299-308.

Hyotylainen T, Oikari A. 1999. The toxicity and concentrations of PAHs in creosote-contaminated lake sediment. Chemosphere 38: 1135-44.

Kaplan E.L., Meier P., 1958. Nonparametric estimation from incomplete observations, Journal of the American Statistical Association 53: 457-481.

Kilikidis S., Kamairanos A., Karamanlis X, Gianakou U. 1994. Determination of polycyclic aromatic hydrocarbons in the effluents of an urban waste treatment plant and the water, sediment and mussels of the receiver Themairkos Gulf (N. Greece). Fresenius Environmental Bulletin 3: 95-100.

Krajcar V. 2003. Climatology of geostrophic currents in the Northern Adriatic. Geofizika 20: 105-114.

Kraus R., Supić N. 2011. Impact of circulation on high phytoplankton blooms and fish catch in the northern Adriatic (1990.-2004.). Estuarine Coastal and Shelf Science 91: 198-210.

Kungolos A., Samaras P., Koutseris E. 2003. Using Bioassays for Testing Seawater Quality in Greece. Journal of Environmental Science and Health Part 38(3): 533-44.

Kuzmić M., Janeković I., Book J.W., Martin P. J., Doyle J. D. 2006. Modeling the northern Adriatic doublegyre response to intense bora wind: A revisit. Journal of Geophysical Research Oceans 111.

Lammel G., Spitzy A., Audy O., Beckmann S., Codling G.P., Kretzschmann L., Kukučka P., Stemmler I. 2017. Organochlorine pesticides and polychlorinated biphenyls along an east-to west gradient in subtropical North Atlantic surface water. Environmental Science and Pollution Research 24(12): 11045-11052.

Lau-Wong MM. 1990. Assessing the effectiveness of depuration of polluted clams and mussels using the Microtox bioassay. Bulletin of Environmental Contamination and Toxicology 44: 876-883.

Legović T., Justić D. 1997. When do phytoplankton blooms cause the most intense hypoxia in the northern Adriatic Sea? Oceanologica Acta 20: 91-99.

Legović M., Vućak Z. 1981. Kinematics of water exchange in the Rijeka Bay. Thalassia Yugoslavica 17: 155-175.

Lukawska-Matuszewska K., Burska D., Niemirycz E. 2009. Toxicity assessment by Microtox in sediments, pore waters and sediment saline elutriates in the Gulf of Gdansk (Baltic Sea). Institute of Oceanography, University of Gdansk, Poland 37(7): 592-598.

Lyons D.M., Supic N., Smodlaka N. 2007. Geostrophic circulation patterns in the northeastern Adriatic Sea and the effects of air-sea coupling: May-September 2003. Journal of Geophysical Research 112.

Manca B., Giorgetti A. 1999. Flow patterns of the main water masses across transversal areas in the southern Adriatic Sea: Seasonal variability. In: Malanotte – Rizoli P., Eremeev V.N. (eds.), The eastern Mediterranean as a laboratory basin for the assessment of contrasting ecosystems 495-506. Kluwer Academic Publication, Amsterdam.

Marine Strategy Framework Directive - MSFD (European Commision) https://ec.europa.eu/environment/marine/eu-coast-and-marine-policy/marine-strategy-framework-directive/index_en.htm (date of access: 12.6.2020).

Mihanović H., Vilibić I., Carniel S., Tudor M., Russo A., Bergamasco A., Bubić N., Ljubešić Z., Viličić D., Boldrin A., Malačić V., Celio M., Comici C., Raicich F. 2013. Exceptional dense water formation in the Adriatic shelf in the winter of 2012. Ocean science 9: 561-572.

Montero N., Belzunce-Segarra M., Menchaca I., Garmendia J., Franco J., et al. 2013. Integrative sediment assessment at Atlantic Spanish harbors by means of chemical and ecotoxicological tools. Environmental Monitoring and Assessment 185: 1305-1318.

Mozetič P., Solidoro C., Cossarini G., Socal G., Precali R., France J., Bianchi F., De Vittor C., Smodlaka N., Fonda Umani S. 2009. Recent trends towards oligotrophication of the northern Adriatic: evidence from chlorophyll a time series. Estuaries and Coasts 33: 362-375.

Murray L., Mudge S., Newton A., Icely J. 2006. The effect of benthic sediments on dissolved nutrient concentrations and fluxes. Biogeochemistry 81: 159-178.

Naes K., Oug E. 1997. Multivariate approach to the distribution patterns and fate of polycyclic aromatic hydrocarbons in sediments from smelter-affected Norwegian fjords and coastal waters. Environmental Science and Technology 32: 1253-1258.

Najdek M., Blažina M., Djakovac T., Kraus R. 2005. The role of the diatom *Cylindrotheca closterium* in a mucilage event in the northern Adriatic Sea: coupling with high salinity water intrusions. Journal of Plankton Research 27: 851-862.

