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Contribution to the Evaluation of the Biological Cleaning Products of Thessaloniki using Pollution Biomarkers in *Mytilus galloprovincialis*

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Abstract

The purpose of the study was to investigate the effects of the secondary biological purification products on the cellular and genotoxic level in the digestive gland and the haemolumph of mussels *Mytillus galloprovincialis*. The mussels were divided into 3 groups, where the effect of 0.02, 0.2 and 0.5% (v/v) of the biological purification sample was performed respectively after chlorination for 10 and 20 days, while the 4th group was the control animals. The results of the study showed statistically significantly lower NRR times and remarkable sensitivity of the haemocytes to the formation of single-stranded DNA fragments in the mussels exposed at both biological sample purification samples compared to the control ones. Finally, the results of the biomarker "stress assessment" showed statistically significantly less survival time in the exposed mussels compared to controls. Notable is the presence of significant correlations between the values of the biomarkers applied.

Keywords: Biological cleaning; Pollution biomarkers; *Mytilus galloprovincialis*; Biomonitoring

Introduction

The aquatic ecosystem health can be severely affected by municipal wastewater effluents (MWWE) which contain complex mixtures of domestic, municipal and industrial origins [1-6]. The MWWE include solid objects, sand, suspended solid particles, organic-natural ingredients that are consumed by microorganisms causing a parallel reduction of dissolved oxygen in the water body, pathogenic microorganisms which are responsible for transmitting diseases to humans and other organisms and nutrients (such as phosphorus, nitrogen) which can cause eutrophication [7-9]. These discharges contain a wide range of natural and anthropogenic substances, including pharmaceutical products, heavy-metals, ammonia, pesticides, endocrine disruptors and polycyclic aromatic hydrocarbons [7,10,11], many of which have also been measured in the receiving environment [12].

Numerous contaminants can be found in wastewaters, such as heavy metals and persistent organic pollutants. Major industrial sources include surface treatment processes with elements such as Cu, Zn, Ni and Cr, as well as industrial products that, at the end of their life, are discharged in wastes [13]. Persistent organic pollutants (POPs) constitute a wide group of compounds which are either intentionally produced, such as polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCs), or unintentionally or accidentally formed as byproducts of industrial or other human activities, for instance dibenzo-p-dioxins and furans (PCDDs/Fs) and polycyclic aromatic hydrocarbons (PAHs) [14]. These compounds are characterized by pronounced persistence against chemical/biological degradation, high environmental mobility, strong tendency for bioaccumulation in human and animal tissues, significant impacts on human health and the environment even at extremely low concentrations. Their low biodegradability makes them refractory to the biological treatment of wastewater [15,16].

The wastewater treatment plants (WWTPs) are one of the most effective ways of dealing with pollution of water resources. These facilities are designed to remove pollutants from the municipal waste and release a clean product to water recipients. According to the directive issued by the Council of Environment Ministers of the EEC in 1992, large cities should have sewage treatment waste, the extent of which is determined by which of the above harmful ingredients it removes. Sewage treatment waste includes three stages. The primary treatment aims to remove bulky solids, sand and suspended solids, while during the secondary or biological treatment the organic components of biological oxidations are removed. Finally, the tertiary treatment is designed for the removal of nutrients (such as phosphates, nitrates, borates and silicates).

Due to the high cost of biological treatment, several units, including the sewage treatment plant in the city of Thessaloniki, apply up the secondary cleaning of urban wastewater in the product of which a final disinfection with chlorine takes place. The basic parameters usually assessed in the effluent is

- COD corresponding to the amount of dissolved oxygen required to achieve the chemical oxidation of one liter of waste and
- BOD which corresponds to the amount of dissolved oxygen used by microorganisms for the oxidation of organic load in one liter of waste. Most previous studies on WWT products refer among others to the estimation of these two parameters, whereas few studies exist on the evaluation of WWT products' effects on biological systems.

