Olive oil mill wastewater toxicity in the marine environment: Alterations of stress indices in tissues of mussel *Mytilus galloprovincialis*

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**A R T I C L E   I N F O**

Article history:
Received 16 June 2010
Received in revised form 1 November 2010
Accepted 20 November 2010

Keywords:
Olive mill wastewater
Toxicity
Stress-indices
Mussel
Mytilus

**A B S T R A C T**

This study investigated the impact of olive mill wastewater (OMW) as a pollutant of the marine environment, via the detection of stress index alterations in mussels *Mytilus galloprovincialis*. Due to the absence of data concerning the levels of OMW in the receiving waters, mortality test (96 h) was first performed in order to estimate the range of OMW concentration where no mortality occurs. OMW concentrations ranging from 0.01 to 0.1% (v/v) showed no increased mortality and thus were used for the determination of pre-pathological alterations in tissues of mussels. In particular, mussels exposed to either 0.1 or 0.01% (v/v) OMW for 5 days showed significant alterations of stress indices in their tissues. Specifically, decreased neutral red retention (NRR) assay time values, inhibition of acetylcholinesterase (AChE) activity, as well as a significant increase of micronucleus (MN) frequency and DNA damage were detected in haemolymph/haemocytes and gills, compared with values measured in tissues of control mussels. The results of the present study showed that OMW disposal into the marine environment could induce pre-pathological alterations in marine organisms, before severe disturbances, such as disease, mortality, or population changes occur.

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1. Introduction

Olive mill wastewater (OMW), a hardly treated by-product generated during olive oil production, constitutes an important environmental problem, since its disposal into watercourses could lead to deterioration of natural water bodies, pollution and environmental degradation.

Although there are no data concerning the level of OMW in the receiving waters, many OMW constituents have been shown to result in physiological alterations both at the organism and cellular levels. Paixao et al. (1999) emphasized the difficulties in determining which of these parameters were the most significant in causing toxicity. Nevertheless, OMW chemical parameters, such as COD, BOD, low molecular weight phenolic compounds, free fatty acids, as well as phenoxy radicals (Sayadi et al., 2000; Lee et al., 2006), low pH and dissolved oxygen, high solids and organic matter (Amaral et al., 2008) have been investigated for their harmful effects in plants (Aliotta et al., 2002; Cassa et al., 2003; Kistner et al., 2004), aquatic animals (Yesilada et al., 1999; Fiorentino et al., 2003) and bacteria (Yesilada and Sam, 1998; Isidori et al., 2005). Moreover, Labieniec and co-workers reported significant morphological alterations and changes in digestive gland cells of freshwater mussel *Unio tumidus*, after exposure to phenolic compounds located in various plant tissues, such as those of olive trees (Labieniec and Gabryelak, 2006, 2007; Labieniec et al., 2003). However, the effects of OMW substances in marine invertebrates are unclear.

Marine mussels, such as *Mytilus* sp., are commonly used as bioindicators of environmental pollution in coastal waters (Livingstone, 1991), because their physiology is well known and they respond rapidly to the environmental change. In particular, exposure of mussels to xenobiotics, derived from effluents like OMW, can occur through direct contact with contaminated water, thus leading to alterations in their homeostasis. The net result of exposure and toxicity is a measurable endpoint, called biomarker.

Investigation of a battery of biomarkers both in field and laboratory studies, is considered a reliable tool for assessing defensive, genotoxic, clastogenic or even histopathological alterations, before severe disturbances such as disease, mortality, or population changes occur, thus providing a detailed picture of the organisms’ health and/or the status of the surrounding environment (see also Depledge, 1993; Roy and Hanninen, 1993; Bresler et al., 1999; De Lafontaine et al., 2000; Dailianis et al., 2003; Moore et al., 2004). In particular, estimation of lysosomal membrane stability constitutes a very useful index of cellular damage and can be evaluated with the use of the neutral red retention (NRR) assay in lysosomes of different types of cells, such as haemocytes, digestive gland cells and gill cells (Regoli, 1992; Krishnakumar et al., 1994; Petrovic et al., 2001; Dailianis et al., 2003; Domouhtsidou et al., 2006, 2007; Labieniec et al., 2003). However, the effects of OMW substances in marine invertebrates are unclear.

