Pesticide residues in honeybees, honey and bee pollen by LC–MS/MS screening: Reported death incidents in honeybees

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Organic pollution and its effects in the marine mussel *Mytilus galloprovincialis* in Eastern Mediterranean coasts

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ABSTRACT

Persistent chemicals and emerging pollutants are continuously detected in marine waters and biota. Out of these, polycyclic aromatic hydrocarbons (PAHs) and organochlorine pesticides (OCs) are significant contaminants with decades of presence in the marine environment. The Mediterranean Sea is an ecosystem directly affected by a variety of anthropogenic activities including industry, municipal, touristic, commercial and agricultural. The Mediterranean mussel (*Mytilus galloprovincialis*) is a filter feeder, which presents wide distribution. In this regard, the specific organism was used as a biological indicator for the monitoring and evaluation of pollution in the studied areas with focus on the mentioned chemical groups. Pristine Turkish sites with minimum effect from anthropogenic activities, in contrast with Greek sites which were subjected to heavy industrial and shipping activity, were selected. A gas chromatographic tandem mass spectrometric method (GC–MS/MS) was developed and validated to monitor 34 compounds (16 EPA priority PAHs and 18 OCs). Analyses of mussel samples in 2011 from sites with the limited anthropogenic pollution shores have shown the occurrence of 11 pollutants (6 PAHs, 5 OCs), while in the samples from sites with intensive activity and expected pollution, 12 PAHs and 6 OCs were detected. Biochemical and biological responses studied only in mussels samples from the sites with the highest contamination showed a situation that was under strong seasonal influence. The intensity of the response was also influenced by deployment duration. Noteworthy correlations were detected among biochemical/biological effects and between mussel body burden and these effects. Continuous monitoring of priority pollutants of East Mediterranean Sea is vital both for ecological and human risk assessment purposes.

1. Introduction

Environmental contaminants are constantly found in marine waters and biota. Out of these, polycyclic aromatic hydrocarbons (PAHs) are carcinogens or probable carcinogens and they are capable of long term harmful effects in both vertebrates and invertebrates. In the latter, PAHs have been shown to modulate immunological responses (Wootton et al., 2003; Hannam et al., 2010; Croxton et al., 2012), a wide range of enzyme activities (Akcha et al., 2000; Luna-Acosta et al., 2011), protein transcription or expression (Snyder et al., 2001; Hoarau et al., 2006; Xu et al., 2010) as well as to cause DNA lesions (Akcha et al., 2000; Perez-Cadahia et al., 2004; Machella et al., 2005; Giannapas et al., 2012) and cell injury (Aarab et al., 2004; Einsporn and Koehler, 2008). On the other hand, organochlorine pesticides (OCs) constitute a family of persistent, lipophilic compounds that are banned as pesticides since they cause a variety of neurotoxic, hormonal- and immuno-modulating and tumorigenic effects (Androutsopoulos et al., 2013).

Bivalves have been routinely used for environmental monitoring purposes. Mussels in particular are widely distributed in marine waters and are easy to collect. They are filter-feeding and bioaccumulate contaminants (Gielazyn et al., 2003), having a suitable size for biochemical analysis and they are tolerant both to reduced salinity conditions (Wilson et al., 1998) and to a wide range of pollutants. Since mussels are able to bioconcentrate

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various pollutants from their surrounding environment, the use of the organism can provide more information about the presence of the various pollutants than the analysis of the water (Baumard et al., 1999). For these reasons mussels have been used as bioindicators of chemical pollution of coastal waters in the Mussel Watch Programmes (Goldberg, 1975).

The Mediterranean Sea is an ecosystem directly affected by a variety of anthropogenic activities. Biomonitoring in Greek and Turkish coastal waters with the use of the Mediterranean mussel (*Mytilus galloprovincialis*) has been performed by various research groups [indicatively see (Tsangaris et al., 2011; Kucuksezgin et al., 2013)]. In order to proceed from detection of contamination to assessment of its impact detection of effects at subcellular, cellular and organism level is currently proposed (Bellas et al., 2013). In the present study relatively pristine Turkish sites aimed to be of minimum impact from anthropogenic pollution sources-in contrast with Greek sites selected to be of heavy industrial and shipping activity and anticipated high pollution levels. A tiered approach has been followed, focusing on chemical analysis of PAHs and OCs in mussel tissues via the development and validation of a GC–MS/MS methodology. This was accompanied by detection of effects at subcellular, cellular and organism level. The approach of combined methods allows the precise measurement of pollution burden and its correlation to possible biological and toxicological effects on bivalves. In this direction, this integrated approach using transplanted mussels is able to distinguish between sites, seasons and pollution burden, highlighting at the same time the influence of natural factors and other contaminants which affect the biological response.

2. Materials and methods

This section is described in detail in Supplementary Material.