Despite the fact that the effluents of WWTPs contain the previously mentioned pollutants at trace levels, they appear to have toxic effect to living organisms and therefore chemical analysis seems to be inadequate for their characterization. Thus, use of bio monitoring assays may enhance the characterization of such discharges. Among the effects which may be observed in aquatic species exposed to treated

and untreated municipal wastewater are increase of metallo thioneins and mixed-function oxidase activity, vitellogenin induction, lymphocyte proliferation, decrease of phagocytic activity, lower production of byssal threads, modulations in the immune system, lower tolerance to air exposure, DNA damage, decreased lysosome retention and higher mortability [17-22]. Organisms used as bio-indicators include the mussel *Mytilus galloprovinciallis* [16]. Mussels are considered efficient indicators for toxicological studies due to their filter-feeding capability, potential to bioaccumulate contaminants, and wide distribution in coastal and estuarine areas [19,23-26].

A broad-spectrum cellular biomarker, considered in this work, is the stability of lysosomal membrane of haemocytes (retention of dye "neutral red") (NRR). Apart from causing lysosomal destabilization, pollutant may act as endocrine disrupters and cause oxidative stress. Oxidative stress induced by environmental pollutants occurs when the production of reactive oxygen species (ROS) exceeds the tolerance levels of an organism and can lead to a variety of disorders of physiological functions of cells. Small concentrations of ROS are involved in physiological processes of the cell, such as control of cell proliferation and play an important role as messenger in signal transduction pathways [27].

An important target of oxidative stress should be the DNA damage [28]. The loss of integrity of DNA suggests the induction of genetic alterations and other irreversible toxic effects to invertebrates [29], always in relation to water quality of the environment where they live. If DNA damage is not repaired, a cascade of biological effects on cell or organism level and finally on population level can be induced. The damage of DNA in a variety of aquatic animals has been associated with growth attenuation, abnormal development and survival reduction of embryos, larvae and adults [30].

Finally, the ''stress on stress'' response (SOS) has been considered as another possible index of general stress, expressed by a reduction of survival time in air due to marine pollution [31]. According to this test, exposure to air, which represents a natural stressor, is superimposed on mussels that have already experienced the effects of several pollutant stressors, such as heavy metals and organic chemicals. Furthermore, the SOS response has proved effective in both laboratory and field studies, after short- or long-term contaminant exposure, using transplanted or indigenous mussels [31-35].

The present work aims to evaluate the effects of WWT products based on objective criteria used internationally and rely on established biomarkers proposed by international organizations. Additionally, it aims to highlight new data related to the effects of WWT products both in cellular and genotoxic level on mussels *Mytilus galloprovincialis*.

Materials and Methods

Collection of mussels-Plant description-Experimental procedure

The WWTP of the city of Thessaloniki is located in Sindos, near the French river and its operation is supervised by the Company of Water Supply and Sewerage of Thessaloniki (EYATh). It serves about 1 million residents by treating daily 120000-150000 m^3 of raw wastewaters (Figure 1). About 5-10% of the total flow is contributed by industry. The plant also receives the greatest part of the local urban run-off, which is mainly composed of atmospheric deposition, and traffic related emissions deposited on the road surface. The treatment process includes screening, grid removal, and primary sedimentation without use of chemical coagulants, conventional activated sludge treatment and effluent disinfection using chlorine gas (Cl_2). The treated wastewater is discharged in Thermaikos Gulf via a channel. Sewage sludge (primary plus excess activated) is anaerobically digested, thickened, and dewatered [36]. The greatest amount of this sludge is deposited in a municipal landfill, while its use as soil amendment is also under consideration by the local authorities [16].

Figure 1: The waste-water treatment plants (WWTP) of the city of Thessaloniki, located in Sindos.

Mussels *Mytilus galloprovincialis* (5-6 cm long) were collected from Chalastra (west side of Thessaloniki, northern Greece), and transported to the laboratory within 1h after the collection. The mussels were maintained without food supply, for 1 week, in order to be acclimated to laboratory conditions. After the acclimation period, 3 groups of mussels (40 mussels/group) were placed in static tanks, containing 10L of aerated seawater. Each group of mussels was treated as follows: group B of animals was treated with 25% v/v of wastewater treatment product collected after chlorination (WWTP) and group C was treated with 50% v/v of WWTP for 30 days. Control group of mussels (Group A) consisted of non-exposed mussels. The water was renewed every 2 days and new quantities of WWTP and food dissolved in seawater were added.