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2. Materials and methods

2.1. Determination of OMW chemical constituents

Fresh OMW was obtained from an olive oil production plant located in Patras (Western Greece), which uses a three-phase decanter centrifugation process for the extraction of olive oil. Because of its seasonal production and its tendency for fermentation, OMW samples were stored at −18 °C, in order to secure a constant composition throughout the experimentation period.

Prior to the experiment, determination of OMW constituents was performed (Table 1). Total nitrogen content was determined after digestion of the samples by the persulfate method (D’Elia et al., 1977), with the use of a Shimazu TOC-VCS/CP analyzer, equipped with TNM-1 TN unit. Total and dissolved chemical oxygen demand (t-COD and d-COD, measured using open and closed reflux methods respectively), total and volatile suspended solids (TSS and VSS, measured using Whatman GFF filters) were estimated according to the Standard Methods for Analysis of Waters and Wastewaters (APHA, 1995). Total sugars were analyzed spectrophotometrically, via the production of a colored sugar derivative after the addition of L-tryptophan, sulphuric and boric acid (Josefsson, 1983). Total phenolic compounds were analyzed spectrophotometrically according to the Folin–Ciocalteu method (Waterman and Mole, 1994) and the syringic acid was used as a reference for phenolics quantification. For all spectrophotometric methods (phosphates, d-COD, sugars, phenolics) a VARIAN Cary 50C UV-Vis spectrophotometer was used.

Metals were measured using an ICP-MS spectrometer (ELAN 6100, Perkin-Elmer Sciex) or a Perkin Elmer A Analyst 300 Atomic Absorption spectrometer (AAS) using an air-acetylene flame (Ca, Mg, K and Na). The quality of measurements was assured by the use of Dorm-2 dogfish muscle (Certified Reference Material for trace metals, National Research Council of Canada). Prior to metal analysis, OMW was digested following the 3030-H method (nitric acid–perchloric acid digestion), where H₂O₂ was used instead of perchloric acid (APHA, 1999).

Values of each OMW parameter tested ranged within previously reported values for OMW (Fountoulakis et al., 2002; Davies et al., 2003; Amaral et al., 2008; El Hassani et al., 2009).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-COD (g/l)</td>
<td>186.66 ± 12.99</td>
</tr>
<tr>
<td>d-COD (g/l)</td>
<td>88.40 ± 1.60</td>
</tr>
<tr>
<td>BOD₅ (g/l)</td>
<td>21.90 ± 1.41</td>
</tr>
<tr>
<td>TS (g/l)</td>
<td>64.68 ± 1.03</td>
</tr>
<tr>
<td>VSS (g/l)</td>
<td>62.18 ± 1.55</td>
</tr>
<tr>
<td>Total phenols* (g/l)</td>
<td>11.1 ± 0.1</td>
</tr>
<tr>
<td>Total nitrogen (g/l)</td>
<td>0.71 ± 0.02</td>
</tr>
<tr>
<td>Total phosphorus (mg/l)</td>
<td>328.30 ± 8.63</td>
</tr>
<tr>
<td>Orthophosphoric (mg/l)</td>
<td>291.33 ± 1.8</td>
</tr>
<tr>
<td>Total sugar (g/l)</td>
<td>41.65 ± 0.07</td>
</tr>
<tr>
<td>pH</td>
<td>5.62 ± 0.2</td>
</tr>
<tr>
<td>Color</td>
<td>Brown-red</td>
</tr>
<tr>
<td>Opacity</td>
<td>High</td>
</tr>
<tr>
<td>Conductivity (mS/cm)</td>
<td>11.28 ± 2.1</td>
</tr>
<tr>
<td>Density (g/cm³)</td>
<td>1.029 ± 0.04</td>
</tr>
</tbody>
</table>

* Expressed with reference to syringic acid.

2.2. Mussel collection and handling

Mussels (5–6 cm long) were collected from a mussel farm located in the north side of Korinthiakos Gulf (Gulf of Kontinova, Galaxidi, Greece), transferred to the laboratory and acclimated without feeding in static tanks, containing recirculated UV-sterilized and filtered artificial sea water (35–40‰ salinity) for 7 days at 15 °C. No mortality was observed throughout the acclimation period.

