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Assessment of the toxicity of crude oil in *Sinonovacula constricta* clams

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Abstract – This study is aimed at investigating the toxic effects on clams (*Sinonovacula constricta*) exposed to selected concentrations of individual crude oil: water accommodated fraction (WAF) and chemically enhanced (or dispersed) WAF (CEWAF). For this purpose, a study was performed on clams exposed to 0.04 mg L^{-1} and 0.01 mg L^{-1} WAF and CEWAF for 15 days, using parameters of antioxidant defense and oxidative damage. The accumulation of total petroleum hydrocarbon (TPH) in the gills of clams was dependent on time and dose, and CEWAF accumulation was higher than WAF accumulation. Exposure of clams to CEWAF oil resulted in significantly ($p < 0.05$) elevated ethoxyresorufin-O-deethylase (EROD) activity compared with exposure to controls. The level of EROD induction was concentration-related, as indicated by the induction observed in clams exposed to higher concentrations of CEWAF. Oxidative damage indicators (DNA strand breaks) were also measured in gills to assess the effects of the selected crude oil by alkaline unwinding assay. Results showed that DNA damage was significantly induced, except in the low-level groups of WAF, and different trends were detected with time of exposure. Significant correlations between TPH uptake and both EROD activity and DNA damage can be used as suitable tools for integrated levels of study on the biomarkers of crude oil exposure.

Keywords: EROD activity / DNA damage / toxicity / assessment

1 Introduction

In recent decades, development of the offshore oil industry and shipping activities have increased the risk of water contamination by petrochemical products from navigation accidents and oil spills. Petroleum products released from the oil industry are composed mainly of non-cyclic and cyclic hydrocarbons, nitrogen, oxygen, sulfur compounds, produced water, alkylphenols, and heavy metals (Wake 2005). Chemical dispersants are used to diffuse crude oils after spills and discharges to prevent the coating of inshore areas inhabited by a variety of marine organisms (Khan and Payne 2005). Laboratory studies on observed toxicity (Carr and Linden 1984) were further validated by field reports from the oil spills for which dispersants were used (Lewis and Aurand 1997), and many effective and less toxic dispersants have been developed since. Dispersant efficiency is influenced by sea energy, temperature, salinity, and the nature of crude oil (Ramachandran et al. 2004). Risks to aquatic organisms could arise from exposure to both dispersants and dispersed oil, as well as a combination of the two, which may either exacerbate or mitigate toxic effects during chemical dispersion (Getter and Baca 1984).

Assessing the effect of petroleum on the marine environment is complicated because it contains complex mixtures of organic compounds. The chemical pollution of aquatic ecosystems led to the development of analytical techniques that are capable of detecting dispersed pollutants (Pacheco and Santos 1998). The risk of fish poisoning from oil and oil-dispersant treatments may be assessed in terms of exposure to polycyclic aromatic hydrocarbons (PAHs) and changes in exposure of PAHs after the use of dispersants (Ramachandran et al. 2006). Ethoxyresorufin-O-deethylase (EROD) belongs to a family of cytochrome enzymes and is induced in the presence of xenobiotic compounds including PAHs (Hodson et al. 1991). EROD could be a sensitive biomarker in detecting the effects of PAHs (Yanxia Tao 2013). Animals usually possess high levels of this enzyme family in organ tissues involved in food processing, such as the hepatopancreas in crustaceans (Da Silva Rocha et al. 2012a; Snyder 1998). As the most important PAH metabolic enzyme, CYP450 can be assessed by measuring the activities of EROD and aryl hydrocarbon hydroxylase (Cappello et al. 2013; D'Agata et al. 2014). EROD activity is a reliable and repeatable measure of exposure to hydrocarbons from crude oil (Ramachandran et al. 2006).