Nesto N., Bertoldo M., Nasci C., Da Ros L. 2004. Spatial and temporal variation of biomarkers in mussel (*Mytilus galloprovincialis*) from the Lagoon of Venice, Italy, Mar. Environmental Research 58: 287-291.

Nõges P., Argillier C., Borja Á., Garmendia J.M., Hanganu J., Kodeš V., et al. 2016. Quantified biotic andabiotic responses to multiple stress in freshwater, marine and ground waters Science of the Total Environment 540: 43-52.

Olajire AA., Altenburger R., Kuster E., Brack W. 2005. Chemical and ecotoxicological assessment of polycyclic aromatic hydrocarbon-contaminated sediments of the Niger Delta, southern Nigeria. Science of the Total Environment 340: 123-136.

Oliva A.L., Arias A.H., Quintas P.Y., Buzzi N.S., Marcovecchio J.E. 2017. Polycyclic Aromatic Hydrocarbons in Mussels from a Soutth American Estuary. Archives of Environmental Contamination and Toxicology 72(4): 540-551.

OSPAR 2009. Trend Analysis of Maritime Human Activities and their Collective Impacton the OSPAR Maritime Area, Publication Number: 443/2009. London: OSPAR Commission.

Pampanin D. M., Volpato E., Marangon I., Nasci, C. 2005. Physiological mesurements from native and transplanted mussel (*Mytilus galloprovincialis*) in the canals of Venice. Survival in air and condition indeks; Comparative Biochemistry and Physiology, Part A 140: 41-52.

Papadopoulou D., Samara C. 2002. Polycyclic aromatic hydrocarbon contamination and Lumistox® solvent extract toxicity of marine sediments in the North Aegean Sea, Greece. Environmental Toxicology 17: 556-566.

Passarini F., Rampazzo G., Ghirardini AV., Sperni L., Salizzato M., Pavoni B. 2000. Extraction, identification and quantification of heavy metals in Venice lagoon sediments using toxicity tests with microorganisms. Annali di Chimica 90: 91-101.

Pavoni B., Salizzato M., Ghirardini AV. 1998. Organic micropollutants (PCB, PAH, chlorinated pesticides) in sediments of Venice Rii-chemical analysis and toxicity tests using *Vibrio fischeri*. Chimica 88: 189-200.

Petrović S., Semenčić L., Ozretić B., Ozretić M. 2004. Seasonal variations of physiological and cellular biomarkers and their use in the biomonitoring of north adriatic coastal waters (Croatia). Marine Pollution Bulletin 49: 713-720.

Picer M., Picer N., Mikac N. 1982. Crude oil, phenols and PCBs. In: Jeftić M (ed) Ecological study of Rijeka Bay. Institut "Ruđer Bošković", Zagreb, Rovinj, pp 156-201.

Piggott J.J., Townsend C.R., Matthaei C.D 2015. Reconceptualizing synergism and antagonism among multiple stressors. Ecology and Evolution 5: 1538-1547.

Poulain PM., Kourafalou V.H., Cushman-Roisin B. 2001. Northern Adriatic Sea. In: Cushman-Roisin B., Gačić M., Poulain PM., Artegiani A. (eds) Physical Oceanography of the Adriatic Sea. Springer, Dordrecht.

Poulain P.M., Raicich F. 2001. Forcings. In: Cushman–Roisin B., Gačić M., Poulain P.M., Artegiani A. (eds.), Physical oceanography of the Adriatic Sea, 45–65. Kluwer Academic Publishers, Dordrecht, Neederlands.

Privileggio L. 2017. Utjecaj sezone na osjetljivost biomarkera obzirom na veličinu dagnje. Bachelor Thesis, Sveučilište Jurja Dobrile u Puli, Pula, Croatia. pp. 1-42.

Quiniou F., Judas A., Lesquerandre E. 1997. Assessment of potential toxicity in estuary water and sediments in the Bay of Brest using two types of bioassay. Annales de l'Institut Océanographique 73: 35-48.

Ringwood A.H., DeLorenzo M.E., Ross P.E., Holland A.F. 1997. Interpretation of Microtox Solid-phase Toxicity tests: The Effects of Sediment Composition, Environmental Toxicology and Chemistry 16: 1135-1140.

Schiewe MH., Hawk EG., Actor DI., Krahn MM. 1985. Use of bacterial bioluminescence assay to assess toxicity of contaminated marine sediments. Canadian Journal of Fisheries and Aquatic Sciences 42: 1244-1248.

Shepard F.P. 1951. Sediments of the continental shelves. Geological Society of America Bulletin 43: 1017-1040.

Shor Lm., Kosson DS., Rockne KJ., Jang LY., Taghon GL. 2004. Combined effects of contaminated desorption and toxicity on risk from PAH contaminated sediments. Risk Analysis 24: 1109-20.