2.3. Determination of sub-lethal concentrations of OMW in mussels (mortality test)

Since there are no data concerning the levels of OMW in the receiving waters, thus making it difficult to predict the effects of OMW effluent, mortality test (96 h) was first performed in order to estimate the range of OMW concentration where no mortality occurs. OMW concentrations similar to those used in the present study have been previously used for the investigation of OMW toxic effects (Rouvalis et al., 2004; Martins et al., 2008). Briefly, 4 groups of mussels (10 mussels/group) were placed in static glass-tanks, containing aerated (dissolved oxygen 7–8 mg/l at 15 °C and 35‰ salinity) artificial seawater (1 l/mussel) and exposed for 4 days to different concentrations of OMW (1, 0.2, 0.1 and 0.01% v/v OMW), while one group of mussels was maintained under the same conditions without adding OMW (control group of mussels).

Mussels that gaped wide and did not respond to the touch were con-
sidered dead and removed from the tanks. According to the results, mortality obtained in mussels exposed to low OMW concentrations (0.1 and 0.01%, v/v) was negligible, compared with those occurred in mussels exposed to higher OMW concentrations (1 and 0.2%, v/v) in each case. Moreover, there were no pH changes in tanks containing 0.1 or 0.01% (v/v) OMW, thus reducing the potential interference of pH with the obtained results (Amaral et al., 2008).

It is worth noting that mortality test was carried out with the use of only one tank for each OMW concentration tested, thus representing a typical case of pseudoreplication and the LC50 value (0.255% v/v as determined by Probit analysis; data not shown) was based on a three point regression analysis, while the accuracy of the LC50 is 10% at best. Despite these uncertainties, OMW concentrations ranging between 0.1 and 0.01% (v/v) could be considered as non-lethal concentrations of OMW and finally were used for the investigation of pre-pathological effects of OMW on mussels, before other disturbances, such as mortality, occur.

2.4. Experimental procedure for biomarkers’ evaluation in tissues of mussels

Two groups of mussels (20 mussels/tank) were placed in static glass-tanks (301) under conditions mentioned above and exposed for 5 days to either 0.1 or 0.01% (v/v) OMW, while a third group was maintained, without adding OMW, during the exposure period (control group of mussels). Seawater was changed every day and new quantities of OMW and food were added in each tank. During exposure period, no mortality was found in control or OMW exposed groups of mussels.

After the exposure period, mussels were removed and immediately prepared for tissue collection. In brief, haemolymph was withdrawn from the posterior adductor muscle of 10 individuals (control and OMW-exposed mussels respectively), using a sterile 1 ml syringe with an 18G1/2 needle, containing Alseve buffer (ALS buffer; 20.8 g/l glucose, 8 g/l sodium citrate, 3.36 g/l EDTA and 22.5 g/l NaCl, pH 7 and 1000 mOsm) and 40 μl of haemolymph suspension from each individual per experimental and/or control group of mussels was spread on slides, transferred to a lightproof humidity chamber and allowed to attach. The aforementioned process was repeated twice and all slides were prepared for either NRR or OMW exposed groups of mussels.

For the estimation of MDA and MT content, AChE activity and DNA damage, animals from either control or OMW-exposed mussels respectively, were used. One ml of pooled haemolymph from each group was centrifuged (1200 rpm, 10 min, at 4°C) and the cell pellet was resuspended in 40 μl ALS and 360 μl of 0.1% low-melting-point agarose (0.1% LMA) in Kenny’s salt solution (0.4 M NaCl, 9 mM KCl, 0.7 mM K2HPO4, 2 mM NaHCO3) was finally added. The whole cell suspension was layered on 4 glass slides (100 μl cell suspension/slide), coated with 200 μl of 2% normal-melting-point-agarose (NMA) (NMA) in TAE solution (40 mM Tris-acetate, 1 mM EDTA), and covered with a coverslip. After solidification of agarose, the coverslip was removed and slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% N-lauryl sarcosine, 1% Triton X-100, 10% DMSO, pH 10) at 4°C for 1 h. In dark. Slides were rinsed in distilled water, placed on horizontal gel electrophoresis tray and covered with freshly prepared electrophoresis buffer (0.075 M NaOH, 1 mM EDTA, pH > 12) for 20 min to allow the DNA to unwind. After the end of electrophoresis (25 V/cm, 300 mA for 15 min), slides were placed on a staining tray and was neutralized neutralizing solution (0.4 M Tris, pH 7.5) for 5 min. This procedure was repeated three times, changing the solution between washes. Then slides were drained and 50 ml of filtered ethidium bromide (20 mg/ml) was added with a coverslip overlaid for further comet analysis. The presence of comets was examined in haemocytes using an Axio Carl Zeiss fluorescent microscope x 100 magnification (Carl Zeiss Micro-imaging, Germany), equipped with Isis fluorescence imaging system analysis (Metasystems, Germany), (Fig. 1B). All slides were coded and randomly scanned.