The PAHs and OCs analyzed are depicted in Table 1s. The biomarkers analyzed were Filtration Rate (FR), Neutral Red retention time (NR), Reduced Glutathione levels (GSH), Malondialdehyde levels (MDA), 4-Hydroxy-nonenal levels (4-HNE), Protein Carbonyl levels (PC), Ethoxy-*O*-resorufin Deethylase activity (EROD), Vitellogenin levels (VI).

3. Results and discussion

3.1. Validation of GC–MS/MS method

No certified reference material for the validation of the method was used due to problems of encountering it from sources. However the method was fully validated with all criteria being acceptable. The complete validation results are presented in Supplementary material (Table 4s).

3.1.1. QuEChERS applicability

As regards the QuEChERS procedure that was applied, based on literature reports (Application; Ramalhosa et al., 2009), it must be pointed out that its applicability domain seems rather limited when compared to other cutting edge state of the art methods for the specific measurements, such as microwave assisted extraction combined with Florisil purification step [see (Cortazar et al., 2008) and references therein]. Vorkamp et al. has proposed the use of Soxhlet extraction with distinct purification steps for OCs and PAHs [see (Vorkamp et al., 2010) and references therein]. The latter proved efficient in the determination of analytes minimizing matrix interferences. In this context the usefulness of the presented analytical method can be tolerated in the context of an integrated approach (combined with effect studies) as the one presented in this paper. However we need to stress that the concomitant presence of OCs and PAHs in one analysis without choosing different sample preparation-extraction pathways is an advantage of the presented analytical method, that if optimized can be a valuable tool for analysis. Its optimization can be further pursued according to a recently published work on the effective use of this technique in PAHs analyses in wild grown and commercial mussels (Madureira et al., 2014).

3.2. Contaminants levels – comparison with other studies

Eleven compounds (naphthalene, acenapthylene, acenapthene, phenathrene, benzo(a)anthracene, indeno(1,2,3-cd)perylene, a-BHC, b-BHC, pp-DDD, pp-DDT and pp-DDE) were detected in 8 samples from the Turkish sites. Concentrations in samples above LOQ varied from 20.3 to 34.8 ng g^{-1} dw as (see Table 2s, Supplementary material) with the highest levels attributed to naphthalene. In all sampling spots of 2012 and 2013, in the Greek estuarine coasts detectable levels of the contaminants were exhibited with a clear trend of higher accumulation in the winter season of 2013 for both Megara and Salamina (for results see Tables 1 and 2). As expected the second month of sampling the concentrations of detected contaminants was higher than the respective of 1st month, with the exception of indeno(1,2,3-cd)pyrene for Salamina region. In Salamina fluoranthene, chrysene and pyrene were encountered in concentrations above 100 ng g^{-1} dw. In this regard, PAHs were the dominating contaminants of Salamina and Megara stations. In Volos region OCs were the predominant contaminants with naphthalene being the only PAH detected. Salamina sampling point is regarded as the highest polluted sampling point as it is in the Gulf where the oil refineries of the country are located.

Megara is relatively the most "pristine" site among the examined estuarine points of this study. Despite that, PAHs were detected due to its proximity to Attiki prefecture (metropolitan region of Athens) however in lower concentrations and number of analytes than in Salamina region. Volos harbor where the sampling point was established was dominated by OCs. The only detected PAH was naphthalene.

When comparing the sum of OCs (ΣOCs) for Salamina and Megara regions the summer of 2013 we observed that ΣOCs is almost twofold times higher the 1st month than in the second. The latter can be attributed in both cases to point sources of pollution which resulted in increased concentrations of OCs in the first sampling period. As regards the sum of PAHs (ΣPAHs) during the 2013 summer we observed a substantial decrease in concentrations of PAHs the 2nd period only for Salamina. In Megara the ΣPAHs at the second sampling period was almost 15-fold higher than the respective sum in the first period. In this view we observed that for Salamina region the ΣPAHs is higher in both summer sampling periods compared to the respective periods of 2012. The latter is associated with the high concentration observed in winter 2013 which implies that the ΣPAHs after its peak concentration in the 2nd sampling period of winter 2013 it gradually decreases reaching a low level of 47.9 ng g^{-1} dw. For OCs the picture was different. Starting from 2012, OCs were not detected in Salamina, however in summer 2013 low but not negligible levels of OCs were detected. Despite that, it has to be stressed that this concentration is decreased in the second sampling period with the exception of pp-DDE.

On the whole ΣPAHs and ΣOCs ranged accordingly: in Greek samples ΣPAHs and ΣOCs ranged from 5.7 to 518.1 ng g^{-1} dw and from 6.6 to 140.8 ng g^{-1} dw respectively. In a previous study in Greece, Valavanidis et al. (2008) has reported the ΣPAHs in mantles and gills of mussels collected in 2005 and 2006 (Valavanidis et al., 2008). In Elefsina (near to Megara) mean ΣPAHs in the mantle of mussels was 1288 ng g^{-1} dw, while in gills it was calculated at 1482 ng g^{-1} dw. In our study the maximum

Table 1
LOD–LOQ values and concentration range of contaminants detected in summer 2012 and winter 2013 Greek samples.

