Comparison between Resident and Caged Mussels: Polycyclic Aromatic Hydrocarbon Accumulation and Biological Response

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Abstract

The aim of this study was to compare the capability of "passive" and "active" biomonitoring to determine the environmental pressure. For this purpose, PAHs content and several biological responses in resident and caged mussels (*Mytilus galloprovincialis*) at five sampling sites (Rijeka Bay, Adriatic Sea) were analsed. Resident mussels were found better in reflecting the level of PAH loads at particular sites while only caged mussels could detect input of HMW PAHs. When data of each investigated parameter were compared separately, the majority of differences between resident and caged mussels' results were site-specific. Integration of biological response patterns expressed as Index of Biological Response (IBR) resulted with different sampling sites ranking for resident and caged mussels. Multiple Factor Analysis (MFA) based on integration of tissue PAH concentration and biological response revealed resident mussels as more powerful for detection of environmental pressure. The use of resident mussels is recommended as appropriate and less costly approach for monitoring the effect of pollution.

Key words: biomarkers, caged mussels, monitoring, multiple factor analysis, polycyclic aromatic hydrocarbons, resident mussels

1. Introduction

Design of ecotoxicological surveys monitoring spatial and temporal trends of chemical contamination in coastal and estuarine areas is important for interpretation of collected data. Alongsides monitoring of pollutant level in marine organisms due to its possible transfer to humans following seafood consumption, the concept of integration of chemical, biochemical and physiological parameters has been accepted for ecotoxicological characterization of the marine environment (Galloway et al. 2002; Bihari et al. 2007; Fernandez et al. 2010, Barhoumi et al., 2014, Regoli et al., 2014). Biomonitoring has the advantage of an integrated evaluation in time and space of the presence and the effect of the bioavailable pollutants. Additionally, it enables the evaluation of possible causal relationship between contaminant presence and observable biological effects in aquatic organisms dwelling in a contaminated environment. Under environmental stress, marine life responds at all levels of biological organization (molecular, cellular, organism, population, community, and ecosystem). Responses at molecular and cellular levels, as the earliest signals of environmental disturbance before community and ecosystem responses can be detected, have been commonly used to evaluate the health effects of environmental contamination in marine ecosystems (Michel et al. 2000; Nigro et al. 2006; Fernandez et al. 2010). Assessment of PAH-induced cellular damage generally includes determination of genotoxic effect by means of measuring DNA damage or DNA integrity (Galloway 2002; Bihari et al. 2006) and activities of enzyme affected by PAH metabolites (Devier et al. 2005; Fernandez et al. 2010). At the same time, integrated monitoring studies usually include determination of mussel physiological status (Thomas et al. 1999; Bihari et al. 2007). In “mussel watch”, monitoring programs resident (passive biomonitoring) and/or caged mussels (active biomonitoring) as bioindicators of chemical contaminants could be used. Resident mussels have better discriminative capacity in reflecting the cumulative effects (Nigro et al. 2006), display lower seasonal variability of biomarkers (Lehtonen et al., 2016) and due to the long –term exposure are more sensitive to tissue contaminant accumulation (Marigómez et al., 2013, Martínez-Gómez et al., 2017). On the other hand, due to the short-term exposure influences of genetic differences and adaptive phenomena in caged mussels are reduced leading to higher sensitivity (Garmendia et al., 2011). Moreover, determination of biological response in caged mussels is invaluable in the investigation of areas where they better represent the diffused contamination (Martínez-Gómez et al., 2017) and/or native populations are absent. Due to the aforementioned abilities and limitations, several authors agreed that a parallel analysis of caged and resident mussels will improve the assessment of pollution and its effects on

ecosystem health; especially in case of chronic pollution and long-term biomonitoring programmes (Nigro et al., 2006, Hunt and Slone, 2010, Marigómez et al., 2013, Lehtonen et al., 2016). Taking into the account financial side, the use of native mussels as cost-effective was indicated as appropriate except in cases of site-point or time-point pollution when caging remains more useful.

The aim of this study was to examine the advantages/disadvantages of the caging approach for determination of environmental contamination in the urbanised area of Rijeka bay, Croatia, and compare the results with the results obtained using resident mussels. Due to its coastal population, influence of tourism and industrial facilities, Rijeka Bay was identified as affected by human activities and has been included in MEDPOL and national monitoring programmes. The amount of polycyclic aromatic hydrocarbons in the marine sediment indicated this coastal area as an environment with a high PAH input (Bihari et al. 2007; Traven et al. 2008). To asses the input of PAHs in marine environment and the impact on marine organisms, both the concentration of accumulated PAHs and their biological effects (biomarkers) were determined in caged and resident mussel *Mytilus galloprovincialis*.

Polycyclic aromatic hydrocarbons (PAHs) are widespread chemicals in the marine environment. PAHs are present at high concentration in most urbanized coastal areas as a consequence of numerous human activities mostly related to combustion or disposal of fossil fuels (pyrolytic sources) and offshore drilling or petroleum shipping activities (petrogenic sources). They can also originate from natural events such as forest or grass fires, volcanic eruptions and petroleum seeps as well as from natural synthesis by organisms (biogenic source). Once released into the marine environment PAHs tend to adsorb on suspended particles in seawater column and sediment becoming bio-available to the marine organisms. Accumulation of PAHs in biota depend upon the proximity to the sources of pollution, their bioavailability and species ability for PAH biotransformation. PAHs affect living organisms through their toxicity. The reactive products of PAHs metabolism such as epoxides and dihydrodiols bind to cellular proteins and DNA causing the biochemical disruption and cell damage. The toxicity of individual PAHs increases as a molecular weight increases and four-, five-, and six-ring PAHs have the highest mutagenic and carcinogenic potential (reviewed in: Neilson 1998; Douben 2003). Due to their harmful biological effects, they are listed as priority substances in the EU Water Framework Directive (WFD).

In the attempt to link the exposure of marine organisms to chemicals and *in situ* biological response, several biomarkers were determined in both resident and caged mussels. The testing included quantification of the level of toxic content in mussel tissue (potential

toxicity), specific biomarkers of genotoxicity (DNA integrity) and general neurotoxicity (acetylcholine esterase activity) as well as determination of mussel physiological status ("stress-on-stress"). Considering that the level of toxic compounds in an organism relies on the contaminant concentration in the environment, determination of mussel biological extract's potential toxicity enables detection of toxic contaminants presence and discrimination of polluted environment (Cotou et al. 2002). Toxicity of many chemicals, including PAHs lies in their capacity to interact with DNA.The genotoxicity of environmental pollutants could be observed as a decrease in DNA integrity. Some genotoxic agents, e.g. PAHs contribute to the inhibitory effect of pesticides on acetylcholine esterase activity (Jett et al. 1999). Besides, other types of pollutants such as heavy metals, surfactants and algal biotoxins display anti-choline esterase activity and thus AChE activity has been suggested as an useful biomarker of general stress (Guilhermino et al. 1998, Lehtonen et al. 2003). In addition to referred molecular biomarkers that are more or less responsive to narrow range of contaminants, this study also included the determination of mussel health status. Measured as "stress-on-stress" (de Zwan et al. 1995, Pampanin et al. 2005, Bihari et al. 2007), mussel physiological status reflects the integrative impact of complex contaminant mixture related to the survival potential of an organism.

Simple and multivariate statistical methods were used to investigate the relationships among biological and chemical variables. The focus was put on the difference of the biological response between caged and resident mussels and the relevance of the particular biological response for design and interpretation of the data in future ecotoxicological studies of the area. Integrated biomarker response index (IBR) that combines investigated biological responses and counteracts the influence of the natural variability as well as adaptive responses of individual biomarkers was used to characterize the pollution load at investigated sampling sites. Moreover, multiple factor analysis (MFA) that analyses data sets of variables (PAHs tissue concentrations and biomarkers) collected on the same set of observations (resident or caged mussels) was performed to analyse differences between those two observations.

2. Materials and methods

2.1. Study area

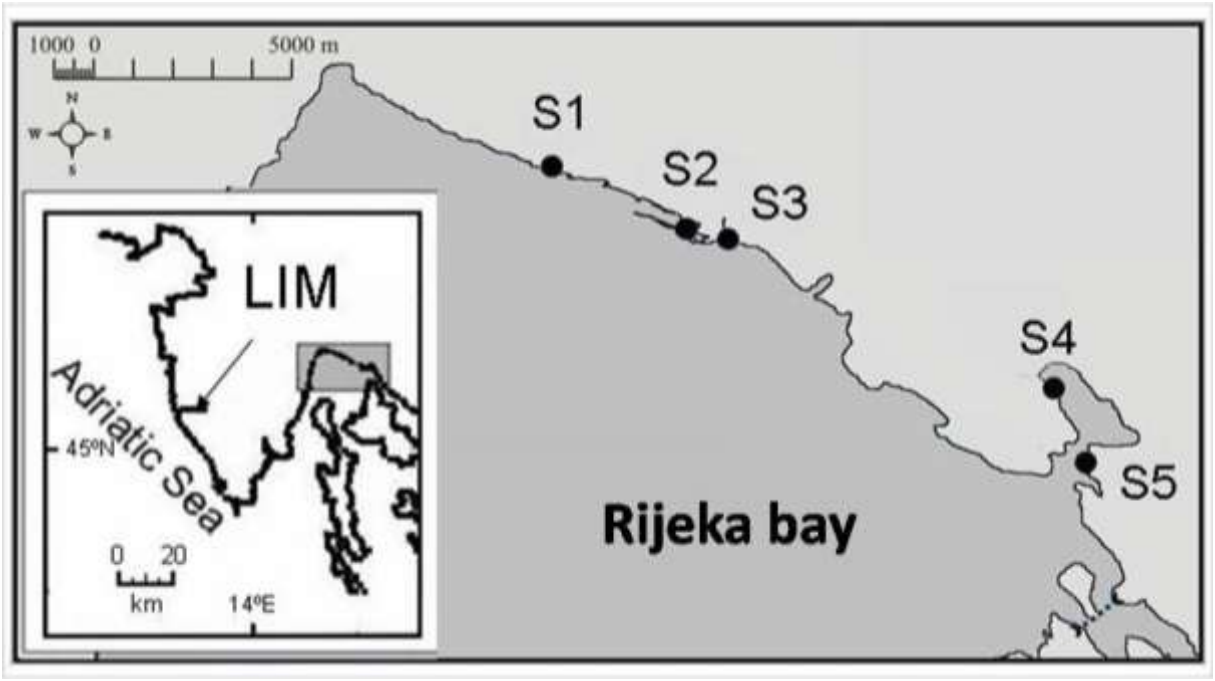

Figure 1. Investigated area: sampling sites S1-S5 in Rijeka Bay and reference site in Lim Bay. (1,5 column fitted figure)

Investigation was carried out at 5 sites from Rijeka Bay, Adriatic Sea, Croatia, (Fig1) representing areas with high level of human activities: shipyard (S1, S5), harbour (S2), river mouth (S3) and industry (S4). Basic environmental parameters of seawater at investigated sites were given in Table1.

