The role of lysosomal membrane stability, malondialdehyde levels and DNA damage as pollution biomarkers of terrestrial environments using Eobania vermiculata

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Research Article

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Abstract

The current work investigates the role of neutral red retention assay, malondialdehyde contents and DNA damage through comet assay as biomarkers of terrestrial environmental pollution using the land snail *Eobania vermiculata*. Snails were collected near a lignite power station located about 6 km near the city of Ptolemaida in the district of Kozani. The results of the study showed significantly lower neutral red retention times, higher malondialdehyde contents and higher formation of single-stranded DNA fragments in the haemolymph of snails collected from the polluted area compared to control animals. In support of our data, the values of pollution biomarkers showed significant correlations, thus encouraging their use in terrestrial pollution biomonitoring studies and highlighting the effects of pollution in terrestrial environments.

Introduction

Lignite is a conventional fossil fuel for electricity generation, releasing various air pollutants such as particulate matter (PM), sulphur dioxide (SO2), nitrogen oxides (NOx), PAHs, heavy metals, and fly ash especially during winter and spring when the prevailing winds are weak to hold them (Triantafyllou et al., 2006). Greece is positioned among the beat places for the lignite generation within the European Union, Europe, and around the world (Chatzitheodoridis et al., 2008). Western Macedonia has six lignite steam control plants which produce in total 4440 MW electricity. The impact of the environmental pollution on the area from these lignite power plants has widely been analyzed (Evagelopoulos et al., 2022a; Ziouzios et al., 2021; Samara et al., 2018; Iordanidis et al., 2008). Moreover, previous studies on lignite miners (Sichletidis et al., 2004) and/or children living in the city of Ptolemaida (Sichletidis et al., 2005; Spyratos et al., 2015) correlated atrophic rhinitis, infectious bronchitis, nasal obstruction, chronic nasal symptoms and upper respiratory system disorders in general with the environmental pollution caused by lignite power stations of the area. Previous works of our team have indicated the toxic cellular and biochemical effects of lignite on snail tissues (Itziou and Dimitriadis, 2009; 2011).

Land snails such as *Archchcatina papyracea* (Owojori et al., 2022), *Helix aspersa* (Sahraoui et al. 2021; Tyrki et al., 2022), *Cantareus aspersus* (Petitjean et al., 2021), *Achatina fulica* (Song et al., 2020) and *Eobania vermiculata* (Itziou and Dimitriadis, 2011; Cofone et al., 2020) may accumulate various heavy metals derived from industrial pollution, and thus they are used as valuable bioindicator organisms.

The biomarker neutral red retention assay (NRR) refers to the retention time of the dye into the lysosomes. Several studies have shown that the time is mainly influenced by the presence of pollutants thus enhancing the application of this biomarker for the evaluation of heavy metal or organic effects both in field (Sturba et al., 2018) and in laboratory studies (Itziou and Dimitriadis 2012; Pappa et al., 2021). The main lipid peroxidation product is malondialdehyde (MDA) and is known to cause DNA damage or carbonylation of cellular proteins (Ma et al., 2014). MDA lipid peroxidation products may be commonly used for estimating the levels of oxidative stress in land snails (Sidiropoulou et al., 2018; Sturba et al., 2018). An important target of oxidative stress in land snails is DNA damage (Angeletti et al.,...
If DNA damage is not repaired, a cascade of biological effects on cell or organism level and finally on population level can be induced. Comet assay is used as an established biomarker in pollution biomonitoring studies (Sebbio et al., 2014). Previous studies supported that early signs of exposure to genotoxicants may be detected by comet assay, showing DNA strand break damage (Reinecke and Reinecke, 2004; Dallas et al., 2013).

The main goal of the present study was to evaluate NRR times, MDA levels and DNA damage through comet assay in the hemolymph of snails collected from a polluted area, as a result of environmental stress. Furthermore, the correlation analysis between NRR time values, MDA levels and DNA damage was carried out in order to reinforce the use of these established cellular, biochemical and genotoxic pollution biomarkers on land snails. Finally, more study is needed in order to improve the general ecosystem health, including human health especially in polluted areas, such as the one studied.

Materials And Methods

Snail collection

Snails were collected near a lignite power station (about 20-50m) near the city of Ptolemaida at the district of Kozani (West Macedonia, Northern Greece), since the areas near the mines of the lignite power station in the prefecture of Kozani are characterized as highly polluted, according to chemical monitoring data (Triantafyllou et al., 2006). An area in the district of Grevena (West Macedonia, Northern Greece) was considered as the reference station since it is a non-industrialized region (Fig. 1). Twenty-five individuals were sampled from each station. The mean shell size of the snails was 28.14 ± 1.44 mm length and 15.08 ± 1.17 mm width. Sampling of snails was followed by their immediate transfer to the laboratory in plastic cages. Between sampling and analysis there was no starvation of the snails collected for the field study.

Neutral red retention assay (NRR assay)

The NRR assay was performed according to Lowe and Pipe (1994), with small modifications (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.