Compound	Concentrations (ng g^{-1} dw)												LOD (ng g^{-1} dw)	LOQ (ng g^{-1} dw)
	Salamina				Megara				Volos					
	Summer 2012 1st	Summer 2012 2nd	Winter 2013 1st	Winter 2013 2nd	Summer 2012 1st	Summer 2012 2nd	Winter 2013 1st	Winter 2013 2nd	Summer 2012 1st	Summer 2012 2nd	Winter 2013 1st	Winter 2013 2nd		
Naphthalene	5.7	6.9	<LOD	<LOD	6.1	17.3	11.6	13.3	3.5	18.8	nd	nd	0.8	7.9
β-BHC	nd	nd	nd	nd	nd	6.6	nd	nd	6.9	6.9	nd	nd	0.4	4.1
δ-BHC	nd	nd	nd	nd	nd	Nd	nd	nd	6.1	6.1	nd	nd	0.4	4.0
pp-DDE	nd	nd	nd	nd	nd	Nd	nd	nd	nd	26.3	nd	nd	0.5	5.1
pp-DDD	nd	nd	nd	nd	nd	Nd	nd	nd	nd	28.3	nd	nd	0.6	5.9
Benzo(b)fluoranthene	nd	nd	19.9	31.6	nd	Nd	nd	21.6		22.7	nd	nd	0.5	5.2
Benzo(g,h,i)perylene	nd	nd	nd	9.7	nd	Nd	nd	7.3	nd	nd	nd	nd	0.5	4.9
Indeno(1,2,3-cd)pyrene	nd	nd	16.1	14.6	nd	Nd	nd	11.4	nd	nd	nd	nd	0.7	6.8
Fluoranthene	nd	nd	nd	113.5	nd	Nd	nd		nd	nd	nd	nd	2.0	19.7
Pyrene	nd	nd	nd	150.5	nd	Nd	nd	61.9	nd	nd	nd	nd	2.0	19.9
Benzo(a)anthracene	nd	nd	nd	43.0	nd	Nd	nd	14.6	nd	nd	nd	nd	1.0	10.3
Chrysene	nd	nd	nd	153.5	nd	Nd	nd	nd	nd	nd	nd	nd	1.2	11.6
ΣPAHs	5.7	6.9	37.6	518.1	6.1	17.3	11.6	130.1	3.5	41.5	–	–		
ΣOCs	–	–	–	–	–	6.6	–	–	13	67.6	–	–		

Table 2
LOD–LOQ values and concentration range of contaminants detected in summer 2013.

Compound	Salamina Concentrations (ng g^{-1} dw)		Megara Concentrations (ng g^{-1} dw)		LOD (ng g^{-1} dw)	LOQ (ng g^{-1} dw)
	Summer 1st	Summer 2nd	Summer 1st	Summer 2nd		
Naphthalene	47.7	9.7	16.3	14.7	0.8	7.9
Aldrin	48.6	nd	94.5	31.8	0.8	8.2
Fluorene	30.2	7.1	0	10.3	0.3	3.4
pp-DDE	47.5	51.1	46.3	33.5	0.5	5.1
γ-BHC	7.2	6.4	nd	7.4	0.2	2
Benzo(a)pyrene	20.8	nd	nd	nd	0.7	6.6
Phenanthrene	90.2	31.1	nd	41.4	1.3	12.9
Fluoranthene	ND	nd	nd	73.7	2.0	19.7
Pyrene	96.7	nd	nd	94.4	2.0	19.9
Dibenzo(a,h)anthracene	6.0	nd	nd	nd	0.8	8.1
ΣPAHs	291.6	47.9	16.3	234.5		
ΣOCs	103.3	57.5	140.8	72.7		

concentration of ΣPAHs obtained in mussels tissue was 234 ng g^{-1} dw in Megara [second sampling of summer 2013 (Table 2)]. In Salamina region in the study by Valavanidis et al. (2008) the ΣPAHs was ranging from 1759 to 2454 in mantle and gills. In our study maximum ΣPAHs in Salamina was reported in winter 2013 at 518.1 ng g^{-1} dw, again lower than the one reported by Valavanidis et al. In another study of Tsangaris et al. (2011) PAHs and OCs levels were monitored in caged mussels including Saronikos Gulf and Pagasitikos Gulf (Volos harbor is situated in that Gulf) in sampling stations (Tsangaris et al., 2011). In this study PAHs concentration in Saronikos Gulf was 75 ng g^{-1} dw while in Pagasitikos Gulf was slightly above 50 ng g^{-1} dw. From our results we conclude that ΣPAHs levels in Saronikos Gulf in some cases are above the levels reported by Tsangaris et al. (2011) while for Volos only in summer of 2012 the ΣPAHs are below 50 ng g^{-1} dw but comparable. As regards ΣOCs, concentrations are much higher than the ones reported by Tsangaris et al. (2011).