Table 1. Brief description of the investigated area.

Investigated site	Description	T ^{o}C	pH	DOM[a] mg/l	Salinity	DO[b] mg/l
REF	Protected area	19.0	7.99	27.10	35.71	8.20
S1	Shipyard	19.5	8.10	26.90	35.29	8.33
S2	Harbour	19.0	8.06	21.40	27.30	8.34
S3	River mouth, urban runoff	17.5	8.16	13.49	16.40	10.85
S4	Urban sewage, industrial outflow	19.0	7.74	11.26	13.38	10.04
S5	Shipyard, urban sewage	19.0	7.88	21.74	27.78	7.67

[a]DOM - dissolved organic material

[b]DO - dissolved oxygen

2.2. Sampling strategy

Mussels (*Mytilus galloprovincialis* Lmk, 1819), shell-length from 5 to 6 cm, referred as reference mussels, were taken from a fish-farm located in the Lim bay and within 3 hours transferred by car in humid atmosphere (18^{o}C) to five locations in Rijeka bay (cca 300 specimens per site)(Table 1). They were immerged 1 – 2 m from the surface and caged for 30 days. Resident mussels (5-6 cm), dwelling in vicinity of the caged mussels were collected from near the air-water interface (0.3 – 0.5 m) together with caged mussels and transferred to lab immediately after collection. Random sub samples of 30 resident and 30 caged mussels were separated for "stress-on-stress" determination. For PAH analysis and toxicity assay, soft tissue of 100 resident and 100 caged mussels was dissected and for AChE activity and DNA integrity measurement, gills from 15 resident and 15 caged specimens were dissected and immediately frozen in liquid nitrogen.

2.3. PAH analysis

For PAH determination mussel tissue (20 – 30 g) was heated at 70-80^{o}C in 200 ml 1M KOH dissolved in ethanol for 5 h. After addition of 200 ml cyclohexane and heating for 15 min. several washing with distilled water were performed until complete removal of KOH. The sample was evaporated to 2 ml and adding 0.5 – 1 g Al_2O_3, removed fat. After evaporation to dryness, samples were dissolved in 1 ml methanol (Stijve and Hischenhuber 1987). The determination of PAH amount was performed with high performance liquid chromatography using Thermo-Finnigan (ThermoQuest Corp., USA) equipment and a LiChrospher RP-18 (AGILENT, USA) column (250 * 4 mm). The flow rate was adjusted to 1.5 ml/min and the mobile phase mixture was carried out in the following conditions: time = 0 min., 80:20 (%) methanol/water, time = 10 min., 100 % methanol. The maximum elution time was 16 min. For simultaneous determination of different PAH compounds UV-VIS and fluorescence detector was used. The excitation and emission wavelengths were changed during the analysis according to the program shown below.

	Wavelengths	
Time	Excitation	Emission
(min)	(nm)	(nm)
0	252	402
7.6	238	398

8.5	268	398
11.5	300	466
16.0	300	466

The peak heights and relative areas were recorded with ChromQuest Software Ver 2.51 (ThermoQuest Corp., USA). The detection limits were 1 ng/l for seawater sample, 1 µg/kg dry weight for sediment samples and 0.1 ng/g wet weights for mussels. 10 of 16 US EPA PAHs were analyzed (ACE-acenaphthene, PHE-phenanhtrene, ANT-anthracene, FLU-fluorene, PYR-pyrene, CHR-chrysene, B(a)A-benzo(a)anthracene, B(b)F-benzo(b)fluoranthene, B(a)P-benzo(a)pyrene, IND-indeno(1,2,3,c.d)pyrene). The recoveries of PAHs were from 64 % for anthracene to 98 % for acenaphthene.

2.4. Toxicity assay

The potential toxicity of mussel biological extracts was measured by the Microtox® bioassay (Cotou et al. 2002). The bioassay measures the reduction of bacterial (*Vibrio fisheri*) luminescence following the exposure to toxic extract. The biological fluid was extracted from 3 pooled mussels (~ 1g wet weight) and homogenized in a dilution buffer (BioFixLumi, Macherey-Nagel, Germany), 1:3 (w/v) with a Potter-Teflon homogenizer. The homogenate was centrifuged at 5000g for 15 min and the supernatant was used for toxicity testing. The decrease in luminescence was measured for serial dilutions of mussel extracts according to the BioFix® Lumi procedure prescribed by the manufacturer (Macherey-Nagel, Germany) in Microtox® Model 500 luminometer (AZUR Environmental, U.S.A.). EC_{50} (ml) was estimated using Mictox®Omni Software. The potential toxicity was expressed as $1/EC_{50}$ x 100.

2.5. DNA integrity

To determine DNA integrity in mussel cells, gill tissue (100 mg) was homogenized in 2 ml TE buffer (1 mM EDTA, 10 mM TrisHCl, pH 7.4) and DMSO (9:1) in liquid nitrogen. DNA denaturation rate in mussel gill lysates at alkaline pH was measured by FAST Micromethod ® (Batel et al. 1999, Bihari et al. 2005) in the Fluoroscan Ascent microplate reader (Labsystem, Finland).The procedure is based on the ability of fluorochrome Picogreen, (Molecular Probes Inc., U.S.A.) to preferentially interact with double-stranded DNA in alkaline conditions. Briefly, 25 µl (100 ng DNA/ml) of gill homogenate was lysed in 25 µl lysing solution (4.5 M urea, 0.1% SDS, 0.2 M EDTA, pH 10.0) supplemented with Picogreen (20 µl/ml lysing solution) in the dark for 30 minutes. Analyses were performed in quadruplicates of 5 gills samples. DNA denaturation conditions were achieved by adding

250µl NaOH-EDTA (pH 11.5). Afterwards, the decline in fluorescence of the dsDNA-Pico-Green complex was measured in microplates at room temperature. The decrease in DNA integrity (genotoxicity) reflected as loss of dsDNA after 5 minutes of denaturation time was expressed as ΔF/min.

2.6. Acetylcholine esterase activity

Acetylcholine esterase (AChE) activity in mussel gills was measured by the method of Ellman et al. (1961), adjusted to microtitar plates (Bocquene and Galgani 1998). The tissue was homogenised in cold (4°C) 0.1M Tris HCl buffer, pH 8.0 (1:4, w/v) using Teflon Potter homogeniser. After centrifugation of homogenate at 10000g, for 30 min the supernatant was taken for immediate measurement of enzyme activity. The reaction mixture contained 14 µl 0.1M Tris HCl (pH 8.0), 14 µl 8 mM 5,5'-dithiobis-2-dinitrobenzoic acid and 298 µl gill tissue homogenate. The enzyme reaction was initiated with 14 µl of substrate (45 mM acetylthiocholine, ACTC). The absorbance increase at 415 nm was recorded every 30 seconds in microplate reader (Labsystems, Multiscan Ascent®, Finland) using Ascent Software ™, version 2.4 and specific AcHE activity was expressed as nanomoles of hydrolysed ACTC per minute per mg of protein ($nmol/min^{-1}$ mg^{-1}). Tissue protein concentration in homogenates was determined according to the method of Lowry (1951) with bovine serum albumin (BSA) as the protein standard.

2.7. "Stress-on-stress"

Resident mussels, caged mussels and referent mussels were placed in boxes (30 animals/ box) in humid atmosphere at 19^{o}C. Survival was assessed daily. Death mussels were recognized according to their specific smell, absence of any muscular activity and open valves. The average survival time (LT_{50}) was determined for each sample (box) using probit analysis (Toxicologist ver. 1.0).

2.8. Statistical analyses

All the statistical data analyses were performed with STATISTICA 8.0 (StatSoft Inc, U.S.A.). Wilcoxon matched pair test was used for the detection of significant difference between two sets of data non-parametric. Cluster analysis using Euclidean distance and complete linkage was performed to determine the similarity between distributions of accumulated PAHs. Non-parametric Spearman test was used to determine the degree of correlation between investigated parameters. To determine the relationship between biomarkers and accumulated PAHs, PCA was performed. To present the difference in biological response between resident and caged mussels and pollution status of each location in a simple way, an integrated biomarker response (IBR) was used. It was calculated as an

average (IBR/n) of different arrangements of biomarkers in the set (Beliaeff and Burgeot, 2002; Broeg and Lehtonen, 2006, Lehtonen et al., 2006, Marigómez et al., 2013) and visualized using a star plot graphic tool.

Multiple factor analysis (MFA) was applied to derive an integrated picture of both biomarker response and PAH level as well as the relationship between observations obtained in resident and caged mussels. MFA (Abdi and Valentin, 2007, Abdi et al., 2013) was used to analyse set of observations (sampling sites) described by several groups (resident and caged mussels) of variables (measured parameters). Each data set was pre-processed (centred and standardized). MFA was performed in two steps. First a principal component analysis (PCA) was performed on each data set (resident and caged mussels) which is then normalized by dividing its elements by the square root of the first eigenvalue (matrix equivalent of the standard deviation) obtained from its PCA. Second, the normalized data sets were merged to form unique matrix and a global PCA was performed on the matrix. The individual data sets were then projected onto the global analysis to analyze communalities and discrepancies.