Neutral red retention assay (NRR assay)

This assay for the loss of the dye from the lysosomes to the cytosol was used in at least 50% of the examined cells. The NRR assay was performed according to Lowe and Pipe [37], with small modifications. Haemolymph was withdrawn from the posterior adductor muscle of 10 mussels in physiological saline so as to obtain a 50/50 of cell/physiological saline suspension. The physiological saline, pH 7.3 contained 4.77 g/l HEPES, 25.48 g/l NaCl, 13.06 g/l $MgSO_4$, 0.75 g/l KCl, 1.47 g/l $CaCl_2$. Suspensions were spread on slides, transferred to a lightproof humidity chamber, and allowed to attach. Then, 40 µl of the neutral red (NR) probe were added to the cell monolayer. After a 15 min incubation period, slides were examined systematically under a light microscope every 15 min. The NRR time was measured individually for 10 mussels as the time when the NR dye leaked towards the cytoplasm and the mean derived for each experimental group.

Alkaline single-cell gel electrophoresis (Comet) assay

The procedure used follows the method described by Singh et al. [38] with some modifications described by Dailianis et al. [39]. The presence of comets was examined in haemocyte suspension using a fluorescent microscope, Zeiss axovert inverted fluorescent microscope 200x magnification and WANG epifluorescence microscope (WANG BioMedical, The Netherlands). Four slides were analysed for each group. All slides were coded and the whole slide was randomly scanned. At least 250 cells per slide were analysed. Comets on each slide were scored visually as belonging to one of five predefined classes (Figure 2) according to tail intensity and were given a value of 0, 1, 2, 3, or 4 (from undamaged, 0, to maximally damaged, 4). The percentage of DNA in tail was estimated using ''TriTek Cometscore version 1.5'' software.

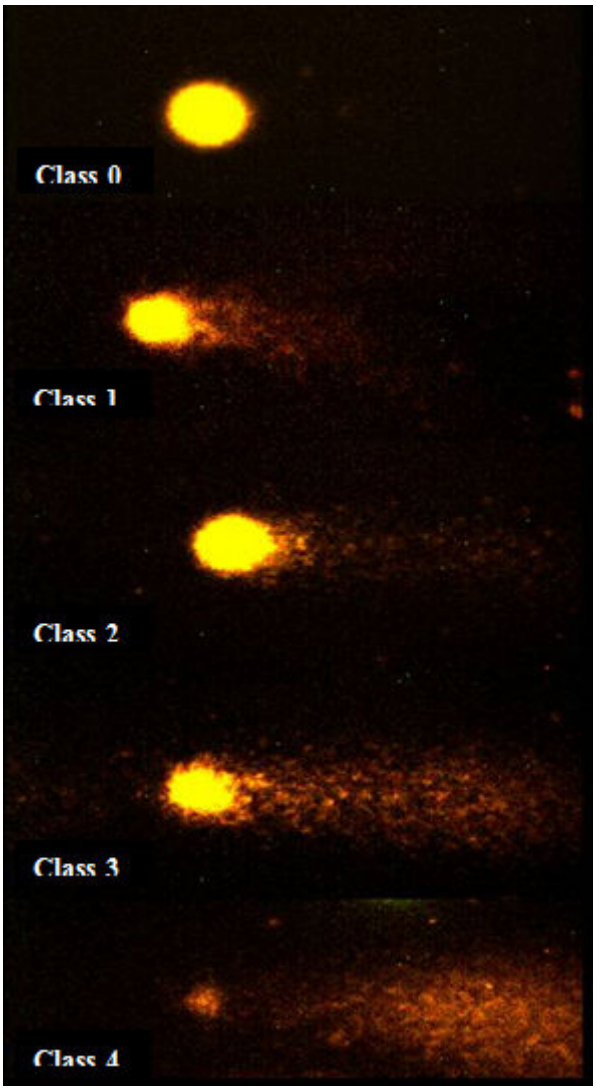

Figure 2: Representative images of comets classified within the five classes of damage (200x magnification).

Stress on stress (SOS)

On their arrival in the laboratory, mussels were placed on trays and were exposed to air at 18°C. Mortality was checked every day. Mussels were considered dead when they did not respond to the squeezing of the valves, after the valves have gaped, or did not recover when placed in seawater.

Data analysis

Data on NRR assay and the SOS response were tested using Duncan's test ($p<0.05$), breakdown and one-way ANOVA), based on previous studies [16]. Tukey test (one way ANOVA, $p<0.01$) was used for the comparison of the grade of DNA damage between control and exposed cells, according to Itziou and Dimitriadis [40,41]. Statistical correlation between the measured parameters (biomarkers) was assessed using Pearson test ($p<0.05$). Analysis was carried out using the STATISTICA statistical package (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 (Figure 3). Determination of NRR times in mussels indicated lower values in haemocytes of mussels treated with both concentration of WWT product (ANOVA, Duncan's test, $p<0.05$) (Figure 4).

