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Multiple pollution biomarker application on tissues of *Eobania vermiculata* during two periods characterized by augmented and reduced snail activity

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ABSTRACT

In the present study a package of biomarkers was applied on land snails *E. vermiculata* collected from polluted areas, as well as from an unpolluted reference one. Snail collection was performed during two different sampling periods characterized by reduced and augmented organism activity, October and May, respectively. The biomarkers applied were lysosomal membrane stability on digestive cells (LMS), neutral red lysosomal retention assay on haemocytes (NRR), morphometric changes of the lysosomal system (VDL, NDL), morphometric alterations of the neutral lipids (VDLP, NDLP), acetylcholinesterase activity on digestive gland and hemolymph (AChE), metallothionein content on digestive gland (MTs) and cyclic AMP content on digestive gland (cAMP). The results revealed significant differences in biomarker values between the two sampling periods. Significant differences were also detected among the sampling groups. The fluctuation of the parameters applied indicated that spring is a more suitable period for sampling conduction compared to autumn and that biomonitoring studies should be performed with special attention during the last mentioned period.

1. Introduction

One of the most significant aspects which has come in for attention in recent times is the variation of pollutant accumulation and biomarker response during different seasons. Their study is considered highly important, in order to get a proper and more comprehensive interpretation in biomonitoring studies (Sheehan and Power, 1999). It is sometimes difficult to explain whether organisms' responses are owed to pollution or to physiological changes (Kagley et al., 2003). Application of pollution biomarkers during certain periods may give misleading results, since changes in animal tissues derived from physiological factors could mistakenly be attributed to pollutant presence. Several studies have been performed on marine mollusks (Marigomez et al., 1996; Cancio et al., 1999; Petrovic et al., 2004; Guerlet et al., 2007), while, information regarding this aspect in land snails is very scanty (Niyogi et al., 2001).

The battery of biomarkers applied in the present study included lysosomal membrane stability, extension of lysosomes, neutral lipids and black silver deposits, acetylcholinesterase activity, metallothionein content and cAMP content. Lysosomal membrane stability was estimated at first on digestive cells using lysosomal membrane stability test (LMS), which has previously been proposed as an effective pollution biomarker in land snails *E. vermiculata* (Itziou and Dimitriadis, 2009). Lysosomal membrane stability was also estimated on haemocytes using neutral red retention assay (NRR), which constitutes one of the most reliable biomarkers of general stress conducted in field (Siboni et al., 2004), and laboratory studies (Lowe et al., 2006). Moreover, changes in lysosomal structure were studied, since they have been used as general markers of pollutant-induced stress using molluscs as sentinel organisms, in a number of field and laboratory studies (Cajaraville et al., 2000; Koukouzika and Dimitriadis, 2005; Itziou and Dimitriadis, 2011). Another biomarker, which is still under investigation, refers to the morphometric alterations of neutral lipids detected in snails' digestive gland. Several field studies on mussels or snails suggest that both heavy metal (Najimi et al., 1997) and organic pollution (Lowe, 1988; Moore, 1988; Itziou and Dimitriadis, 2012) is responsible for the accumulation of neutral lipids. Autometallography (AMG), a very sensitive and extremely precise histochemical technique (Danscher and Montagnese, 1994) was the next technique studied, which provides information about the cellular and subcellular localization of metals, as well as the cellular response to toxic metal uptake (Raftopoulou et al., 2002; Soto et al., 2002; Raftopoulou and Dimitriadis, 2011). Another biomarker used was the measurement of the acetylcholinesterase (AChE) activity, demonstrated by previous studies, mainly due to its inhibition in the presence of organophosphorus compounds and carbamates (Coeurdassier et al., 2001; Gambi et al., 2007; Laguerre et al., 2009; Panda and Sahu, 2004). Furthermore, the synthesis of the

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low molecular weight cystein-rich, cytoplasmic, metal-binding proteins, known as metallothioneins (MT), is indicative of a specific response of organisms either from polluted areas, or after exposure to metals (Dallinger et al., 2004). cAMP acts as a second messenger and is involved in signal transduction pathway. Changes in cAMP have previously been considered as a remarkable index of heavy metal (Sutherland et al., 1974; Dailianis et al., 2003; Fabbri and Capuzzo, 2006; Dailianis and Kaloyianni, 2007) or organic (Evanson and Van Der Kraak, 2001; Zhang et al., 2002) bioaccumulation.

The aim of the present work was to study potential differences between biomarker values applied on snails collected during two different sampling periods. The sampling was performed on May and on October, since during these periods snails are characterized by augmented and low activity, respectively. The ability of the biomarkers to evaluate terrestrial pollution was investigated, and thus the most suitable period for the conduction of biomonitoring studies was determined. The application of a package of parameters aimed to give further information on their simultaneous changes.

2. Materials and methods

2.1. Snail collection

Snails were collected during two different sampling periods: October 2006 and May 2007, based on previous studies indicating augmented activity of animals during spring compared to autumn (Lazaridou-Dimitriadou and Kattoulas, 1991). Snails were sampled from graduated distances of the major road Agiou Dimitriou near Aristotle University of Thessaloniki (5, 20 and 150 m). Snails were also collected from graduated distances of the phosphate fertilizers industry in the prefecture of Kavala (20 and 700 m). Finally, snails were collected from graduated distances (20 and 700 m from the main stack sources) of a lignite power station at the district of Kozani (West Macedonia, Northern Greece). An area in the district of Pella (Central Macedonia, Northern Greece) was considered as the reference station based on rather low levels of heavy metal contents in snail digestive gland, according to measurements using Flame Atomic Absorption Spectrophotometry (Itziou and Dimitriadis, 2011). The mean shell size of the snails collected for the present work on October 2006 was 19.03 ± 1.1 mm length and 11.4 ± 1.23 mm width, while on May 2007 the corresponding values were 27.03 ± 1.2 mm length and 13.07 ± 1.21 mm width. Sampling of snails was followed by their immediate transfer to the laboratory in plastic cages.