Results and discussion

3.1. Polycyclic aromatic hydrocarbons

The total concentration of the 10 PAHs determined in the resident and caged mussels from 5 investigated sites in Rijeka Bay is presented in Table 2. The total PAH content in resident mussels at all investigated sites was higher than in mussels from the pristine Mediterranean areas but in the range determined for mussels collected in highly urbanised areas (Baumard et al. 1999, Barhaumi et al., 2014) or in vicinity of gas-drilling activities in the northern Adriatic (Gomiero et al., 2015). The total PAH content in caged mussels was higher than at 123 stations around the coast of the NW basin of the Mediterranean Sea (Galgani et al, 2011) but lower than in urbanized Adriatic coastal areas (Fabbri et al., 2006) . In both, resident and caged mussels PAH levels were higher than previously reported for resident mussels in surrounding areas (Bihari et al. 2007) although neither individual PAH concentration exceeded EAC value (OSPAR, 2013).

At one sampling site (S3) situated at the Rječina river estuary and characterised by low salinity (16.4) PAH content in caged mussels decreased during 30 days, probably, due to the high influence of freshwater with low contaminant concentration that had mixed with seawater and had reduced the bioavailability of present contaminants. Decrease in PAH concentration would classify this area as not influenced by recent PAHs input. In contrast, PAH content in resident mussels at S3 was higher than in referent mussels and therefore this site should be classified as an area contaminated by PAHs. Since PAH concentration in resident mussel represented a time-weighted average it is evident that PAH dynamics in the mussels is not only influenced by the presence of PAH but also by the adaptation of indigenous mussel to particular environmental situation (Galgani et al., 2011). The level of PAH content in mussels caged at all other investigated locations was higher than before transplantation indicating accumulation of bio-available PAHs during 30 days period as a consequence of PAHs presence in surrounding water.

Table 2. Concentration of individual PAH compounds (ng/g ww), selected PAH ratios in resident (r1-r5), and caged (c1-c5) mussels *Mytilus galloprovincialis* from referent (REF) and 5 investigated sites in the Rijeka Bay.

	REF	r1	c1	r2	c2	r3	c3	r4	c4	r5	c5
Acenaphthene	64	146	58	157	102	88	59	121	85	136	88
Phenanthrene	32	49	38	50	40	39	35	41	40	45	36

Anthracene	0.7	1.4	0.8	1.5	1.0	0.9	d.l.	1.4	0.9	1.5	0.8
Fluoranthene	15	50	18	60	22	19	7.1	23	10	49	8.2
Pyrene	5.2	24	12	30	17	8.4	d.l.	13	0.8	25	d.l.
Chrysene	2.5	50	7.5	69	6.3	7.9	2.9	10	7.2	34	3.9
Benzo(a)anthracene	1.0	20	4.3	62	23	2.7	2.6	5.9	3.2	15	4.7
Benzo(b)fluoranthene	d.l.	41	d.l.	57	19	6	d.l.	d.l.	d.l.	d.l.	d.l.
Benzo(a)pyrene	d.l.	d.l.	d.l	d.l.	1.6	d.l.	d.l.	d.l.	d.l.	d.l.	d.l.
Indeno(1,2,3-*cd*)pyrene	d.l.	d.l.	d.l.	d.l.	2.0	d.l.	d.l.	d.l.	d.l.	d.l.	d.l.
Σ PAHs	120	381	139	486	234	172	108	215	147	305	142
Σ COMB[a]	23.7	144	41.8	278	159	44	12.6	52	21.3	40.3	12.1
Σ CARC[b]	3.5	111	9.9	188	51.9	16.6	5.5	15.9	10.4	16.0	20.3
BAPEs[c]	0,25	6,86	0,64	12,95	6,14	1,15	0,4	0,91	0,54	2,14	0,65
Ratios											
LMW/ Σ PAHs	0.80	0.51	0.69	0.53	0.61	0.31	0.87	0.75	0.85	0.59	0.88
LMW/HMW	4.00	1.08	2.33	0.75	1.56	3.00	6.69	3.17	6.14	1.50	7.33
COMB/PAHs	0.20	0.48	0.30	0.57	0.39	0.25	0.13	0.24	0.14	0.40	0.12
CARC/PAHs	0.03	0.29	0.09	0.39	0.22	0.10	0.06	0.08	0.07	0.16	0.06

d.l. below detection limit
[a] PAHs produced by combustion of fossil fuels
[b] carcinogenic PAHs proposed by EPA (USEPA, 1984)
[c] carcinogenic potential expressed as equivalents of BaP based upon relative carcinogenic potency of each individual PAH proposed by Nisbet and LaGoy, 1992.

At all sampling sites tissue concentration of 10 PAHs was higher in resident than in caged mussels. Previous studies suggested that the equilibrium time depended on the pollution level, with a faster balance when the environmental levels of pollutants were high indicating that 3-4 weeks was enough to reach the equilibrium between environmental and tissue levels of pollutants (Payne et al, 2008, Marigómez et al., 2013). Observed difference of PAH content between resident and caged mussels in Rijeka Bay may be explained by longer caging time needed to reach equilibrium (Serafim et al, 2011) or irregular PAH input dynamics in the area. When four sampling sites (S2, S3, S4 and S5) were taken into consideration high correlation (R^2 = 0,908) between the amount of accumulated PAHs during 30 days and PAH content in resident mussels was found suggesting the existence of dynamic equilibrium between matrices in resident mussels and related PAH accumulation kinetics in transplanted mussels. The only exception was S1 where low amount of accumulated PAHs could not be related to relatively high PAH content in resident mussels. This result evidenced site-specific irregular PAH input at S1 that could not be detected by caged mussels.

In investigated area the majority of PAHs could have come from motor vehicle exhausts, residential and industrial heating sources, coal, crude oil and natural gas processing. The predominance of combustion PAHs was noticed only at S2. In resident mussels it exceeds 50% that is in accordance with intense accumulation determined in caged mussels (2 times over the initial value) reflecting chronic as well as recent input of combustion PAHs. Compared to other sampling stations S2 is located in a harbour, in the city centre, where, due to the concentrated transportation activities, combustion-related PAHs were the expected main source of contamination. However, in caged mussels at S3, S4 and S5, the amount of combustion PAHs after 30 days decreased (50% of initial value at S3 and S5). This decrease indicated that rapid elimination of previously accumulated combustion PAHs (coal and wood burning during winter) occurred during the caging period. However, their absence in May when caging was performed, changed the equilibrium point at those sites.

Moreover, only in caged mussels from site S2 Benzo(a)pyrene (BaP)and Indeno(1,2,3-*cd*)pyrene (IND) were detected indicating their presence during the caging period. Bihari et al. (2007) reported IND presence in sediment and absence in resident mussel from the areas southwest and southeast from the S1 – S5 sampling sites. Absence of BaP and IND in resident mussels could be attributed to the biotransformation and elimination efficiency arising from genetic predisposition of indigenous population to cope with particular environmental conditions in harbour (Lacroix et al, 2017). Caged mussels could have dissimilar metabolic (biotransformation and elimination) efficiency due to the different origin and genetic predisposition for adaptation to a new environment. Nevertheless, the fraction of high molecular weight hydrocarbons was higher in resident than in caged mussels at all sampling sites. However, when compared to initial status, caged mussel accumulated HMW PAHs only at S1and S2 (Fig. 2) while at S4 and S5 mussel accumulated LMW PAHs.

In resident mussels, the LMW/HMW ratio was related to the total PAH content (Fig3). The increase of total PAH amount was followed by a decrease of LMW/HMW suggesting the presence of a dynamic equilibrium between bio-available PAH uptake from the medium and its elimination. The LMW/HMW ratio in caged mussels was ΣPAH independent. Exposure of caged mussel to new environmental condition for a period of 30 days significantly changed the LMW/HMW ratio.

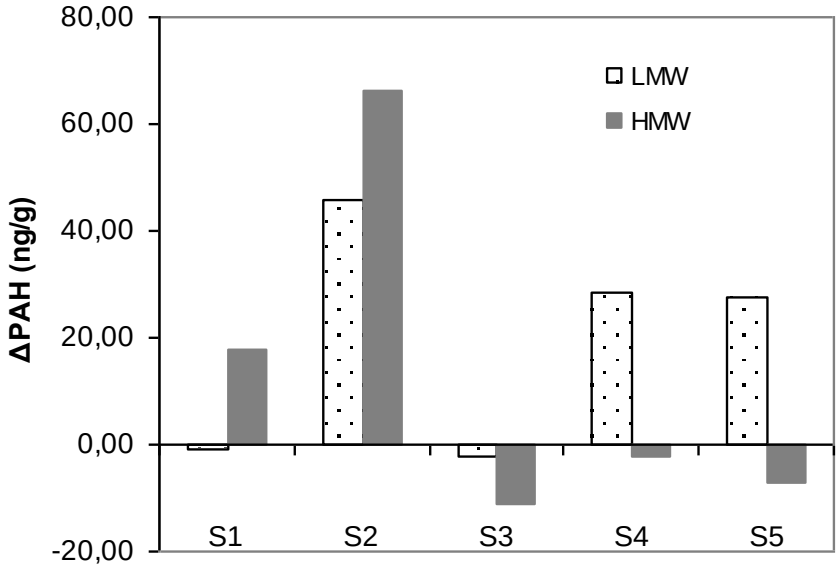

Figure 2. Accumulation/elimination of low molecular weight (LMW) and high molecular weight (HMW) PAHs during caging (30 days) at 5 investigated sites in Rijeka Bay. (1 column fitted figure)

Predominance of bio-available LMW at S4 and S5 PAHs could be ascribed to the absence or HMW and faster uptake of LMW. Mussels can directly absorb lower weight PAHs through interstitial filtered water, while heavier hydrocarbons are mainly ingested in particle form from the digestive system. Moreover, differing effect between resident and caged mussels was detected at S2 where B(a)P and IND were found only in caged mussels. Higher efficiency of PAH biotransformation and elimination pathways in resident compared to caged mussels and suggests existence of resident mussel population adapted to a polluted environment (Lacroix et al., 2015). When compared to caged mussels, resident mussels were chronically exposed to contamination and thus have developed metabolic adaptations for efficient elimination of harmful compounds that would enable their survival in the contaminated environment. This contrasting effect observed at S2, S4 and S5 indicated complex PAH dynamics between matrices (Picardo et al., 2001, Bihari et al., 2007) leading to particular PAH partitioning in distinct environment.