Determination of MDA

Haemolymph of five animals was collected from snail heart cavity with a sterilized syringe and placed in plastic tubes. Haemolymph was pooled and centrifuged for 10 min at 3000 g at 4°C, and the supernatant was collected. MDA was detected in the supernatant, according to previous described method by Niehaus and Samuelsson (1998).

Alkaline single-cell gel electrophoresis (Comet) assay
The procedure used follows the method described by Singh et al. (1988) with some modifications as described by Itziou et al. (2011). Comets on each slide were scored visually as belonging to one of five predefined classes according to tail intensity and were given a value of 0, 1, 2, 3, or 4 (from undamaged, 0, to maximally damaged, 4) (Fig. 2). The percentage of DNA in tail was estimated using “TriTek Cometscore version 1.5” software.

Data analysis

Data on NRR assay was tested using Duncan's test (p < 0.05), breakdown and one-way ANOVA). For the determination of MDA contents statistical analysis was carried out using Instat 2 software (GraphPad InStat), post-hoc multiple comparison (Bonferroni, p < 0.05), where significant differences were detected in the ANOVA. Tukey test (one way ANOVA, p < 0.01) was used for the comparison of the grade of DNA damage between control and exposed cells. Simple linear correlation (Pearson's test) conducted with mean values was used to establish significant relationships between the biological responses. The analysis was carried out using STATISTICA 7 (STATISTICA, Microsoft Co.).

Results

Neutral red retention assay (NRR assay)

The NRR time for each group was the time after the NR probe application, when there was a loss of the dye from the lysosomes to the cytosol, in at least 50% of the examined cells. Determination of NRR times in snails indicated lower values in haemocytes of snails collected near the lignite power station compared to the ones collected from the reference site (ANOVA, Duncan's test, p<0.05) (Fig. 3).

MDA contents

The results of the study showed statistically higher MDA contents in snails collected near the lignite power station, compared to controls (Bonferroni, p<0.05) (Fig. 4).

Alkaline single-cell gel electrophoresis (Comet) assay

The statistical analysis of the results (Tukey's test, p<0.01) indicated marked susceptibility of haemocytes to DNA damage in snails collected from the polluted site, compared to the control ones (Fig. 5).

Correlation analysis

The results of the correlation coefficient analysis among the applied oxidative stress parameters (Pearson's test, p<0.05 are summarized as follows:

1. There was a strong negative correlation between the NRR times and the MDA contents (r=-0.99).

2. There was a strong negative correlation between the NRR times and the levels of
3. There was a strong positive correlation between the levels of DNA damage and the MDA contents ($r=0.99$).

**Discussion**

The present study focused on the investigation of NRR, MDA and comet assay in order to enhance their role as biomarkers of terrestrial pollution monitoring, using the snail *E. vermiculata*. The biomarker changes recorded in snails collected near the main sources of the lignite power station (Ptolemaida, Northern Greece), could be related to the fact that the areas close to the mines are more polluted than the distant ones (Triantafyllou et al., 2003; 2006). Besides, Western Macedonia is generally overwhelmed by lignite mining, lignite-fired control plants, and area warming frameworks. Previous works concerning pollution monitoring near the lignite power station revealed high levels of particulate matter (PM), sulphur dioxide (SO2), nitrogen oxides (NOx), PAHs, heavy metals, and fly ash in the air and/or the soils of this area (Evagelopoulos et al., 2022b; Kaldellis et al., 2004; Petaloti et al., 2006; Stalikas et al., 1997).

Lysosomal membrane stability has widely been studied in assessing cellular toxicity caused by pollutants, with neutral red retention assay to be used as a well-known biomarker in pollution studies under field or laboratory conditions, in various organisms such as earthworms (Maboeta et al., 2004), isopods (Nolde et al., 2006), freshwater snails (Brown et al., 2004) and land snails (Snyman et al., 2002; Itziou and Dimitriadis, 2011). The results of the present study further support the effect of chemicals on haemocyte lysosomal membrane in the land snail *E. vermiculata*. This was confirmed by a significant reduction of NRR times in snail lysosomes from the polluted area. In accordance with our results, a significant decrease in lysosomal membrane stability was previously observed in land snails *Cantareus apertus* exposed either to polluted soil (Leomanni et al., 2016) or to vaporized CdCl$_2$ (Sturba et al., 2018). Several studies have linked the toxic effects of heavy metals with the production, even indirect, of reactive oxygen species (ROS) (Pinto et al., 2003; Valko et al., 2005), which may then alter the efficiency of membrane-bound proton pumps, increasing membrane permeability and eventually results in the loss of acid hydrolases into cytosol, as it has also been supported by Moore et al. (2004). Moreover, consistent with our results are the findings by Radwan et al. (2019) supporting a decrease in lysosomal membrane stability in snails *Theba pisana* exposed to the industrial chemical acrylamide, further expanding the use of the biomarker in terrestrial pollution biomonitoring studies. The response of *E. vermiculata* to the effect of pollution as shown by the augmented lysosomal membrane permeability enhances its role as a biomarker for estimating pollution of terrestrial environments.