In Turkish samples ΣPAHs and ΣOCs ranged from 3.8 to 39.9 ng g^{-1} dw and 9.5 to 20 ng g^{-1} dw respectively (see Table 2s). In another recent work by Kucuksezgin et al. (2013) ΣPAHs in Saros and Canakkale-similar to our sampling stations-were 41.6 and 44.4 ng g^{-1} dw respectively (Kucuksezgin et al., 2013). Thus the maximum ΣPAHs value obtained in our work in Kabatepe bay is comparable to the value given by Kucuksezgin et al. (2013). Regarding OCs the ΣOCs value in Saros was estimated by Kucuksezgin et al. (2013) at 7.5 ng g^{-1} dw (referring to the compounds of our method) while in Canakkale was 8.6 ng g^{-1} dw. In our study the maximum ΣOCs is 20.0 ng g^{-1} dw, higher than the one reported by Kucuksezgin et al. (2013) however the overall range is comparable.

3.2.1. PAHs sources discrimination

Study of PAHs distribution and PAHs concentration ratios can be used to discriminate PAHs of petrogenic and pyrolytic origin. In general higher proportion of high molecular weight (HMW, 4-6 ring molecules) PAHS suggests pyrolytic input, while a high proportion of low molecular weight (LMW, 2-3 ring molecules) PAHs indicates petrogenic sources. This approach should be treated with caution when studying mussels and deducting conclusions because various PAHs have different uptake and depuration rates which can change their profile.

Diagnostic ratios were used for summer 2012 samples (Gogou et al., 1998). In this context in Salamina petrogenic input dominated due to the sole presence of naphthalene residues. The winter of 2013 HMW PAHs prevailed, suggesting pyrolytic input. Similar was the picture for Megara with the exception of the 1st winter sampling where petrogenic character was evidenced. Conclusively, in all sampling seasons an alternation of an initial petrogenic to pyrolytic profile was obtained, with the exception of winter 2013 in Salamina where the petrogenic profile remained constant and predominant.

For summer of 2013 using diagnostic ratios of PAHs it was observed that the LMW/HMW ratio was >1 for Salamina in the 1st and 2nd sampling period. The latter indicates petrogenic input. In Megara, although the 1st sampling period only naphthalene was detected the situation was changed dramatically the second sampling period where a LMW/HMW ratio of 0.40 was evidenced indicating pyrolytic contribution. In Megara ΣPAHs was higher in summer 2013 sampling. Same was the conclusion for OCs however a declining trend was observed since the overall concentration was decreased to half.

In case of Volos sampling we observed the concomitant presence of p,p-DDD and p,p-DDE. It is reported that p,p-DDD/p,p-DDE ratios <1 indicate that p,p-DDT is biotransformed to DDE under aerobic conditions. However in this case the respective ratio was 1.07 thus such biotransformation cannot be hypothesized.

3.3. Biochemical responses

3.3.1. Correlations among biochemical markers and contaminant levels

Significant correlations were revealed (see Supplementary Material, Table 3s) in summer 2012 between the biomarkers FR and NR as well as between MDA and PC at medium level according to Cohen (1988). A significant negative correlation was found between contaminants level (PAHs) and physiological responses (FR) at strong level according to Cohen (1988). More correlations were found for 2013 sampling, probably also because of the higher sampling frequency and the subsequent increase in the analyzed sample size. A positive correlation was noted for FR and NR, for 4-HNE and MDA and for 4-HNE and PC, at medium level. Regarding pollutant levels, GSH was highly correlated ($P < 0.01$) at medium level with PAHs, according to Cohen (1988).

Oxidative damage in lipids and proteins is the common outcome of the damage of reactive oxygen species (ROS) to the cells. Injury to lipids by ROS consists of a chain reaction of oxidations, resulting in peroxidation of the unsaturated fatty acids of the cell membrane. The short-lived lipid hydroperoxides decompose to a variety of end products namely MDA, hydrocarbons, cyclic endoperoxides, isoprostanes and unsaturated aldehydes (Kohen and Nyska, 2002). Damage to proteins by ROS may lead to formation of carbonyl groups (aldehydes and ketones) on aminoacid side chains (Dalle-Donne et al., 2003). It is probable that a number of existing pollutants in the selected sites were capable of eliciting ROS production, which led to unprejudiced damage in all cell macromolecules and as such, to correlations between markers of oxidative damage. This relationship was evident both for 2012 (for MDA and PC) and for 2013 (for MDA and 4-HNE and for MDA and PC). PAHs metabolism entails continuously redox-cycling quinones (besides genotoxic hydrodiol epoxides) (McCoull et al., 1999), while OCs are capable of induction of CYP2B isoforms which then undergo extensive futile oxidation/reduction cycling (Stevenson et al., 1999). Of course, a number of additional pollutants including heavy metals are also implicated in oxidative stress and its effects.