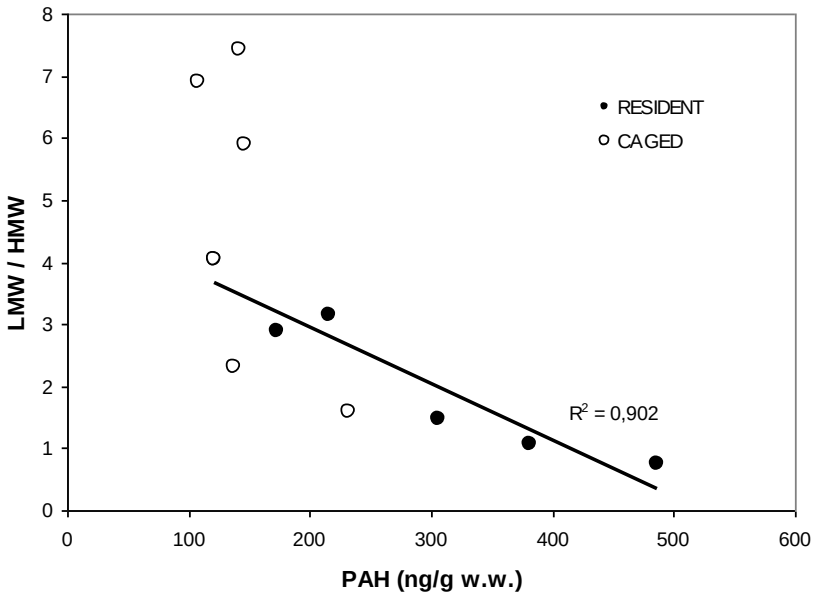

Figure 3. Relation of low molecular weight (LMW) and high molecular weight (HMW) PAHs ratio and total PAH content in resident and caged mussels *Mytilus galloprovincialis*. (1 column fitted figure)

In order to compare the PAHs distribution in resident and caged mussels the sets of data containing fractions of accumulated individual PAHs were submitted to similarity analysis. A hierarchical cluster analysis of individual PAH content (Fig4) revealed 3 groups of mussels with statistically different PAH distribution (ANOSIM). The first group comprise c2, r2 and r1, the second group r4, r5, c4, c5, ref, r3 and the third group by c1 and c3. Similar PAH distribution between resident and caged mussels indicated similar time-weighted relations of bioavailable PAHs at 3 investigated sites; S2, S4, and S5. This corresponding PAH content between caged and resident mussels at the same site could be related to the amount of accumulated PAHs (Seraphim et al., 2011, Marigómez et al., 2013). Significant correlation between PAH content in caged and resident mussels was found for sampling sites S2 ($r=0.69$, $p<0.05$) and S4 ($r=0.63$, $p<0.05$). Irrelevantly of pollution level at these sites (486 ng/g in resident and 234 ng/g in caged at S2; 215ng/g in resident and 147 ng/g in caged at S4), it indicated the equilibrium between environmental and tissue level of pollutants was reached during caging period. On the contrary, at S1 this equilibrium point was not reached and PAH distribution in caged mussels differed from PAH distribution in resident mussels.

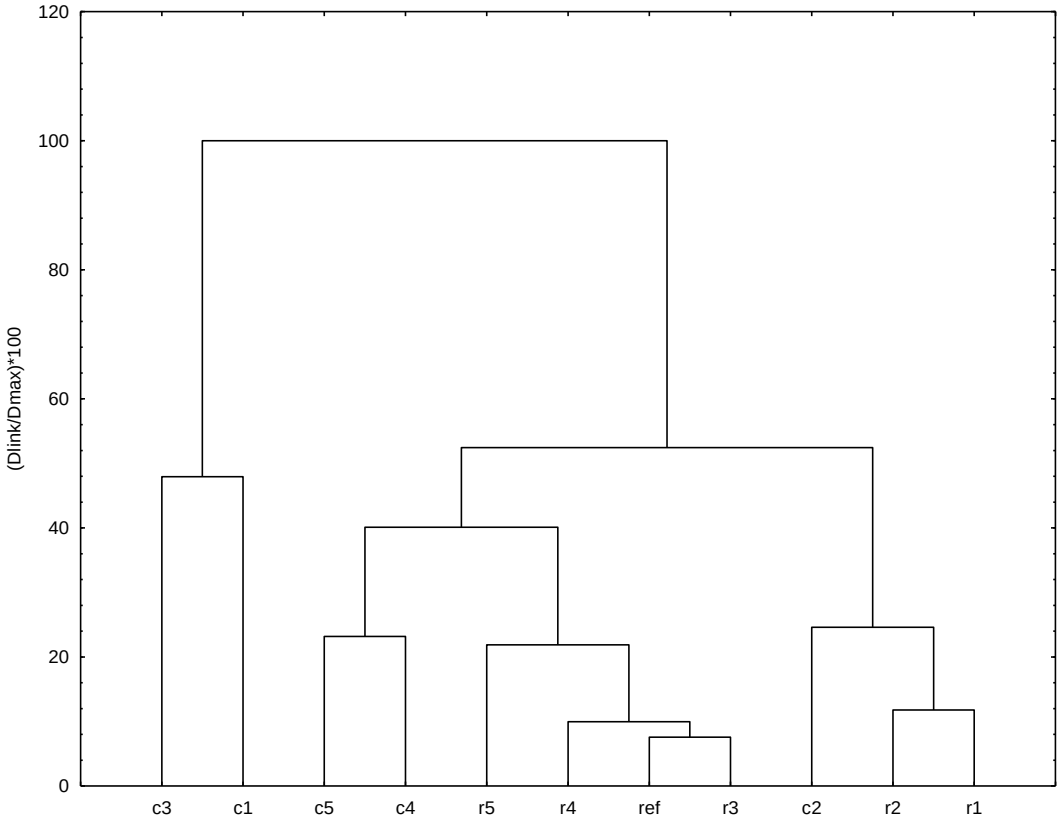

Figure 4. Similarity of accumulated PAHs distribution between resident (r1-r5) and caged (c1-c5) mussels *Mytilus galloprovincialis* from 5 investigated sites in Rijeka Bay. (1,5 column fitted figure)

During the transplantation period, caged mussels at S1 accumulated the lowest fraction (36%) and mussels at S3 next to the highest fraction (63%) of PAH amount found in resident mussels. Therefore, similarity/dissimilarity of PAH content between caged and resident mussels from the same site could not be related to the amount of accumulated PAHs as observed by Seraphim et al. (2011) and Marigómez et al.(2013). In comparison to r1 and r3, c1 and c3 samples are characterized by the absence of BbF, that suggests slow uptake of HMW PAHs by caged mussels in specific environmental conditions. Besides the differences of decontamination kinetics for each individual PAH (Rantamaki, 1997) and mussel adaptation capacity, PAH pattern differences between resident and caged mussels could be explained by the variability of sources and related input dynamics linked to either site-specific irregular activities (S1-shipyard) or freshwater inflow (S3-estuary). .

3.2. Biological response

Biomarkers were initially analysed separately (Fig5) and statistically significant differences were determined between referent and investigated sites for resident and caged mussels as well as between caged mussels before and after transplantation to particular investigated site. Modulations of biological responses were detected in both resident and caged mussels at all investigated sites indicating the existence of a chemical pressure in explored area.

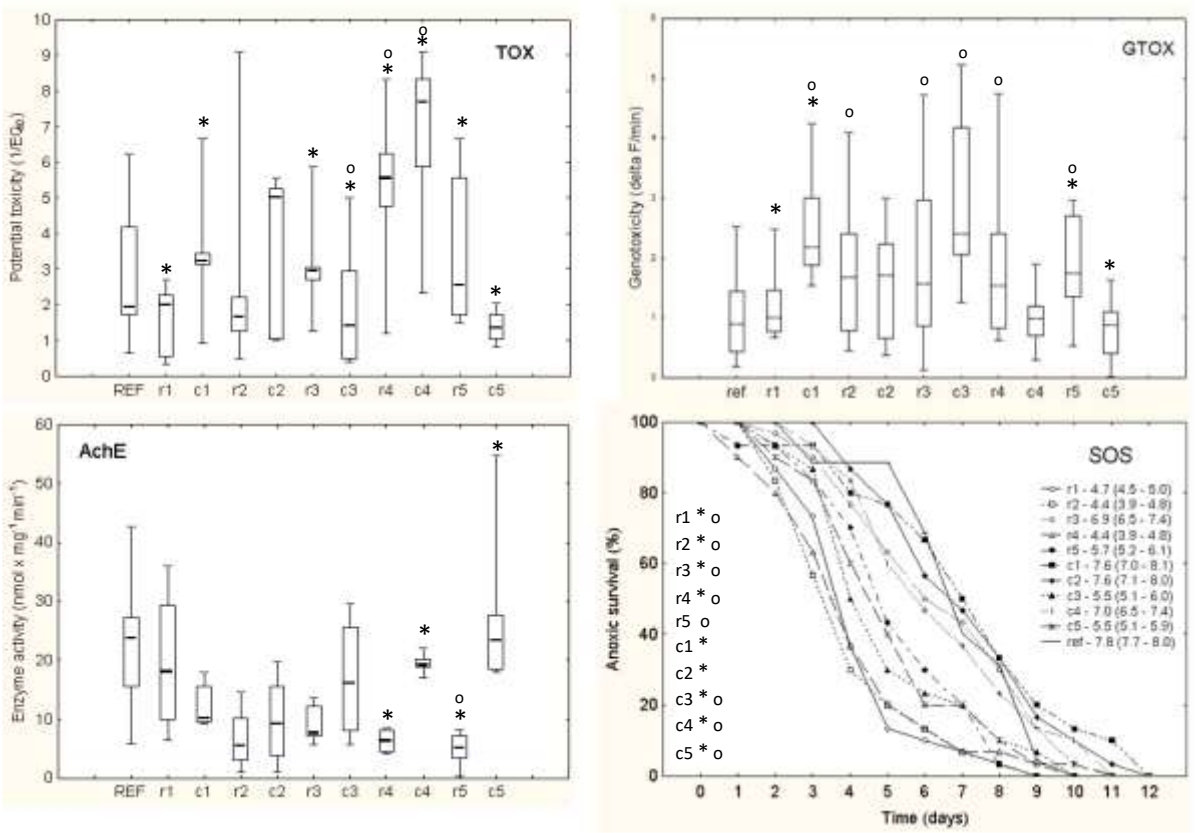

Figure 5. Biological response of resident (r1-r5) and caged (c1-c5) mussels *Mytilus galloprovincialis* to environmental conditions at referent site (ref) and 5 sites in Rijeka bay (TOX – potential toxicity of biological fluids, GTOX- 1/DNA integrity in gills cell, AChE- acethylcholine esterase activity in gills, SOS – "stress-on-stress").p <0.05 *vs* ref (o), p <0.05 r_i *vs* c_i (*) . (2 columns fitted figure)

In resident mussels, the median potential toxicity ranged from 1.7 (r2) to 5.6 (r4), while for caged mussels the median values were from 1.3 (c5) to 7.7 (c4) It corresponded well to previous findings for the Rijeka bay area (Bihari et al. 2007). Toxicity, significantly higher than in referent mussels, was determined in resident and caged mussels collected at S4.