The results of the present study indicated elevated MDA levels in haemolymph of snails *Eobania vermiculata* collected from a polluted site near the lignite power station compared to controls. Our findings on MDA are consistent with the ones by previous works regarding the results of pollutant bioaccumulation in snail tissues (Radwan et al., 2010; Siwela et al., 2010). In fact Abarikwu et al. (2017)
found significant elevated levels of MDA in soft tissues of snails *Achatina achatina* from metal polluted sites, compared to controls. Moreover, Radwan et al. (2019) reported an increase in MDA values in tissues of snails *Thesa pisana* exposed to a widespread industrial chemical acrylamide. MDA increase was also noticed by Song et al. (2019) after an exposure of snails *Achatina fulica* for 28 days to microplastic fibers in soil environments suggesting that oxidative damage is one mechanism of microplastic toxicity. Moreover, aromatic hydrocarbons are known to cause oxidative stress in various invertebrates (Livingstone, 2001; Regoli et al., 2003). A previous study on freshwater molluscs exposed to domestic heating oil showed that antioxidant enzymes SOD and CAT couldn't protect them against lipid peroxidation and thus caused MDA production, which further enhances its use as a pollution biomarker (Al-Fanharawi et al., 2018). Moreover, Mnkandla et al. (2019) suggested that persistent exposure of freshwater snails *Lymnae natalensis* to heavy metals Cu, Cd, Pd or Hg induced the antioxidant defense system, and decreased lipid peroxidation. In summary our results highlight MDA levels elevation in snails collected from the polluted site, which indicates that oxidative stress is involved in the toxic mechanisms and the pollution caused by lignite power stations.

The results from the current work, as shown by the comet assay, presented elevated levels of DNA damage in snails collected from the polluted area compared to the control snails. The formation of DNA fragments could be induced either indirectly via interaction with oxygen radicals, or directly via the inhibition of the activity of repair enzymes (Sarker et al., 1995). This probably explains the elevated levels of DNA damage in snails from the polluted site compared to the control snails, as shown by the comet assay. Previous studies reported significant differences between the digestive glands of freshwater mollusks *Unio tigrdis* and *Viviparous bengalensis* exposed to domestic heating oil and controls, recorded by comet assay (Al-Fanharawi et al., 2018). Moreover, accumulated concentrations of As, Cd, and Hg in sub-adult land snails *Cantareus aspersus* caused various DNA damages (Louzon et al., 2021). Mutagenic effects of pollution from a coal plant were previously recorded by Filippi et al. (2018), as showed by MN test on haemocytes of land snails *Helix aspersa*. In fact higher values of MN were observed in snails collected within a distance of 10 km from the plant, which is in compliance with comet assay results of the current study in snails *E. vermiculata* collected nearby the power plant (20–50 m). Our results are also in accordance with a recent work of de Souza et al. (2015) which showed that land snails *Helix aspersa* exposed to soil samples collected either at 1,4 and/or 2,9 km from a coal plant had significantly higher DNA damage values than the control group at several times of exposure, as depicted by comet assay.

**Conclusion**

In the present study, an investigation of pollution biomarkers has been performed on tissues of land snails *E. vermiculata* collected from a polluted site near a lignite power station in Ptolemaida, Kozani, Greece. The overall results further display the usefulness of this land snail as a bioindicator organism and support the application of this ecotoxicologic approach for assessing the biologic impact of pollutants. Moreover, our results showed that chronic exposure of snails to polluted conditions induced persistent responses on each biomarker as well as significant correlations among the biomarkers used.
Declarations

All authors have read, understood, and have complied as applicable with the statement on "Ethical responsibilities of Authors" as found in the Instructions for Authors

Funding declaration

The authors declare that they did not receive any funding for the present study.

Author contributions

The author Itziou Aikaterini conceptualized the study, designed and performed the experiments and data collection, and wrote the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing interests that could have appeared to influence the work reported in this paper.

Data availability statement

All data generated or analyzed during this study are included in this published article.

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Figures
Figure 1

a. The reference area in the district of Grevena (West Macedonia, Northern Greece) which is a non-industrialized region.

b. The contaminated area near a lignite power station (about 20-50m) near the city of Ptolemaida at the district of Kozani (West Macedonia, Northern Greece).
Figure 2

Representative images of comets classified within the five classes of damage (200x magnification)
Figure 3. NRR values (min) of the haemocytes of snails *E. vermiculata* collected from reference area and near the lignite power station. In each experiment, haemocytes of ten animals were analysed. ★, indicate significant difference between between the two groups (Duncan’s test, p < 0.01).

Figure 3

See image above for figure legend.
Figure 4. MDA content in haemolymph of snails *E. vermiculata* collected from reference area and near the lignite power station. ★, indicate significant difference between the two groups (Bonferroni, p<0.05).

Figure 4

See image above for figure legend.
Figure 5. DNA damage of isolated haemocytes of snails *E. vermiculata* collected from reference area and near the lignite power station. In each experiment, the tissue of four animals was used and 250 cells per incubation per slide were analysed. ★ indicate significant difference between the two groups (Tukey test, p < 0.01).

Figure 5

See image above for figure legend.