FR and NR were also two markers which were also positively correlated both in 2012 and in 2013. Both of these biomarkers are commonly used as indicators of generalized physiological stress (Karacik et al., 2009). This was also verified in the present research where more stressful situations (i.e. winter 2013) were characterized by low values in both biomarkers. PAHs are supposed to decrease FR, as shown by recovery of feeding rates in *Mytilus edulis* which had been previously exposed to crude oil (Widdows et al., 1985). This was also corroborated by the strong inverse correlation between PAH body burden and FR values in the present study for 2012. Interestingly, this correlation was not repeated for the 2013 sampling. This was also evident in the study of Karacik et al. (2009) on *M. galloprovincialis*, where high PAH body burden was accompanied by low FR values in the Asian part

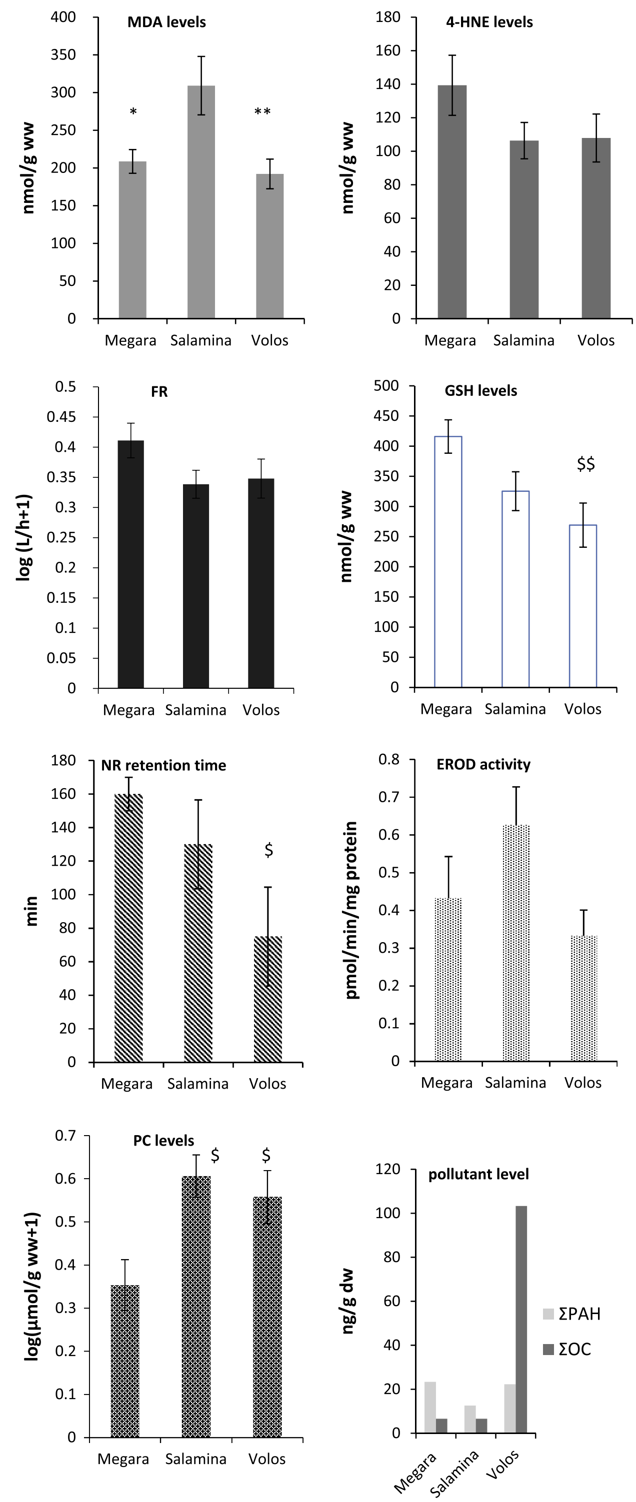

Fig. 1a. Differences between sites for 2012 sampling (summer 2012). ANOVA for 4-HNE, EROD, GSH, FR and PC followed by Tukey's HSD post hoc test; Kruskal–Wallis for MDA and NR followed by Mann–Whitney U-test. $^{*}P < 0.05$ in relation to Salamina, $^{**}P < 0.01$ in relation to Salamina, \$ $P < 0.05$ in relation to Megara, \$\$ $P < 0.01$ in relation to Megara. No VI levels shown because assay not performed for summer 2012.

of Istanbul Strait whereas significant PAH pollution did not exert the same response in the European part. Regarding OCs, relationships were found between DDT and PCB body burden and destabilization of lysosomal membrane for zebra mussels in a polluted confluence (Binelli et al., 2010). In the present study, despite substantially low values of NR in the OC polluted area of Volos, these low values were not repeated in the OC polluted samples of 2013. Nevertheless, our overall results corroborate the use of this highly sensitive, low cost biomarker for initial pollution screening as also proposed by others Viarengo et al. (2007a,b).