Sampling site S4 was in a part of Rijeka suburb situated in a closed bay with limited water exchange (Degobbis, 1981) under the influence of nearby industry (oil-refinery, charcoal transport) and inflow of freshwater from urban and agricultural area (Frančišković_Bilinski, 2013, Fafanđel et al., 2015). The potential toxicity of mussel biological extracts could not be related to total PAH content suggesting that PAHs were not the dominant toxic pollutants accumulated in the mussel tissue.

At three sampling sites (S1, S2, S4) potential toxicity of caged mussels' biological fluids increased and after caging period was higher than potential toxicity of resident mussels' at the particular site. It indicated rapid accumulation of toxic compounds (including PAHs) in caged mussels during the transplantation period and the presence of elimination mechanisms in resident mussels. When compared to the reference, only mussels at S4, both caged and resident expressed statistically higher potential toxicity, thus, showing similar capacity to detect affected environment.

As expected, DNA integrity in resident mussels from S2, S3, S4 and S5 was significantly lower than DNA integrity in referent mussels. However, after the transplantation period the decrease in DNA integrity was observed only in caged mussels from S1 and S3. In spite higher inter-individual differences in resident than in caged mussels, chronic exposure to contaminated environment had greater impact on DNA integrity resulting in better sensitivity of resident mussels to express genotoxic effect.

There was no overall correlation between DNA integrity and total PAH content or between DNA integrity and genotoxic potential expressed as benzo(a)pyrene equivalents (BaPEs). Similar results were previously reported suggesting that genotoxic impact derived not from the PAH parental compounds but rather from their reactive metabolites and/or other DNA-interacting chemicals (Thomas et al. 2007; Fernandez-Tajes et al.; 2010). It is still worth mentioning that referent mussels express the highest DNA integrity while the lowest DNA integrity was determined in the resident mussels containing the highest PAH amount and the highest BaPEs. It is very likely that PAHs are not dominant genotoxins at all sampling sites and other environmental factors affected the mussel DNA integrity. Therefore, low DNA integrity found in resident and caged mussels at S3 characterized by low salinity and low PAH input could be related to intense transcriptional activities associated with mussel' adaptation to brackish waters at the Rječina river mouth (Hamer, 2008). The significant differences in DNA integrity between resident and caged mussels was observed at S1 and S5. At S1 the genotoxic effect was detected in caged but was absent in resident mussels. This higher sensitivity of caged mussels has probably arisen from the modification of gene

expression rhythm as a consequence of adaptation to a more contaminated environment (Venier et al.2006, Banni et al, 2011). On the contrary, in mussels caged at S5 DNA integrity has not changed after transplantation and was higher than in resident mussels suggesting current lack of genotoxic compound input linked to irregular technological processes associated with local activities (shipyard).

The highest median acetylcholine esterase (AChE) activity (25.5 nmol/mg min) was determined at a referent site. It is in the accordance with the previously measured spring activities at the same site (Semenčić 2004) and comparable to activities measured in Adriatic areas not impacted by pesticides (Corsi et al. 2002). The median activities in resident mussels ranged from 5.1 nmol/mg min at S5 to 18 nmol/mg min at S1while the median AChE activities in caged mussels were from 9.3 nmol/mg min at S2 to 23.3 mnol/mg min at S5. The significant difference in enzyme activity between referent and resident mussels was determined at S5. Although the change of AChE activity in caged mussels during a 30 days period did not express statistical significance, transplanted organisms showed decrease in enzyme activity at all sampling sites. Statistically significant difference of AChE activity between caged and resident mussels was found at S1 and S5. Lower activity in caged mussels could be linked to seasonal/irregular activities, e.g. heavy metals input from the shipyard (S1) while higher activity indicate the absence of inhibiting agents during the transplantation period due to or dilution by intense freshwater inflow (S5). Finally, although caged mussels displayed responsiveness of AChE to new environment, only AChE activity in resident mussels could discriminate an environment impacted by AchE-inhbiting pollutants. In addition, the significant correlation between AChE inhibition and genotoxic effect was determined only in resident mussels ($r = 0.94$, $p<0.05$). It is not surprising since many environmental pollutants have anti-cholinesterase as well as genotoxic activity. In resident mussels where biological effects are related to the chronic exposure the level of particular response is set by adaptation/elimination mechanisms to some optimal value. In contrast, caged mussels when confronted to the specific pollutants present in a new environment displayed acute response.

The “stress-on-stress” survival test revealed stressed conditions at S3, S4 and S5 in both resident and caged mussels. However, at S1 and S2 reduced survival time was detected only in resident mussels designating them as more sensitive. Discrepancy in physiological status between resident and mussels caged for 4 weeks were previously reported for Venice lagoon (Pampanin et al. 2005) as well as for mussels from Galician coast (Marigómez et al., 2013) where caged mussels appeared to be less resistant, than native mussels. It is very likely

that lack of change of mussel's physiological status after transplantation at S1 and S2 reflected current absence of environmental stressors.

To reach a general agreement about sensitivity of biological response in resident *vs.* caged mussels a simple table (Table 4) was created. It presents sensitivity of each biomarker to detect the biological effect (express value statistically higher than referent for each investigated sampling site). At sampling site S3 equal sensitivity was noted. At sampling sites S2, S4 and S5 resident mussels were more responsive. At sampling site S1 sensitivity of biological response was biomarker-specific. Biomarker-specific sensitivity was already observed by Marigómez et al. (2013) who reported that several biomarkers exhibited significant differences between resident and caged mussels and stressed the importance of multi-biomarker approach. It seems that the sensitivity did not originate only from the mussels themselves but also from the site-specific conditions.

Table 4. Biological response sensitivity of resident and caged mussels at five investigated sites in Rijeka Bay.

Biological response	Sampling sites				
	S1	S2	S3	S4	S5
Potential toxicity	-/-	-/-	-/-	R/C	-/-
DNA integrity	-/C	R/-	R/C	R/-	R/-
AChE activity	-/-	-/-	-/-	-/-	R/-
"Stress-on-stress"	R/-	R/-	R/C	R/C	R/C

"R/-" - biological response was detected only in resident mussels (significantly differ from reference)
"-/C" -biological response was detected only in caged mussels (significantly differ from reference)
"-/-"- biological response was not detected (does not significantly differ from reference) neither in resident nor in caged mussels.
"R/C" - biological response was detected (significantly differs from reference) both in resident and caged mussels.

For both resident and caged mussels no correlation has been found between any of measured biological effects. This variability of biological response patterns confirms the importance of the use of several biomarkers since their combination would provide invaluable information beyond that given by individual biomarker. To quantify multi-biomarker effect the integrated biomarker response index (IBR) was calculated. It took into account the level

of each investigated effect, and was presented as a star-plot (Fig. 7). Based on cumulative effect integrated in IBR for each investigated site, caged mussels have identified S1 and S2 as the most affected sampling sites while resident mussels have identified sampling sites S4 and S5.

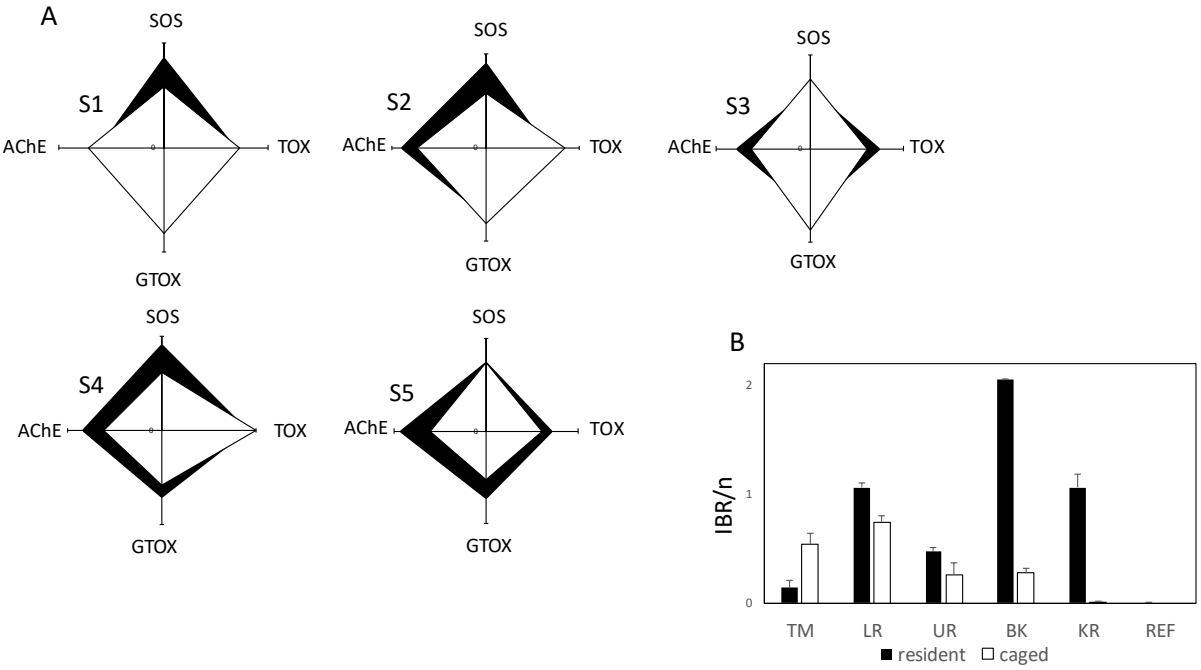

Figure 6. (A) IBR of biological effects (TOX, GTOX, AChE, SOS) presented as star-plot for each sampling site, (B) IBR index calculated for 4 biological effects (■– resident mussels, □- caged mussels) (2 columns fitted figure)

It is very likely that the caged mussels, when compared to resident mussel at S1 lack the adaptation capacity and therefore their transplantation to the new environment containing increased level of contaminants (including PAHs) caused greater response. Moreover, apparent difference of biological response between resident and caged mussels at S4 (TOX, AChE, SOS) and S5 (TOX, GTOX, AChE) due to the low level of effect in caged mussels could be related to the discontinuous presence of environmental stressors confirming low sensitivity of caged mussels to time-point pollution.