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D. Danellakis et al. / Aquatic Toxicology 101 (2011) 358–366

361

Fig. 1. Micronucleus and Comet assay techniques. A: Light microscope views of haemocytes, showing the presence of micronuclei (arrows), (magnification 100 ×); B: the presence of comets, showing different levels of DNA damage in haemocytes, as detected with comet analysis method (Axio Carl Zeiss fluorescent microscope, equipped with Isis fluorescence imaging system analysis).

and Dailianis, 2010) and other cell types of mussels (Dailianis et al., 2005).

2.4.3. Determination of AChE activity in haemolymph and gills of mussels

For estimation of AChE activity, 1 ml of pooled haemolymph from each group was centrifuged (10,000 rpm, 10 min at 4 °C) and stored at −80 °C no more than 1 day before measurement. In addition, 1 g of gills from each group was homogenized in 4 volumes of 0.1 M Tris buffer, pH 8, centrifuged at 20,000 rpm for 30 min and the crude supernatants were stored at −80 °C until analysis. Measurement of AChE activity was carried out according to the procedure described by Dailianis et al. (2003), with the use of acetylthiocholine iodide as substrate to initiate the enzymatic reaction. Specific activity measured in tissues of mussels was expressed as Units/mg protein from 4 measurements (biological replicates). Each measurement was verified in triplicate (technical replicates).

2.4.4. MT determination in gills of mussels

MT content in gill extraction was estimated spectrophotometrically, using a biomolecular MT assay proposed by Viarengo et al. (1997). This method is based on the detection of cytosolic sulphhydryl (–SH) residues, obtained after ethanol/chloroform fractionation of each sample homogenate and the improvement of MT recovery after the addition of RNA (as co-precipitant), a procedure that allows the elimination of low molecular weight soluble thiols, such as GSH and cysteine, which could interfere with MT quantification. The amount of MT was defined assuming a cysteine content in mussel MT of 29% (Mackay et al., 1993). The MT concentration was calculated using reduced glutathione (GSH) as a reference standard. Results are expressed as nmol MDA/mg protein from 4 measurements. Each measurement was verified in triplicate.

2.4.5. Evaluation of lipid peroxidation in haemocytes and gills of mussels

The extent of LPO was measured as thiobarbituric acid reactive substances (TBARS) and expressed as MDA equivalents, which is a major LPO metabolite (along with lipid hydroperoxides, hexanal and conjugated dienes) and considered a reliable indicator of oxidative damage of cellular membranes (Tavazzi et al., 2000). In brief, 1 ml of haemolymph from each group was centrifuged (1200 rpm, 10 min, at 4 °C) and the supernatants were used for MDA measurement. Similarly, 1 g of gills from each group was homogenized with 4 ml of ice cold buffer (Tris–HCl 50 mM, 1 mM EDTA, 0.15 M KCl, pH 7.4), the homogenate was centrifuged at 10,000 rpm for 15 min at 4 °C and the resulting supernatant was used for MDA analysis.