Smaal AC., Wagenvoort A., Hemelraad J., Akkerman I. 1991. Response to stress of mussels (*Mytilus edulis*) exposed in Dutch tidal waters. Comparative Biochemistry and Physiology 100C: 197-200.

Smayda T.J. 1997. What is a bloom? A commentary. Limnology and Ocenaography 42: 1132-1136.

Storelli M.M., Marcotrigiano G.O. 2001. Polycyclic Aromatic Hydrocarbons in Mussels (*Mytilus galloprovincialis*) from the Ionian Sea, Italy. Journal of Food Protection 64(3): 495-9.

Supić N., Orlić M., Degobbis D. 2003. Istrian coastal countercurrent in the year 1997. Il Nuovo Cimento 26: 117-131.

Supić N., Kraus R., Kuzmić M., Paschini E., Precali R., Russo A., Vilibić, I. 2012. Predictability of northern Adriatic winter conditions. Journal of Marine Systems 90: 42-57.

Suresh Kumar C., Jaikumar M., Robin R.S., Karthikeyan P., Saravana Kumar C. 2013. Heavy Metals Concentration of Sea Water and Marine Organisms in Ennore Creek, Southeast Coast of India. The Journal of Toxicology and Health 103: 192-201.

Thomas R.E., Harris P.M., Rice S.D. 1999. Survival in air of *Mytilus trossulus* following long-term exposure to spilled Exxon Valdez crude oil in Prince William Sound. Comparative Biochemistry and Physiology C 122: 147-152.

Turner K., Schaafsma M., Elliott M., Burdon D., Atkins J., Jickells T., et al. 2014. "UK national ecosystem assessment follow-on," in Work Package Report 4: Coastal and Marine Ecosystem Services: Principles and Practice. Cambridge, UK: UNEP-WCMC, LWEC, UK.

Turner R.K., Schaafsma M. (eds.) 2015. Coastal Zones Ecosystem Services: from Scienceto Values and Decision Making. Heidelberg: Springer Ecological Economic Series, Springer International Publications.

Viarengo A., Canesi L., Pertica M., Marcinelli G., Accomando R., Smaal A.C., Orunesu M. 1995. Stress on stress response: a simple monitoring tool in the assessment of a general stress syndrome in mussels. Marine Environmental Research 39: 245-248.

Vigano L., Arillo A., Buffagni A., Camusso M., Ciannarella R., Crosa G., et al. 2003. Quality assessment of bad sediments of the Po River, Italy. Water Research 37: 501-18.

Vilibić I., Grbec B., Supić N. 2004. Dense water generation in the north Adriatic in 1999 and its recirculation along the Jabuka Pit. Deep-Sea Research I51: 1457-1474.

Vilibić I., Supić N. 2005. Dense water generation on a shelf: the case of the Adriatic Sea. Ocean Dynamics 55: 403.

Viličić D. 1991. A study of phytoplankton in the Adriatic Sea after the July 1984 bloom. Internationale Revue der Gesamten Hydrobiologie 76: 197-211.

Viličić D. 2014. Specifična oceanološka svojstva hrvatskog dijela Jadrana. Prirodoslovno matematički fakultet Sveučilišta u Zagrebu Biološki odsjek. Rewiev Paper.

Wall V.D. et al. 2001. The effect of mercury and PCBs on organisms from lower trophic levels of a Georgia salt marsh, Archives of Environmental Contamination and Toxicology. 40: 10-17.

Water Environmental Federation 1997. Biomonitoring in the Water Environment, P07106WW. Prepared by the Task force in the Water Environment, under the direction of the Water Quality and Ecology Subcommittee of the Technical Practice Committee. Water Environment Federation: Alexandria, USA.

WHO/UNEP 1990. Report on the workshop. In: Carcinogenic, mutagenic and teratogenic marine pollutants: impact on human health and the environment. Advances in Applied Biotechnology Series 5: 12-19.

Witt G. 1995. Polycyclic aromatic hydrocarbons in water and sediment of the Baltic sea. Marine Pollution Bulletin 31: 237-248.

Zhang, A., Wang, L., Zhao, S., Yang, X., Zhao, Q., Zhang, X., Yuan, X. 2017. Heavy metals in seawater and sediments from the northern Liaodong Bay of China: Levels, distribution and potential risks. Regional Studies in Marine Science 11: 32-42.

Zhou JL., Maskoui K. 2003. Distribution of polycyclic aromatic hydrocarbons in water and surface sediments from Daya Bay, China. Environmental Pollution 121: 269-281.

Zore-Armanda M. 1969. Water exchange between the Adriatic and the eastern Mediterranean. Deep-Sea Research 16: 171-178.

Žutić V., Svetličić V. 2000. Interfacial processes. In: Hutzinger, O. (ed.), The Handbook of Environmental Chemistry 5: 149-165.