3.3. Integrating PAH content and biological response

When analyzing field data – often no distinguishable correlations between biomarkers and tissue pollutant concentrations can be observed when evaluated individually by linear regression or when subjected to canonical correlation analysis. In search of correlation between PAH accumulation and biological response in mussels exposed to contaminated environment principal component analyses (PCA) of biological responses in resident and caged mussels was performed (Fig6). The plot of scores shows the position of investigated sites (r1-r5, c1-c5) in the ordination plane of the first two principal components (PC1 and PC2), projection of the variables (TOX, GTOX, AChE, SOS) on the PC plane present their contribution to principal components and the size of the bubble represent the PAH concentration. The sequence of eigenvalues was similar from one analysis to the other: the two sets of variables have a strong first direction of inertia (>50%). All the variables determined in resident mussels were significantly negatively correlated to PC1 that explains the 61.4 % of the total variance, and the sampling sites could be classified according to their PC1 score: referent site as the site with the minimal impact and r4 with the major impact. There was no correlation between PC1 and the accumulated PAHs content. This result reflected the cumulative effect of long-term exposure of resident mussels to mixture of environmental factors where PAHs were not the dominant component or the resident mussels have adapted (Lacroix et al., 2017) so the PAH effect has not been easily recognized. Finally, the effect of other pollutants (e.g. heavy metals) present at investigated sampling sites (Perić et al., 2012) that could modulate biological response (S4, S5) should not be neglected.

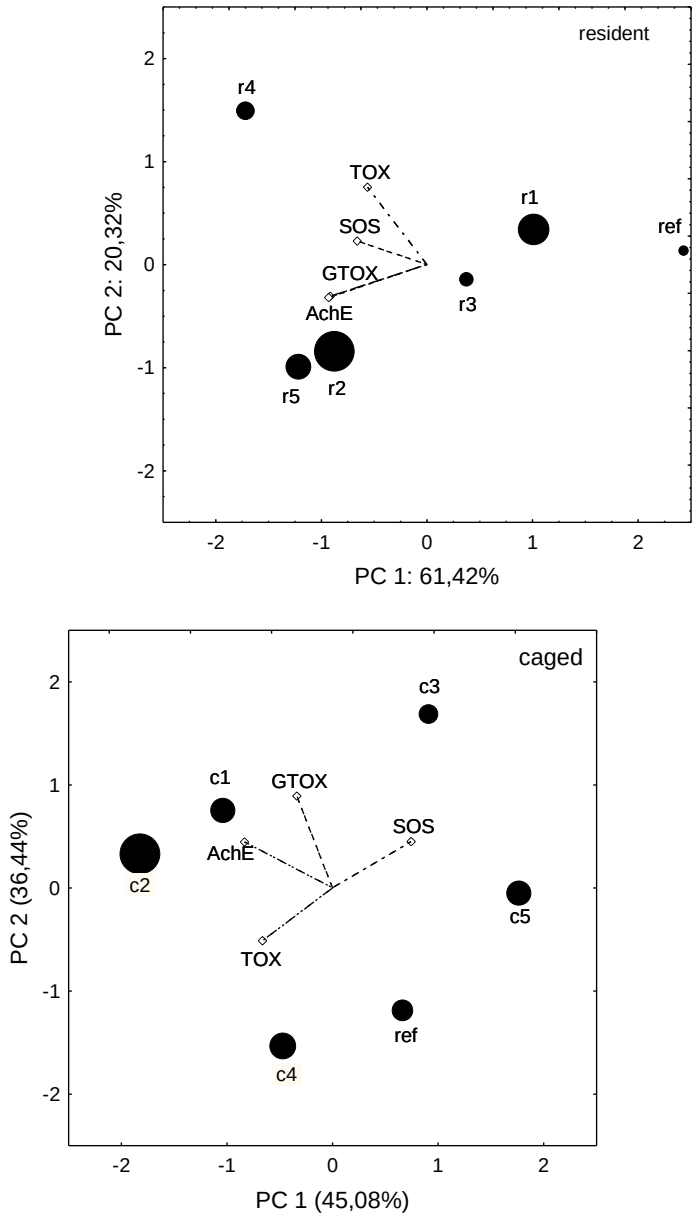

Figure 7. PCA of biological response of resident (r1-r5) and caged (c1-c5) mussels *Mytilus galloprovincialis* to environmental conditions at referent site (ref) and 5 sites in Rijeka Bay (TOX – potential toxicity of biological fluids, GTOX- 1/DNA integrity in gills cell, AChE- acethylcholine esterase activity in gills, SOS – "stress-on-stress"). Superimposed bubble size corresponds to total PAH content. (2 columns fitted figure)

The results of the PC analysis of caged mussels showed that PC1 was negatively correlated to acetylcholine esterase activity, positively correlated to "stress-on-stress" while PC2 was positively correlated to genotoxic effect. Therefore, the position of the sites with minimal impact (ref) would be in the lower right quadrant and the sites with the highest impact (c1, c2)

in upper left quadrant. The position of the sampling site with the lowest PAH content was in the PC plane area that was characterized by the minimal impact while the sampling site with the highest PAH content (c2) was situated in the quadrant corresponding to the highest impact. However, there was no overall correlation between PAH content and any of the first two principal components. Distinct response times and duration for biomarkers and bioaccumulation are in accordance with previously reported field studies results (Garmendia et al., 2011, Marigómez et al., 2013). This supports currently recommended integration of biomarkers and chemical data (OSPAR, 2013), using integrative indices (Regoli et al., 2014) or multivariate analysis (Turja et al., 2014) for the assessment of environmental disturbance caused by chemical stress in coastal areas.

However, the position of sampling sites based on a set of four investigated mussel biological responses clearly shows the difference between PCA results for resident and caged mussels. The observed distinction gave an indication of the different points of view between resident and caged mussels originating from the same biomarkers set. To display differences between resident and caged mussels after integration of all observations (PAH level and biological responses) multiple factor analysis (MFA) was applied. The first step of the analysis that included PCA of each data set revealed that the first eigenvalue of the separate PCA of variables in resident mussels was slightly higher (8.0) than the one of PCA of variables in caged mussels (7.0). Thus, normalization of data allowed balancing and avoided the domination of variables from resident mussels in the construction of the first axis. Taking this way the groups of variables equally into the account, multiple factor analysis (MFA) of chemical and biological data derived an integrated picture of the investigated sampling sites and the relationship between variable groups used to describe them (Fig 8). MFA provided global analysis as a balanced representation of each sampling site according to both resident and caged mussel data set.

Projecting the data set of caged and resident mussels onto the global analysis provided partial representations map that revealed the level of agreement between resident and caged mussels. Partial representations of same sampling site are even closer that they do express the same information. It showed a well-defined partition of sampling sites particularly along the first PC. The first axis is highly correlated to PAH content in resident and caged mussels.

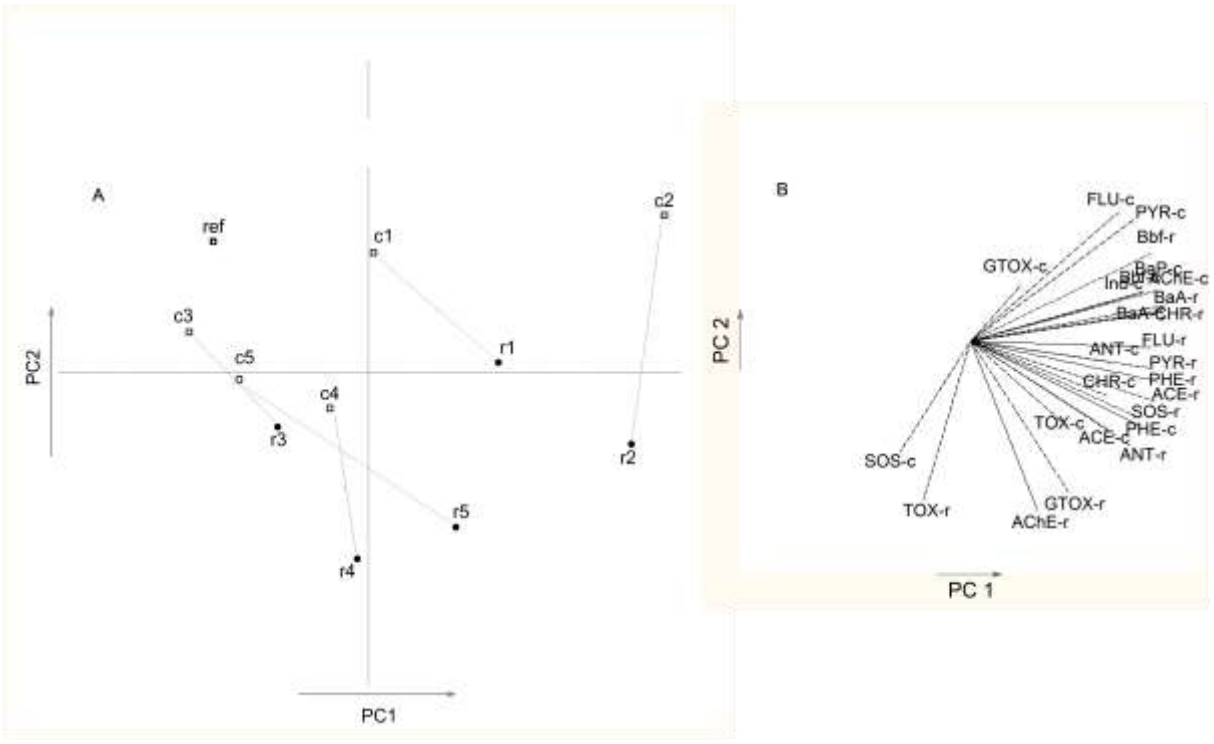

Figure 8. Multiple factor analysis (MFA) relationship between the studies: A) Projection of the resident (●) and caged (□) mussel onto the global analysis. A line segment links the position of the resident/caged mussel to its global position (o). B) Chemical and biochemical descriptors represented as eigenvectors of correlation matrix in the first two planes of PCA-global analysis. (2 columns fitted figure)

Therefore, when considering tissue contaminant load and the observed partition of sampling sites along PC1 caged mussels showed higher discrimination capacity then resident mussels. The partition on axe 1 between resident and caged mussels for S1, S3 and S5 was related to either irregular contaminant input (S1, S5-shipyard) or freshwater inflow (S3-estuary) since caged mussels were limited to recent input and failed to detect contaminant input at sites with irregular activities.