MDA measurement was carried out according to method described by Vlahogianni and Valavanidis (2007). Specifically, supernatants (1 ml) were diluted in 2 ml of trichloroacetic acid (TCA)–thiobarbituric acid (TBA)–HCl (15% w/v TCA, 0.375% w/v TBA in HCl 0.25 N), containing 0.02% butylated hydroxytoluene (BHT) to prevent further peroxidation process and boiled for 15 min at 90 °C. Thereafter, the samples were cooled at room temperature, centrifuged at 3000 rpm for 10 min and were measured spectrophotometrically at 535 nm. A molar extinction coefficient (1.5 × 105 M–1 cm–1) (Wills, 1969) was used for the determination of MDA concentration. The results expressed as nmol MDA/mg protein from 4 measurements. Each measurement was verified in triplicate.

2.4.6. Determination of protein content

When appropriate, protein content in haemolymph and gills of mussels was determined according to the Bradford method (1976) with the use of Bovine Serum Albumin (BSA) as a standard.

2.5. Statistical analysis

Values of AChE activity, MT, MDA and DNA damage are means ± SD from 4 different measurements in each case (each measurement corresponds to each group of either control or OMW-exposed mussels), while NRR and MN values represent the mean values ± SD obtained after analysis of samples derived from 10 individuals of either control or OMW-exposed mussels, in each case. Data sets were checked for homogeneity of variance (Levene’s test of equality of error variances, SPSS Inc. 16) and the statistical significance of differences between stress indices obtained in tissues of control and OMW-exposed mussels were assessed by ANOVA. Post-hoc multiple comparison (Bonferroni test, p < 0.05) was conducted for NRR, MN frequency, AChE activity, MT and MDA, in
order to identify where significant differences occurred at or above the 95% confidence level, while data on the levels of DNA damage in haemocytes of OMW-exposed and control mussels were tested with Kruskal–Wallis non-parametric statistic ($p < 0.05$).

### 3. Results

#### 3.1. Evaluation of NRR in haemolymph/haemocytes of mussels

None of the studied parameters described in the results below changed significantly after 5 days under control conditions (see supplementary material, SM Table 1). Therefore, we used measurements performed in control mussels after 5 days of exposure in the laboratory in all subsequent comparisons with the OMW-exposed mussels. Mussels exposed to either 0.1 or 0.01% (v/v) OMW showed a concentration-dependent decrease of NRR times (43 ± 12 and 22 ± 8 min respectively), compared with values obtained in haemocytes of control group of mussels (93 ± 23 min) in each case (Fig. 2).

#### 3.2. MN test and detection of DNA damage in haemocytes of mussels

High frequencies of either MN or other abnormalities tested (two micronuclei, binucleated and eight-shaped cells) were found in haemocytes of mussels exposed to 0.01 or 0.1% (v/v) OMW for 5 days compared with those in haemocytes of control mussels (Fig. 3A). Total frequency of nuclear abnormalities, apart from MN, was significantly higher in haemolymph of OMW-exposed mussels compared with the control mussels (Fig. 3B).

According to the results of the comet assay, a concentration-dependent increase in levels of DNA damage (expressed as % DNA in tail) were detected in haemocytes of OMW-exposed mussels, compared with levels found in cells of control mussels (Fig. 3C).

#### 3.3. AChE activity in haemolymph and gills of mussels

Significant inhibition of AChE activity was observed in both haemolymph and gills of mussels exposed to 0.1% or 0.01% (v/v) OMW compared with control group of mussels (Fig. 4A and B).

#### 3.4. Determination of MT content in gills of mussels

Mussels exposed to 0.01 or 0.1% OMW showed a slight but significant increase of MT levels compared with the levels in tissues of the control group (Fig. 5A).

#### 3.5. Determination of lipid peroxidation (MDA content) in tissues of mussels

MDA content measured in haemolymph of mussels exposed to different concentrations of OMW (0.1 and 0.01% v/v OMW) showed a concentration-dependent increase, while a slight but significant increase of MDA content was detected in gills of exposed mussels compared with the values measured in the respective tissues of control mussels (Fig. 5B).
Fig. 4. Determination of AChE activity in haemolymph (A) and gills (B) of mussels exposed to 0.01 and 0.1% (v/v) OMW for 5 days. Results, expressed as units/mg protein, are mean ± SD from 4 independent measurements (each measurement was carried out in triplicate). a, indicates significant difference from the control (5 days). Values that share the same symbol are statistically different from each other.