Figure 3: Light micrographs exhibiting the various stages that the haemocytes of *M. galloprovinciallis* undergo during the NRR assay.

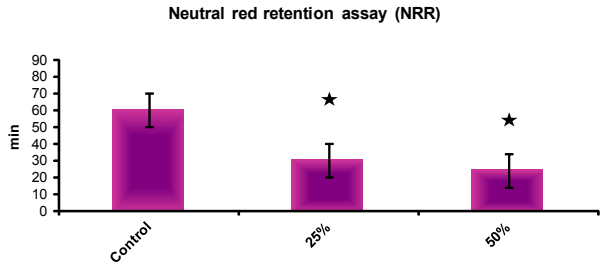

Figure 4: NRR values (min) of the haemocytes of mussels *M. galloprovinciallis* treated with two different concentrations of WWP product (25% and 50%), In each experiment, haemocytes of ten animals were analysed-, indicate significant difference between control value and that observed after the treatment (Duncan's test, $p<0.01$).

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, caused by treatment of mussels with both concentrations of WWT product, compared to the control ones. The DNA damage was further increased in mussels treated with the concentrations of 50% compared to the one of 25%, as showed by the DNA % in tail (Figure 5).

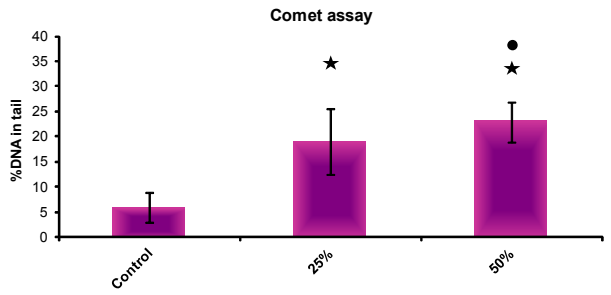

Figure 5: DNA damage of isolated haemocytes of *M. galloprovinciallis* treated with two different concentrations of WWP product (25% and 50%). In each experiment, the tissue of four animals was used and 250 cells per incubation per slide were analysed-, indicate significant difference between control value and that observed after the treatment-, indicate significant difference between values in mussels treated with different concentrations of the WWT product (Tukey test, $p<0.01$).

Stress on stress (SOS)

The results of the study showed statistically lower surveillance time in mussels treated with both concentrations of WWT product, compared to controls (Duncan's test, $p<0.05$) (Figure 6).

Figure 6: SOS of mussels *M. galloprovinciallis* treated with two different concentrations of WWT product (25% and 50%)-?, indicate significant difference between control value and that observed after the treatment (Duncan's test, $p<0.01$).

Correlation analysis

Correlation coefficient analysis among the applied oxidative stress parameters (Pearson's test, $p<0.05$) is listed in Table 1. The results of this table could be summarized as follows:

- There was a strong negative correlation between the NRR times and the levels of DNA damage (r=-1.00).
- There was a strong positive correlation between the NRR times and the values of SOS (r=1.00).
- There was a strong negative correlation between the levels of DNA damage and the values of SOS (r=-0.99).

	NRR	**DNA damage**	**SOS**
NRR	1.00	-1.00	1.00
DNA damage		1.00	-0.99
SOS			1.00

Table 1: Correlation coefficients (r-values, no-parametric Spearman correlation coefficient) for significant correlations between the variables tested in tissues of the tissues of Mytilus galloprovanciallis, the statistically significant correlations are marked with bold.

Discussion

Previous studies have been performed on the effect of WWTPs on aquatic living organisms. It has been reported that these effects may stem from the different chlorinated agents (chlorine gas, chlorine dioxide and sodium hypochlorite) used during sewage cleaning. These chemicals are highly effective in killing the pathogenic microorganisms, but may also cause oxidation of organic and inorganic matter of natural waters, and thus causing a variety of highly toxic disinfection byproducts (DBPs) [42]. The characteristics of natural waters, such as the concentration of natural organic matter (NOM), bromides or iodides, as well as other factors (pH, temperature), together with the type and the quantity of the disinfectant may contribute to the formation of the final DBP mixtures [43-45]. These DBP mixtures may cause adverse effects on marine organisms [46-48], mainly because most halogenated DBPs are metabolized via the oxidative pathway cytochrome P450 [49]. The metabolites generated can be connected with cellular components containing nucleophilic groups, including proteins, phospholipids and glutathione, causing cytotoxicity [50].