2.2. LMS evaluation

The N-acetyl-β-hexosaminidase histochemistry was performed according to Moore (1976) and UNEP/RAMOGE (1999). The determination of LMS values was based on the time of acid labilization period required to produce the maximum staining intensity. The labilization period is the time of acid labilization required to fully labilize the responsive fraction of lysosomal hydrolase in the digestive cells. This was assessed under the light microscope as the first peak. Four determinations were made for each animal by dividing each section into 4 segments and assessing the labilization period in each of the corresponding set of segments. A mean value was then derived for each section, corresponding to an individual digestive gland.

2.3. Evaluation of NRR

The NRR assay was performed according to Lowe and Pipe (1994), with small modifications. The shell of 10 individuals was pierced and hemolymph was withdrawn from a small hole in physiological snail buffer (50/50 of hemolymph/buffer), as it is described by Snyman et al. (2000). The NRR time was measured individually for 10 snails and the mean NRR time of the 10 snails was the NRR time for the whole experimental group.

2.4. Evaluation of lysosomal morphometrical parameters

Digestive glands from five snails from each sampling station, as well as from each container, were dissected out. Five small pieces of five snails' digestive glands were placed on aluminum cryostat chucks, in a straight row across the center of the chuck. The chuck was then placed for 40 s in a small bath of n-hexane, which has been precooled for 2 to 3 min in liquid nitrogen, in order to quench the tissue. All chucks were doubled wrapped in parafilm and stored at $-80\ ^{\circ}C$ until required for sectioning, or alternatively, were kept at $-30\ ^{\circ}C$ and sectioned within 1 week. N-acetyl-β-hexosaminidase was histochemically detected according to Moore (1976) in unfixed cryostat sections, and indicated lysosomes as purple precipitates in the digestive cells of snails. The non pre-treated slides were used for morphometrical analysis of lysosomes (volume density of lysosomes-VDL, numerical density of lysosomes-NDL). The volume density of lysosomes/neutral lipids refers to the volume covered by lysosomes/neutral lipids (μm^3) divided by the volume covered by the cytoplasm (μm^3). This measurement takes into consideration the size of each digestive tubule and that is how it is differerent from measuring only the volume of lysosomes/neutral lipids. The numerical density refers to the number of lysosomes/neutral lipids per μm^3 divided by the volume covered by the cytoplasm (μm^3). That is why this parameter is different from measuring the number of lysosomes/neutral lipids.

2.5. Morphometry of neutral lipids

The neutral lipids' histochemistry was applied according to Moore (1988). Image analysis was used in order to measure the volume density (VDLP) and the numerical density of neutral lipids (NDLP).

2.6. Autometallography (AMG)

Light microscope AMG was performed using the procedure proposed by Danscher (1984) with slight modifications described in Raftopoulou et al. (2002). Glass slides bearing cryosections of digestive gland of snails were covered with a uniform layer of photographic emulsion (Ilford nuclear emulsion-L4) under safelight conditions. After drying in complete darkness for 30 min, the slides were developed with Kodak D-19 for 1–1.5 h, rinsed in tap water and fixed in Acidofix (Agfa) for 10 min. The heavy metals were visualized as black silver deposits (BSD) indicating the presence of silver around them in cellular sites. The cryosections were examined unstained under an Olympus BH-2 light microscope and the BSD extent expressed as volume density of BSD (VDL_{BSD} and NDL_{BSD}) was evaluated by image analysis.

2.7. Image analysis

The quantification of the volume and numerical density of lysosomes, neutral lipids and black silver deposits was performed by the use of image analysis. Slides were examined using a JVC video camera mounted on an Olympus CX41 light microscope with an objective lens of 100x magnification. The image was displayed on the computer screen and captured with Adobe Premiere 5. Binary images segregating lipids and lysosomes from the cytoplasm were obtained by the segmentation procedure, which was manually adjusted in the first measurement of a given section and automatic in the others (EIKONA program). Whenever present, the digestive tubule lumen and the connective tissue surrounding the digestive tubule were eliminated from the analysis. 50 measurements per experimental group of snails were conducted.

2.8. Determination of AChE activity

For the AChE measurements, digestive gland and hemolymph were collected from 5 snails and the procedure described by Dailianis et al. (2003) was followed. The tissues were maintained at $-85\ ^{\circ}C$ for less than a day, before the performance of the enzyme activity. Acetylthiocholine iodide (21.67 mg/ml) was added as substrate to initiate the enzymatic reaction. 4 measurements were performed for the determination of AChE activity.

2.9. MT content

In order to quantify MT in the digestive gland of 5 snails, the spectrophotometric method according to Viarengo et al. (1997) was used. MT content in the samples was measured by evaluating the sulphydryl (-SH) residues content according to Ellman (1961) with DTNB (5, 5-dithiobis-2-nitrobenzoic acid) and reduced glutathione (GSH) as a standard. Four measurements were performed for the determination of MT content.

2.10. cAMP determination

cAMP content in the digestive gland of snails was estimated with the Amersham, [8-3H] adenosine 3′–5′ cyclic phosphate radioimmunoassay kit, according to Dailianis et al. (2003).

2.11. Data analysis

Data on LMS, AChE activity, MT content and cAMP content was tested using non-parametric statistics (Mann–Whitney U-test, $p < 0.05$), while data on NRR