4. Discussion

The impact of OMW disposal in aquatic environments is considered of great interest during the last decades but little is known concerning the effects of OMW effluents on marine invertebrates. To our knowledge, the present study is the first to show OMW-induced toxic effects in tissues of mussel *Mytilus galloprovincialis*, with the use of a battery of well-known stress indices.

4.1. Cytotoxic effects of OMW in haemocytes of mussels

Lysosomes constitute main sites of sequestration and detoxification of toxic inorganic and organic pollutants (Moore et al., 2006). Their functional membrane integrity has been linked with many aspects of pathology associated with toxicity, thus representing a good diagnostic biomarker of individual health status (Moore et al., 2006; Koukouzika and Dimitriadis, 2008). In the present study, haemocytes of OMW-exposed mussels showed increased loss of lysosomal membrane integrity, a fact that is in accordance with previous studies, which demonstrated that lysosomal destabilization reflects gradients of complex mixtures of chemicals in water and sediments (Da Ros et al., 2002; Pisoni et al., 2004; Schiedek et al., 2006).

Since seawater physicochemical parameters, such as pH, dissolved oxygen and salinity, were maintained constant during the exposure of mussels to low OMW concentrations, lysosomal destabilization is most likely to be related to the presence of OMW toxic substances. In fact, phenolic compounds which are regarded among substances which are primarily responsible for OMW toxicity (Sayadi et al., 2000) could mediate the loss of lysosomal membrane integrity in haemocytes of mussels, due to the ability of phenols to enhance membrane fatty acid saturation process, thus decreasing membrane fluidity (Melin et al., 1988). In addition, there is evidence that during phenolic compound degradation, hydroxyl radicals, as well as highly reactive metabolites can be generated, thus leading to cellular damages, such as destabilization of membranes (Seiler, 1991; Fahr et al., 1999). Moreover, the presence of metal ions in the OMW, such as copper and cadmium could contribute to the loss of lysosomal membrane integrity, since their involvement in the induction of membrane destabilization in cells of mussels has been reported in previous studies (Viarengo et al., 1985, 1987; Cajaraville et al., 2000; Kohler et al., 2002).

4.2. Neurotoxic effects of OMW in tissues of mussels

AChE is commonly located as a transmembrane protein in different cell membranes of invertebrates, such as gills and digestive gland and significantly elevated levels of AChE activity have been also reported in the haemolymph of mussels (Dailianis et al., 2003). Despite the fact that AChE involvement to nerve impulse transmission in tissues with high neuromuscular activity is well-established (Najimi et al., 1997; Walker and Thompson, 1991), there is less knowledge about the existence of cholinergic transmission in the peripheral nervous system in molluscs (Heyer et al., 1973; Mercer and McGregor, 1982). However, due to AChE sensitivity to neurotoxic substances, determination of its activity has been widely used as a sensitive stress biomarker in mollusc tissues (Bocquene et al., 1997; Galloway et al., 2002; Dailianis et al., 2003; Valbonesi et al., 2003; Rickwood and Galloway, 2004).
A wide range of contaminants could affect AChE activity (for more information see Lehtonen et al., 2003; Jebali et al., 2006; Vioque-Fernandez et al., 2007; Bonacci et al., 2008) and the present study showed for the first time a significant inhibition of AChE activity in tissues of OMW-exposed mussels, suggesting the presence of neurotoxic substances in OMW. Indeed, enzymatic activity could be affected by certain organic and inorganic substances, such as phenolic compounds and metals, as well as nitrogen-derivatives, such as ammonia, a hypothesis that is in accordance with previous studies on the effects of contaminant mixtures (Chukwu, 1993; Yadav et al., 2007). In particular, ammonia of polluted water has been reported to be neurotoxic to aquatic organisms (Ekweozor et al., 2001), while Basu et al. (2009) showed that components, such as polyphenols, derived from pulp and paper mill effluents, could impair fish reproduction via inhibition of neurotransmitter receptors and enzymes, such as AChE. Moreover, although there is evidence that phenolic compounds have similar properties to humic substances, especially the ability to chelate metal ions (Cruz et al., 2000), thus minimizing metal-mediated toxic effects, their antioxidant activity and oxidation potential is not simple. In fact, previous studies showed that concentrations of phenolic compounds being present at 1% (v/v) OMW could lead to membrane dysfunction, thus leading to necrotic cell death and some neuronal degenerative diseases (Eckert et al., 2003; Martins et al., 2008), while there is evidence that high concentrations of phenolic compounds and metal ions, such as copper, could enhance oxidative and genotoxic effects in cells of freshwater mussel U. tumidus (Labieniec et al., 2003; Labieniec and Gabryelak, 2007), as well as neurotoxicological effects, since some metals like zinc, copper and cadmium, have been reported to exert inhibitory effect on AChE activity (Najimi et al., 1997; Chen and Liao, 2003).