The results of the present study showed reduced NRR values in mussels treated with both concentrations of WWTP compared to control mussels. Since seawater physicochemical parameters, such as pH, dissolved oxygen and salinity, were maintained constant during the exposure of mussels to WWTP concentrations, lysosomal destabilization is most likely to be related to the presence of toxic substances. It is possible that the destabilization of the lysosomal membrane is caused by the oxidative action of both decontaminating agents and their by-products mentioned above, resulting in the production of ROS in the endolysosomal system. Moreover, it is likely that heavy metals, which remain in the treated wastewater in very small concentrations, may exert some effect on the values of biomarkers studied.

More specifically, the low NRR values in mussels treated with sewage sample may be due to the ability of heavy metals to alter the efficiency of the membrane-connected proton pumps. This causes an increase in membrane permeability and ultimately leads to loss of acid hydrolases in the cytoplasm, as it has been supported. Besides, the results of chemical analyses conducted by Karvelas et al. [36] concerning Cr, Pb, Ni, Cd and Zn, showed that almost 50% of their

daily input to the WWTP ends up in the sludge and the other 50% is released with the final effluent stream. This suggests that large sized WWTPs may be significant sources of heavy metals to aquatic recipients.

The presence of WWT effluents in the marine environment could lead to the enhancement of oxidative and genotoxic damage in mussels and possibly other marine organisms. ROS production in the endolysosomal system may cause damage to membranes, proteins and DNA. The formation of DNA fragments could be one of the results and could be induced either indirectly via interaction with oxygen radicals, or directly via the inhibition of the activity of repair enzymes. This probably explains the elevated levels of DNA damage in mussels exposed to WWTP compared to the control mussels, as shown by the comet assay. Besides, the statistically significant correlation between the NRR values and the levels of DNA fragments (Pearson's test, $p<0.05$) further enhances the above results. Furthermore, elevated levels of DNA damage in mussels treated with sewage sample may come from some interaction between the metals and the proteins, leading to the formation of ROS, which in turn react with DNA and cause single strand fragments, as has been suggested. These results are in agreement with other studies which refer to the ability of heavy metals to cause damage to DNA, due to their reaction with repair processes of DNA.

The decreased LT50 values recorded in mussels exposed to sewage sample are probably related to the stress that they undergo due to the above factors, as shown by the statistically significant correlation between the values of the biomarker SOS with NRR values and levels of DNA damage (Pearson's test, $p<0.05$). This results in reduced ability of mussels to adapt to further environmental pressure. According to the SOS test, exposure to air, which represents a natural stressor, is superimposed on mussels that have already experienced the effects of several pollutant stressors. The ability of mussels to keep valves closed and survive under aerial exposure is related to the amount of energy (in the form of ATP) delivered to the adductor muscle. In mussels undergoing stress, a part of this energy is used in detoxification processes. Therefore, the amount of energy available in other physiological functions is depleted, resulting in less chance of survival when the mussels are removed from the water. Thus, the survival of mussels in air or ''stress on stress'' response, treated with sewage sample was significantly lower probably as a consequence of exposure to a mixture of remaining contaminants in the surrounding water as mentioned earlier.

Conclusion

In conclusion, investigation of stress indices in tissues of mussels provides a detailed picture of both the health of the organisms and the status of the surrounding environment. The results of the study showed increased levels of stress in organisms treated with sewage samples. The exposure of freshwater mussels to chlorinated effluents reveals that municipal wastewaters have the potential to cause damage to living organisms. Analyses of treated wastewater from the exit of the Wastewater Treatment Plant of Thessaloniki (WWTP of Thessaloniki) held by the Company of Water Supply and Sewerage of Thessaloniki (EYATh) indicated certain remaining residual heavy metal ions after processing (provided by EYATh, personal communication). Our results are probably related to the results of these analyses. However, more parallel biological and chemical monitoring studies are needed to further examine the effects and toxic mechanism of WWTP constituents, minimize its toxicity, and improve the performance of biological cleaning.

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