GSH is the first line of non-enzymatic defense of the cells. It is synthesized via a two-step procedure and it is continuously regenerated from its oxidized form by Glutathione Reductase (GRd). Pollutants (heavy metals and organics) may affect the suite of enzymes implicated in GSH metabolism and utilization as well as its reduced and oxidized levels in complex and intercalating ways [e.g. see Canesi et al., 1999]. A 7-d exposure of *Unio tumidus* to a polluted site caused significant depletion of GSH and suppression of GRd (Doyotte et al., 1997). Based on these results and another relevant study (Cossu et al., 1997), the authors concluded that GSH levels and the enzymes implicated in GSH formation, regeneration and utilization are promising biomarkers of pollution. Similar results were noted in an *in vivo* exposure of *M. galloprovincialis* to high concentrations of anthracene and/or phenanthrene with depletion of GSH and other thiols after 7 d (Grintzalis et al., 2012). On the other hand, deployment of mussels (*Perna viridis*) to sites characterized by a pollution gradient (PAHs, OCs and PCBs) in Hong Kong markedly increased GSH in gills and digestive gland tissues at 14 at 28 d post deployment (De Luca-Abbott et al., 2005). *P. viridis* had also been previously deployed in Hong Kong harbors where its GSH in gills was strongly correlated with PAH and with BaP body burden and GSH in digestive gland was also positively correlated with BaP body burden after 30 d of deployment (Cheung et al., 2001). Finally, a recent study on *P. viridis* also showed that in most PAH and OC exposure regimes tested *in vivo*, digestive gland GSH was induced. Significant correlations were found between all PAHs and OCs (as individual chemicals or as sum of substances) and digestive gland GSH. Similar results have been noted for catfish exposed to PAH-polluted sediment and to flatfish chronically residing in PAH/PCB contaminated sites (Richardson et al., 2008). In summary, there are strong indications that GSH as well as the enzymes implicated in its formation and