4.3. Oxidative and genotoxic effects of OMW in haemocytes of mussels

The presence of OMW effluents in the marine environment could lead to the enhancement of oxidative and genotoxic damage in mussels and possibly other marine organisms. Increased levels of MDA and MT measured in tissues of exposed mussels could be related to the presence of pro-oxidants in OMW effluent. In particular, OMW compounds, such as phenolics, have similar properties to humic substances, especially the ability to chelate many metal ions (Cruz et al., 2000), which in turn could mediate their autoxidation, the generation of semiquinone and reactive oxygen species, such as O₂⁻ and *OH and the concomitant enhancement of lipid peroxidation process (Khan et al., 2000; Kobayashi et al., 2004). Lipid peroxidation products, such as MDA, could exert extremely deleterious effects on cells and tissues, by inducing damages to DNA or cellular proteins (Leuratti et al., 1998). Moreover, since a number of studies indicated the induction of MT either directly by metals or indirectly by organic aromatic compounds, via the induction of oxidative stress in tissues of mussels (Kling et al., 1996; Daliliani and Kaloyianii, 2007), it could be suggested that MT induction in gills of mussels could be a result of metal ions, such as zinc, copper and cadmium, being present in OMW.

The present study also showed increased levels of nuclear abnormalities and DNA damage in haemocytes of OMW-exposed mussels. The appearance of either MN or other abnormalities within cells could be a result of incomplete incorporation of fragment chromosomes and chromatid breakages which may be caused by clastogens or spindle dysfunctions, and objectively demonstrates the level of genotoxic damage in response to the presence of mutagenic substances (Heddle et al., 1983; Carrano and Natarajan, 1988). Moreover, disturbance of DNA integrity/DNA damage) could be induced either indirectly via interaction with oxygen radicals, or directly via the inhibition of the activity of excision repair enzymes (Eastman and Barry, 1992; Speit and Hartmann, 1995). In fact, since phenolic compounds and chelating metal ions could enhance genotoxic effects, as it was early found in HL-60 cells (Kobayashi et al., 2004) and in tissues of mussels (Labieniec et al., 2003; Labieniec and Gabryelak, 2006), we propose that genotoxic effects of OMW could be primarily due to the high content of phenolic compounds, as well as the presence of metal ions. On the other hand, taking into account the difficulties in determining the derivation of each component in the receiving waters, especially those derived from OMW, its ability to induce genotoxic and oxidative effects is not simple (Jacobi et al., 1998; Hotta et al., 2001) and further investigation is needed in order to clarify the mechanisms of OMW toxicity.

5. 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. Although the present study showed OMW ability to induce cytoxic, oxidative, neurotoxic and genotoxic effects in tissues of mussels, more studies are need to test the hypothesis that phenolic compounds and trace metals are responsible for toxicity of OMW, and to determine the effects and toxic mechanism of different constituents of the complex OMW mixture. Furthermore, the investigation of pre-pathological alterations in tissues of mussels could be applied in parallel with various methods performed for minimizing OMW toxicity, in order to verify both the treatment method efficacy and the concomitant environmental impact of OMW effluents.

Acknowledgements

The authors express their thanks to Dr. Demopoulos, N.A. and Dr. Stephanou, G. (Professors in the Department of Genetics, Cell and Developmental Biology, Department of Biology, University of Patras) for kindly providing the laboratory equipment for conducting comet analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2010.11.015.

References