Among the current environmental genotoxicity tests, the micronucleus (MN) test is one of the most frequently used, serving as an index of cytogenetic damage for over 30 years (Fenech et al. 2003). The alkaline version of the comet assay (pH > 13) is capable of detecting single-strand breaks, double-strand breaks, alkali-labile sites (apurinic/apyrimidinic sites),

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cross-linking, and incomplete DNA repair sites (Valverde and Rojas 2009). It is a quick and sensitive method for the detection of DNA damage in single cells, induced by a variety of genotoxic agents (Tice et al. 2000). DNA strand breakage in bivalves using alkaline unwinding has been approved as an effective biomarker to assess the genotoxicity of pollutants (Ching et al. 2001). Exposed Atlantic cod (*Gadus morhua*) exhibited DNA damage as a result of PAH-induced formation of bulky DNA adducts (Aas et al. 2000). PAHs in oil may also damage DNA through the formation of reactive oxygen species (ROS), such as highly reactive hydroxyl radicals (-OH) (Regoli et al. 2002). However, oil is a complex mixture and other mechanisms and components may influence DNA (Taban et al. 2004). Oil spills can widely distribute petroleum hydrocarbons in the marine environment and seriously affect the DNA of filter-feeding bivalve populations (Lyons et al. 2011). Bivalves exposed to oil spills in inshore waters are at risk of DNA damage and interspecies sensitivity (Hamoutene et al. 2002). After previous oil spills, several studies evaluating PAH content (Nahrgang et al. 2010; Pérez-Cadahía et al. 2004; Xiu et al. 2014), total hydrocarbon levels, and immune response (total hemocyte number, superoxide generation, and phagocytosis) (Nogueira et al. 2009) in tissues of some aquatic organisms have been reported.

Oil can occur at sea as water-soluble fractions, dispersed oil droplets, particle-bound bodies, or emulsions (Aas et al. 2000) The bioavailability and toxicity of oil greatly depend on the type of oil and degree of physical, chemical, and biological degradation (Rice 1985). These are important points to consider when performing realistic studies on oil toxicity in the laboratory. Because of their feeding behavior, bivalve mollusks readily take up lipophilic organic contaminants, such as PAHs from the marine environment, which cause various physiological effects (Livingstone et al. 1995) In the present study, we used an Agamaki clam (*Sinonovacula constricta)*, an economically important shellfish in Asia, as a model organism to investigate total petroleum hydrocarbon (TPH) concentrations, EROD activity, and DNA strand breaks in the gills of clams exposed to selected concentrations of individual crude oil; Water accommodated fraction (WAF) and chemically enhanced (or dispersed) WAF (CEWAF) were used during the 15-day laboratory experiments. This study was conducted to measure the toxic effects of the collected oil spill samples from sea water on the accumulation in tissue, the enzyme activity and the genetic damage (by means of the alkaline unwinding assay) in the gills of *S. constricta* under laboratory conditions. These three parameters are the first steps in assessing the damage caused by oil spills to rich coastal environments, which offer preliminary information for environmental contamination monitoring. Moreover, because *S. constricta* is a commercial species, bioaccumulation findings from juvenile scallops may contribute to studies on resource protection.

2 Materials and methods

2.1 Crude oil and dispersant

Pinghu sour crude (viscosity of 45.42 cP at 30 °C) is a medium light crude oil from the East China Sea. Weathering was achieved by bubbling compressed air through the oil until 20.5% of the volatiles in the oil had been driven off. Dispersant-type Jiefeite 001, which is used on highly viscous oils and emulsions, was used to disperse the oil. This chemical dispersant includes aliphatic hydrocarbons, propylene glycol, and some sulfonic acid salts. Jiefeite 001 is a carbonated-aqueous surfactant, which was purchased from Xinluo Filter Material Company (Shanghai, China), and is to be used on higher viscosity oils and emulsions.

2.2 Preparation of WAF and CEWAF

Fresh WAF was prepared daily by mixing crude oil with seawater at a ratio of 1:10 for 24 h at 20 °C in sealed containers with minimum head space. The vortex was adjusted to no more than a third of the height of the mixture from the oil-water interface (Singer et al. 2000). This particular ratio of oil to water has been reported as the optimal ratio to maximize the TPH content of the water column (Gagnon and Holdway 2000). The mixture was allowed to settle for 1 h to allow the separation of water and oil phases. The water phase was drained through a tap at the bottom of the tank for testing.