The second axis was correlated to biological response (TOX, GTOX, and AChE in resident mussels only confirming that resident and caged mussels did not respond with comparable patterns to the same environment. This absence of correlation of biological response between resident and caged mussels indicated the presence of different adaptation

traits of the indigenous mussel population when compared to caged mussels. It is in accordance to different sample sites ranking obtained by IBR. Moreover, eigenvectors of correlation matrix of chemical and biological descriptors indicated no correlation between bioaccumulation and biological response in resident mussels. Distinct response between bioaccumulation and biomarkers was already observed (Garmendia et al., 2011, Marigómez et al, 2013) and attributed to different response times. Based on their results in Galicia, Marigómez et al. (2013) concluded that the suite of biomarkers was more sensitive after caging (short-term response) whereas tissue pollutant concentrations were more sensitive in native mussels (long-term response). Our case confirmed that long-term exposure of resident mussels were more reliable for overall contaminant bioaccumulation assessment. At the same time due to the adaptation mechanisms resident mussels expressed reduced discrimination capacity and were prone to false negative results (absence of HMW PAHs at S2). It put forward the importance of mussel origin to reflect particular environmental conditions (Pampanin et al., 2005, Viarengo et al., 2007). Indeed, when considering the level of contaminant bioaccumulation, discrimination capacity was higher in caged mussels but resident mussels were more sensitive than caged mussels in expressing biological effects.

Therefore, it was important that MFA integrated chemical and biological data and derived an integrated picture. By the comparison between the representations of partial individuals, the opposition between each sampling site and referent site was much bigger from a resident mussel's point of view than from a caged mussel's point of view. It means that when analysed together tissue PAH concentrations and multiple biological response, resident mussels were more powerful for the detection of environmental pressure in the investigated areas. It can be concluded that despite the observed shortcomings, when contaminant bioaccumulation and biological endpoints were integrated, resident mussels could provide reliable results. Future use of resident mussels in environmental risk assessment that combine chemical and biological measurements by multivarate analysis (Turja et al., 2014), complex modelling tools such as weight of evidence (WOE) (Regoli et al., 2014) or integrated assessment framework (Martinez-Gomez, C. et al., 2017) is supported for long-term monitoring.

4. Conclusion

Comparison of resident and caged mussels' results revealed site-specific differences for each investigated parameter. The correlation between total PAH content in resident and caged mussels was found at majority of sampling sites. The absence of HMW PAHs in resident mussels at S2 indicated limitation of indigenous population and higher sensitivity of caged mussels to reflect PAH load in the environment. There was no correlation of biological response between resident and caged mussels. Resident and caged musssels expressed different biological response sensitivity's patterns and different sample site ranking according to the cumulative biological effect. Integration of all biological effects confirmed low sensitivity of caged mussels to time-point pollution.

For detection of environmental pressure, it was important to combine multiple biomarkers as well as to integrate contaminant content and biological response due to the limitation of individual investigated parameter. MFA was successfully applied in an ecotoxicological study enabling comparison of complex response in two investigated monitoring systems.The visualization of specific and common structures provided achievement of a comprehensive answer about differences between resident and caged mussel's response. MFA analysis of tissue PAH concentrations together with multiple biological response displayed resident mussels as more powerful for detection of environmental pressure in the investigated area.

The results of this study confirmed resident mussels as reliable bioindicator. Their use as an appropriate and less costly approach for monitoring the effect of pollution is recommended. Yet, caged mussels are more suitable in case of areas with chronic contaminant input, but should be avoided at sites characterised by irregular contaminant input.

This investigation contributes to comprehension of advantages and limitations connected to the "resident or caged mussels" dilemma before designing a monitoring strategy as well as during the interpretation of acquired information and provision of data that would be beneficial to its final users.

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References

Abdi H, Valentin D. Multiple correspondence analysis. In: Salkind NJ, ed. Encyclopedia of Measurement and Statistics. Thousand Oaks, CA: Sage; 2007, 651 – 657.

Abdi, H., Williams, L. J., Valentin, D. Multiple factor analysis: principal component analysis for multitable and multiblock data sets. WIREs Comp Stat 2013, 5: 149-179.

Banni, M., Negri, A., Mignone, F., Boussetta, H., Viarengo, A., Pondero, F. 2011.Gene expression rhythms in the mussel *Mytilus galloprovincialis* (Lam.) across an annual cycle. PloS One 6, e18904

Barhoumi, B., Le Menach, K., Clerandeau, C., Ben Ameur, W., Budzinski, H., Driss, M.R., Cachot, J. 2014. Assessment of pollution in the Bizerte lagoon (Tunisia) by the combined use of chemical and biochemical markers in mussel, *Mytilus galloprovincialis*. Marine Pollution Bulletin 84: 379-390

Batel, R., Jakšić, Ž., Bihari, N., Hamer, B., Fafanđel, M., Chauvin, C., Schrőder, H.C., Műller, W.E.G.1999. A microplate assay for DNA damage determination (Fast Micromethod) in Cell Suspensions and Solid Tissues. Analytical Biochemistry, 270,195-200

Baumard, P., Budzinski, H., Garrigues, P., Narbonne, J.F., Burgeot, T., Michel, X., Bellocq, J.1999. Polycyclic aromatic hydrocarbon (PAH) burden of mussel (*Mytilus* sp.) in different marine environments in relation with sediment PAH contamination, and bioavailability. Marine Environmental Research, 47, 415-439

Beliaeff, B., Burgeot, T. 2002. Integrated biomarker response: a useful tool for ecological risk assessment. Environmental Toxicology and Chemistry. 21, 1316-1322

Bihari, N., Fafanđel, M., Jakšić, Ž., Pleše, B., Batel, R. 2005. Spatial distribution of DNA integrity in mussels, *Mytilus galloprovincialis,* from the Adriatic Sea, Croatia. Bulletin of Environmental Contamination and Toxicology ,75, 845-850

Bihari, N., Fafanđel, M., Hamer, B., Kralj-Bilen, B. 2006. PAH content and genotoxicity of coasteal 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 Toxicology, 52, 379-387

Bocquene, G., Galgani, F. 1998. Biological effects of contaminants: Cholinesterase inhibition by organophosphate and carbamate compounds. ICES Tech Marine Environmental Science, 22, 1-12

Broeg, K., Lehtonen, K.K. 2006. Indices for the assessment of environmental pollution of the Baltic Sea coasts: Integrated assessment of multi-biomarker approach. Marine Pollution Bulletin 53, 508-522

Corsi, I., Mariottini, M., Menchi, V., Sensini, C., Balocchi, C., Focardi S. 2002. Monitoring a marine coastal area: use of *Mytilus galloprovincialis* and *Mullus barbatus* as bioindicators. P.S.Z.N. Marine Ecology, 23 (Suppl 1), 138-153

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 in eastern Mediterranean (Greece). Environmental Pollution, 119, 141-149

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

Devier, M-H., Augagneur, S., Budzinski, H., Le Menach, K., Mora, P., Narbonne, J-P., Garrigues, J.P. 2005. One-year monitoring survey of organic compounds (PAHs, PCBs, TBT), heavy metals and biomarkers in blue mussels from the Arachon Bay, France. Journal of Environmental Monitoring 7, 224-240

DeZwaan, 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

Douben, P.E.T. 2003. *PAHs: An Ecotoxicological Perspective*. Southern Gate,Chichester,West Sussex, England; John Wiley and Sons Ltd,

Ellman, G.L., Courtney, K.O., Andres, V., Featherston, R.M. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology, 7, 88-95

Fabbri, D., Baravelli, V, Giannotti, K., Donnini, F., Fabbri, E. 2006. Bioaccumulation of cyclopenta[*cd*]pyrene and benzo[*ghi*]fluoranthene by mussels transplanted in a coastal lagoon. Chemosphere 64, 1083–1092

Fafanđel, M., Piljagić, J., Smodlaka Tanković , M., travizi, A., Bihari, N. 2015. Nutrients vs Toxicity in sediments: a case study of two semi-closed basins in Rijeka Bay, Croatia. Fresenius Environmental Bulletin 24,288-2896

Fernandez, B., Albentosa, M., Viñas, L., Franco, A., Gonzales, J.J., Campillo, J.A. 2010. Integrated assessment of water quality of the Costa da Morte (Galicia, Spain) by means of mussel chemical, biochemical and physiological parameters. Ecotoxicology,19,735-750

Fernandez-Tajes, J., Florez, F., Pereira, S., Rabade, T., Laffon, B., Mendez, J. 2010. Use of three bivalve species for biomonitoring a polluted estuarine environment. Environmental Monitoring and Assessment 177, 289-300

Frančišković-Bilinski S, Cuculić V, Bilinski H, Häusler H, Stadler P 2013. Geochemical and stable isotopic variability within two rivers rising under the same mountain, but belonging to two distant watersheds. Chemie der Erde - Geochemistry 73: 293-308.