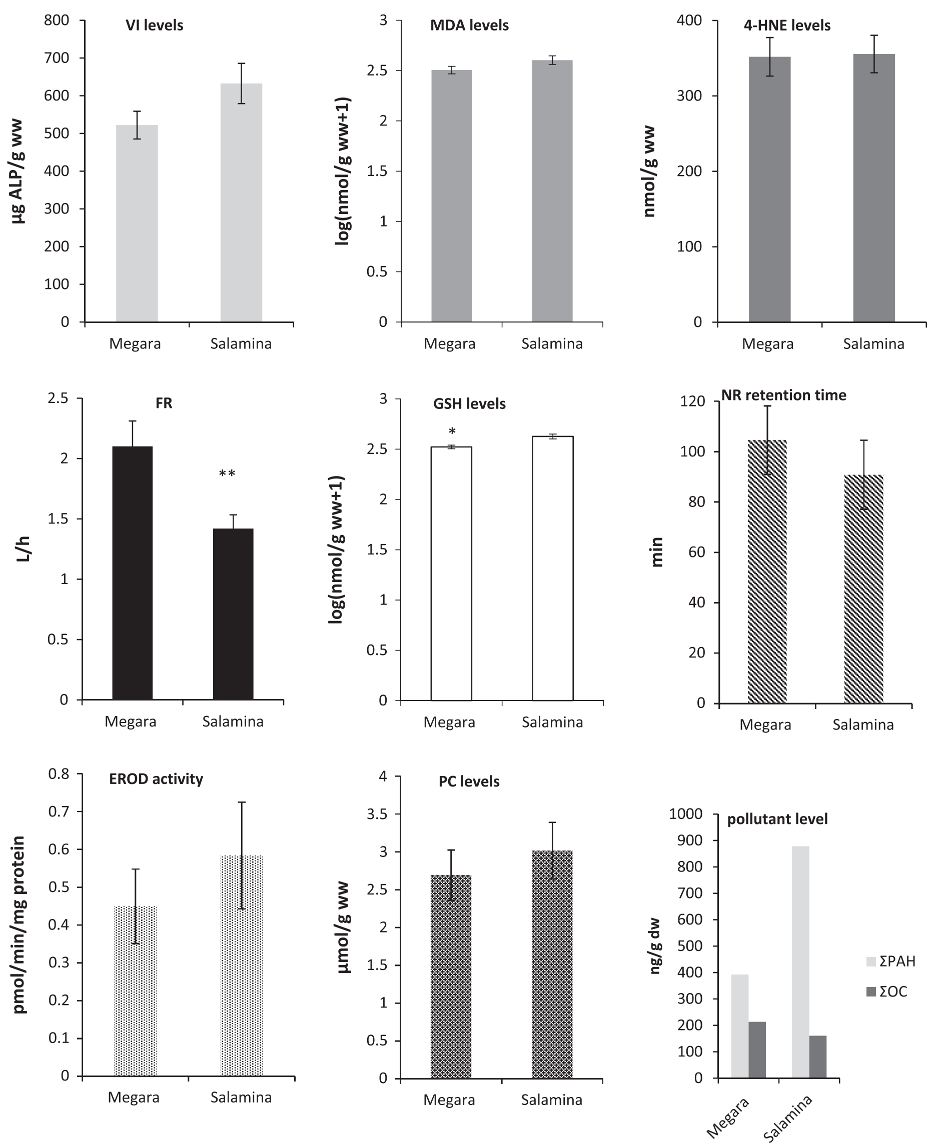

Fig. 1b. Differences between sites for 2013 sampling (winter and summer 2013). ANOVA for 4-HNE, EROD, GSH, VI and MDA; Kruskal–Wallis for PC, FR and NR, *$P<0.05$, **$P<0.01$.

regeneration are distinctly affected by organic pollution. Duration of exposure is crucial in terms of induction or suppression of GSH and that a relatively short duration may elicit an initial decreased response which can be followed by an induction of antioxidant systems. Two more remarks worth mentioning are the apparent lack of induction in EROD activity by increased PAH pollution and the apparent lack of endocrine disrupting properties of OCs in mussels. EROD activity was not induced by PAH body burden corroborating the theory of lack of an inducible CYP1A isoform in bivalves. On the contrary, a CYP4 isoform is present in bivalves and its transcription has shown to be regulated by PAH (Snyder et al., 2001). Furthermore, no differences in VI levels were found in the sampling characterized by higher OC body burden. It is still not certain that OCs exert endocrine disrupting properties in bivalves as they do in mammals. Furthermore, the role of vitellogenin in mussels still needs to be elucidated as it is also implicated in immune reactions (Bouchard et al., 2009).

3.3.2. Differences between sites

Significant differences were detected between sites for the summer of 2012 as shown in Fig. 1a (Megara, Salamina and Volos) and for summer and winter 2013 as shown in Fig. 1b (Megara and Salamina). Results of 2012 showed a compromised situation in the specimens of Volos harbor area when taking into account NR retention time as well as biomarkers of oxidative damage (PC) and GSH levels. Lack of specimens from 2013 (winter and summer) deterred the further analysis of this site. PC, as well as MDA, was also increased in Salamina area which was supposed to be the most polluted in terms of PAHs (refineries in proximity). The situation in Salamina was revealed in more detail in the following year with a number of biomarkers performing worse (FR) in relation to the site of Megara. GSH however was increased in this polluted site. This is in accordance with the previous findings (correlation between GSH levels and PAHs levels).

3.3.3. Differences due to deployment duration and due to season

The duration of exposure (one or two months) had a significant impact on the transplanted mussels' responses (see Supplementary Material, Fig. 4s). For some of the biomarkers (GSH, NR) with a statistical correlation with pollutant levels it may be argued that these differences can be safely attributed to the PAH pollution gradient while for the rest, interference of other, not detected pollutants may be incriminated. It is known that heavy metals exert a number of effects on *M. galloprovincialis* [e.g. (Ciacci et al., 2011; Bouki et al., 2013)] and emerging pollutants such as personal care products and pharmaceuticals also produce detrimental health effects on bivalvian species (Gagne et al., 2006; Martin-Diaz et al., 2009). Therefore, extensive chemical analyses covering a wide range of pollutant classes should each time accompany a mussel biomonitoring program. Besides pollution, adaptation mechanisms even at the short duration of two months may also develop. This may be achieved mostly via epigenetic changes, as postulated by Large et al. (2002) for *M. edulis* chronically exposed to polluted Scottish estuaries even though the metabolic and/or physiological changes necessary for this apparent adaptation are mostly unknown. It is therefore of utmost importance to accommodate the duration of deployment to the type of the biochemical response examined since the later may change in relation to exposure duration (see also Wu et al., 2005 for classification of biomarkers). Despite correlations found between pollutants and biochemical responses, the intensity of the effects was highly dependent on the season of the year (see Fig. 2), with NR and GSH not showing seasonal pattern. For the rest of biomarkers, exogenous factors such as food availability, temperature and growth and endogenous factors such as reproductive activities may affect development and health status of the mussel as also