Galgani, F., Martinez-Gomez, C., Giovanardi F., Romanell, G., Caixach, J., Cento, A., Scarpato, A., BenBrahim, S., Messaudi, S., Deudero, S., Boulahdid, M., Benedicto, J., Andral, B. 2011. Assessment of polycyclic aromatic hydrocarbon concentration in mussels

(*Mytilus galloprovincialis*) from the Western basin of the Mediterranean Sea. Environmental Monitoring and Assessment 172, 301-317

Galloway, T.S., Sanger, R.C., Smith, K.L., Fillmann, G., Readman, J.W., Ford, T.E., Depledge, M.H. 2002. Rapid Assesment of marine pollution using multiple biomarkers and chemical bioassays. Environmental Science and Technology, 36, 2219-2226

Garmendia, I., Soto, M., Ortiz-Zargoitia, M., Orbea, A., Cajaraville, M.P., Marigómez, I. 2011. Application of a battery of biomarkers in mussel digestive gland to assess long-term effects of the Prestige oil spill in Galicia and Bay of Biscay: Correlation and multivariate analysis. Journal of Environmental Monitoring 13, 933-942

Gomiero, A., Volpato, E., Nasci, C., Perra, G., Viarengo, A., Dagnino, A, Spagnolo, A., Fabi, G. 2015. Use of multiple cell and tissue-level biomarkers in mussels collected along two gas fields in the northern Adriatic Sea as a tool for long-term environmental monitorino. Marine Pollution Bulletin 93, 228-44

Guilhermino, T.S., Barros, B., Silva, M.C., Soares, A.M. 1998. Should the use of inhibition of cholinesterases as a specific biomarker for organophosphate and carbamate pesticides be questioned? Biomarkers, 3, 157-163

Hamer; B., Jakšić, Ž., Pavičić-Hamer, D., Perić, L., Medaković, D., Ivanković, D., Pavičić, J., Zilberberg, C., Schröder, H.C., Müller, W.E.G., Smodlaka, N., Batel, R. 2008. Effect of hypoosmotic stress by low salinity acclimation of Mediterranean mussels *Mytilus galloprovincialis* on biological parameters used for pollution assessment. Aquatic Toxicology 89, 137–151

Hunt, C.D., Slone, E. 2010. Long-term monitoring using resident and caged mussels in Boston Harbour yield similar spatial and temporal trends in chemical contamination. Marine Environmental Research 70,343-357

Jett, D.A., Navoa, R.V., Lyons, M.A, Jr. 1999. Additive inhibitory action of chlorpyrifos and polycyclic aromatic hydrocarbons on acetylcholinesterase activity *in vitro*. Toxicology Letters 105, 223–229.

Lacroix C, Richard, G., Seguineau, C., Guyomarch, J., Moraga, D, Auffret, M. 2015.Active and passive biomonitoring suggest metabolic adaptation in blue mussels (Mytilus spp.) chronically exposed to a moderate contamination in Brest harbour (France). Aquatic Toxicology 162, 126-137

Lacroix, C., Duveilbourg, E., Guillou, N., Guyomarch, J., Bassoulet, C., Moraga, D., Chapalain, G., Auffret, M. 2017. Seasonal monitpring of blue mussel (*Mytilus* spp.) populations in harbour area: A focus on responses to environmental factors and chronic contamination. Marine Environmental Research; doi.org/10.1016/j.marenvres.2017.04.008

Lehtonen, K.K., Kankaanpää, H., Leiniö, S., Sipiä, V.O., Pflugmacher, S., Sandberg-Kilpi, E. 2003. Accumulation of nodularin-like compounds from cyanobacterium *Nodularia spumigena* and changes in acetylcholinesterase activity in clam *Macoma balthica* during short-term laboratory exposure. Aquatic Toxicology, 64, 408-421

Lehtonen, K.K., Leiniö,S., Schneider, R., Leivouri, M. 2006. Biomarkers of pollution effects in the bivalves *Mytilus edulis* and *Macoma balthica* collected from the Southern coast of Finland (Baltic Sea). Marine Ecology Progress Series 322, 155-168

Lehtonen,K.K., Turja, R. , Budzinski,H., Devier, M-H. 2016. An integrated chemical-biological study using caged mussels (*Mytilus trossulus*) along a pollution gradient in the Archipelago Sea (SW Finland, Baltic Sea. Marine Environmental Research 119, 207-221

Lowry, D.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, 193, 265-275

Marigómez, I., Zorita, I., Izagirre, U., Ortiz-Zarrgoitia, M., Navarro, P., Etxebarria, N., Orbea, A., Soto, M., Cajaraville, M.P. 2013. Combined use of native and caged mussels to assess biological effects of pollution through the integrative biomarker approach. Aquatic Toxicology 136-137, 32-48

Martínez-Gómez, C., Fernández, B., Robinson, C.D., Campillo, J.A., León, V.M., Benedicto J., Hylland, K., Vethaak, A.D. 2017. Assessing environmental quality status by integrating chemical and biological effect data: The Cartagena coastal zone as a case Marine Environmental Research 124, 106-117

Michel, X., Narbonne, J.-F., Mora, P., Daubeze, M,, Ribera, D., Lafaurie, M., Budzinski H., Garrigues, P.H. 2000. Biochemical indicators of pollution in coastal ecosystems: Experience of the Groupe Interface Chimie-Biologie des Ecosystemes Marins (GICBEM) In: L. Lagadic, T. Caquet, J-C. Amiard, F. Ramade (Eds.), Use of Biomarkers for Environmental Quality Asesment (pp 9-32). New York: Science Publishers

Neilson, A.H. 1998. PAHs and related compound biology. Heidelberg, New York, Berlin: Springer Verlag

Nigro, M., Falleni, A., Del Barga, I., Scarcelli, V., Lucchesi, P., Regoli, F., Frenzilli, G. 2006. Cellular biomarkers for monitoring estuarine environments: Transplanted versus native mussels. Aquatic Toxicology 77, 339-347

Nisbet, C., LaGoy, P. 1992. Toxic equivalency factors (TEFs) for polycyclic aromatic hydrocarbons (PAHs). Regulatory Toxicol ogy and Pharmacology,16, 290-300

OSPAR , 2013. Levels and trends in marine contaminants and their biological effects – CEMP Assessment report 2012. OSPAR Commisssion, 596/2013

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

Payne, J.R., Driskell, W.B., Short, J.W., Larsen, M.L. 2008. Long term monitoring for oil in the Exxon Valdez spill region. Marine Pollution Bulletin 56, 2067-2081

Perić, L., Fafanđel, M., Glad, M., Bihari, N. (2012) Heavy metals concentration and metallothionein content in resident and caged mussels *Mytilus galloprovincialis* from Rijeka Bay, Croatia. Fresenius Environmental Bulletin 21, 2785-2794

Piccardo, M.T., Coradeghini, R., Valerio, F. 2001. Polycyclic aromatic hydrocarbon pollution in native and caged mussels. Marine Pollution Bulletin 42, 951-956

Rantamaki, P. 1997. Release and retention of selected polycyclic aromatic hydrocarbons (PAH) and their derivatives by the common mussel (*Mytilus edulis*) in the brackish water of the Baltic Sea. Chemosphere 35,487-502

Regoli, F., Pellegrini, D., Cicero, A.M., Nigro, M., Benedetti, M., Gorbi, S., Fattorini, D., D'Erico, G., Di Carlo. M., Nardi, A., Gaion, A., Scuderi, A., Giuliani, S., Romanelli, G., Berto, D., Trabucco, Benedetta, Guidi, P., Bernardeschi, M., Scarcielli, V., Frenzilli, G. 2014. A multidisciplinary weight of evidence approach for environmental risk assessment at the Costa Concordia wreck: Integrative indices from mussel watch. Marine Environmental research 96, 92-104

Semenčić, L.2004. Use of biomarker in mussel (*Mytilus galloprovincialis* Lam.) for estimation of coastal pollution. University of Zagreb, Master Thesis (in Croatian)

Serafim, A., Lopes, B., Company, R., Cravo, A., Gomes, T., Sousa, V., Bebbiano, M.J. 2011.A multi-biomarker approach in cross-transplanted mussels *Mytilus galloprovincialis*. Ecotoxicology 20(8):1959-74

Stijve, T., Hischenhuber, C. 1987. Simplified determination of benzo(a)pyrene and other polycyclic aromatic hydrocarbons in various food material by HPLC and TLC. Deutsche Lebensmittel-Rundschau, 9, 276-282

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, 22C, 147-152

Thomas, R.E., Lindeberg, M., .Harris, P.M., Rice, S.D. 2007. Induction of DNA strand breaks in the mussel (*Mytilus trossulus*) and clam (*Protothaca staminea*) following chronic field exposure to polycyclic aromatic hydrocarbons from the Exxon Valdez spill. Marine Pollution Bulletin 54, 726-732

Traven, L., Žaja, R., Lončar, J., Smital, T., Mićović, V. 2008. Cyp1A1 induction potential and the concentration of priority pollutants in marine sediment samples – *In vitro* evaluation using the PLHC-1 fish hepatoma cell line. Toxicology In vitro, 22(6), 1648-1656

Turja, R., Hoher, N., Snoeijs, P., Baršiene, J., Butrimavičiene, L., Kuznetsova, T., Kholodkevich, S.V., Devier, M.-H., Budzinski, H., Lehtonen, K.K. 2014. A multibiomarker approach to the assessment of pollution impacts in two Baltic Sea coastal areas in Sweden using caged mussels. Science of the Total Environment 473-474, 398-409

US EPA. 1984. Health effect assessment for polycyclic aromatic hydrocarbon (PAH).EPA 549/1-86-013.Cincinnati: Environmental Criteria and Assessment Office

Venier, P., De Pittà, C., Pallavicini, A., Marsano, F., Varotto, L., Romualdi, C., Dondero, F., Viarengo, A., Lanfranchi, G. 2006. Development of mussel mRNA profiling: Can gene expression trends reveal coastal water pollution? Mutation Research 602(1-2):121-34.

Viarengo, A., Dondero, F., Panpanin, D.M., Fabbri, R., Poggi, E., Malizia, M., Bolognesi, C., Perrone, E., Gollo, E., Cossa, G.P. 2007. A biomonitoring study assessing the residual biological effect of pollution caused by the HAVEN wreck on marine organsms in the Ligurian Sea (Italy). Archives of Environmental Contamination and Toxicology 53, 607-616