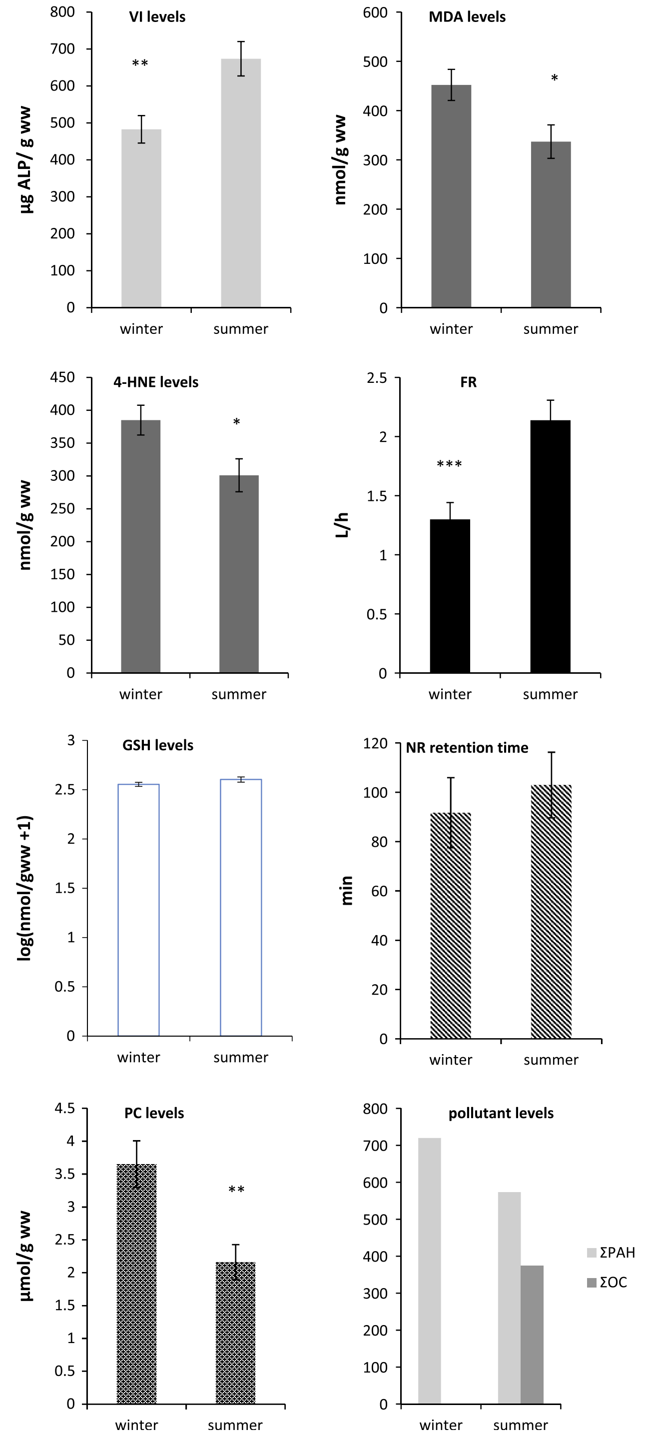

Fig. 2. Differences between seasons for 2013. No EROD activity shown because assay not performed for summer. ANOVA for 4-HNE, VI and GSH; Kruskal–Wallis for MDA, PC, FR and NR, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

mentioned by Power and Sheehan (1996). Despite some of the differences being attributed to a higher PAH body burden in winter, it was clear that the general health of the specimens was significantly worse in winter. Oxidative damage was also higher in winter in the study of Di Salvatore et al. (2013) on *Aulacomya atra* and it was increasing from June to December on *M. edulis*, as mentioned by Nahrgang et al. (2013). Winter time exerts more stressful conditions on the mussel (Power and Sheehan, 1996) probably because of increased dissolved oxygen which produces more ROS and due to reduced efficiency of cellular antioxidant systems (Di

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Salvatore et al., 2013). In order to correct for this possible implication of seasonal stress repeated deployment in distinct seasonal frequencies is proposed for reliable monitoring programs.

4. Conclusions

A GC–MS/MS method based on QuEChERS procedure was developed for the determination of PAHs and OCs in mussels' samples from Greek (sites with high anthropogenic activity and high expected pollution) and Turkish estuaries (sites with no expected pollution). Low but not negligible levels of 6 PAHs and 5 OCs were detected in the Turkish samples. In Greek samples all sampling spots exhibited detectable levels of the contaminants with a clear trend of bigger accumulation of contaminants in winter season for both Megara and Salamina. Hence pollutants with high bio-concentration potential were detected in mussels' samples. The current status of PAHs and OCs potential burden in that part of Eastern Mediterranean verify that pristine sites of the Turkish shores possess very low levels of contaminants. On the other hand the concentrations in Greek estuaries were higher, as expected due to the selection of sampling points in Greece which are subjected to heavy industrial and shipping activity. Biochemical responses in Greek mussels also corroborated a low to intermediate health status of the transplanted specimens. Significant correlations were detected among biochemical/biological responses and between mussel body pollutant burden and these responses. Detailed monitoring of priority pollutants of East Mediterranean Sea is vital both for ecological and human risk assessment purposes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2014.05.078.

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