Pinghu crude oil and Jiefeite 001 dispersant were used along with sand-filtered seawater to prepare CEWAF following procedures described in Ramachandran et al. (2004). The dispersant Jiefeite 001 was added with a Pasteur pipette to the surface of the oil-water mixture and allowed to stir for an additional hour. The resulting solution contained droplets of dispersed oil, and was allowed to settle for another hour before the cloudy emulsion layer at the bottom was drained. Both were securely covered and mixed on a stir plate for 18 h. Following mixing, each mixture was then allowed to settle for 6 h. The water portion was then siphoned out from below the oil slick providing the 100% chemically dispersed water-accommodated fractions (CEWAF).

2.3 Animals and treatments

Healthy *S. constricta* averaging 14.62 ± 2.26 cm in body length was obtained from a commercial farm in Qidong, Jiangsu, China. The clams were subjected to a one-week acclimatization period in a plastic tank of 100 L capacity before the exposure test. The tanks contained aerated seawater (salinity 26‰, pH 8.1) at 23.6 ± 1.5 °C for seven days. During the acclimatization period, the water in each tank was completely replaced once a day and the clams were fed with dried powder of *S. platensis* (30 mg for each individual per day) daily during the experimental period.

After acclimatization, the clams were transferred to aquaria (40 L) and experimental conditions (salinity, pH, temperature, and feeding) were maintained as mentioned in the preceding paragraph. In the treatment aquaria, the clams were exposed to different WAF (0.04 mg L^{-1} and 0.01 mg L^{-1}) and CEWAF (0.04 mg L^{-1} and 0.01 mg L^{-1}) concentrations. Three triplicates were made for each level and 60 clams were used in each aquarium. Seawater containing the same concentrations of crude oil was added to maintain the corresponding concentrations of WAF and CEWAF during the experiment. Water used was replaced every morning.

The WAF and CEWAF concentrations used in this study were selected based on the preliminary tests for acute toxicity, in which the effects of WAF and CEWAF were non-lethal and could be experimentally detected using the present exposure periods. We set two high concentrations of toxicants, which are often used in laboratory exposures to elicit clearly distinguishable effects.

During the experimental period (15 days), clams were sampled 3-days and 15-days, respectively. The gills of each clam were used for further assays. A total of 12 clams, including controls, were sampled and concentrated each day. The clams were sampled and blended for enzyme assays, DNA damage, and TPH uptake analyses.

2.4 Tissue sample preparation

The gill fractions were prepared according to the method described by Bonacci (2003). Pieces of gills, muscle, mantle, and digestive gland samples were excised and added to 50 mM potassium phosphate buffer (pH 7.7) containing 20 mM Tris-HCL, 1.5 mM Na_2EDTA, 1.0 mM dithiothreitol, and 10% glycerol (V:V). Samples were kept on ice during the entire procedure. After centrifugation for 30 min at 4 °C (3000 rpm), the supernatants were collected for the analysis of EROD activity, DNA damage, and protein contents.

2.4.1 EROD assay

EROD activity was measured according to the method by Pohl and Fouts (1980). The reaction mixture contained 100 AL supernatant, 10 μL 0.2 Mmo 7-ethoxyresorufin, 10 μL 6 mM NADPH, and 1.88 mL phosphate buffer (0.125 m pH 7.7, containing Na_2EDTA, 0.05 m, 2–4 °C). The reaction was allowed to proceed for 10 min at RT and was stopped by the addition of 0.5 mL carbinol. Then, incubation vials were centrifuged to remove the precipitated microsomal protein. The supernatants were transferred to vials for the measurement of resorufin concentrations using a luminescence spectrometer (Model LS55, PerkinElmer, UK) at an excitation wavelength of 560 nm and an emission wavelength of 580 nm. Resorufin was identified and concentrations were calculated by comparison with retention times and response of resorufin standards. Blanks corresponded to $t = 0$ min and quantification was achieved with standard additions of resorufin.

2.4.2 DNA damage assay

The alkaline unwinding assay used in the study was previously described by Thilagam (2010). Briefly, gill samples were gently cut into fine pieces and incubated with 1 mL of TNE buffer (0.05 M Tris, 0.1 M NaCl, 0.1 M EDTA, 0.5% SDS, pH 8.0) at 37 °C for 24 h. After incubation, saturated NaCl was added and the mixture was centrifuged at 12 000 g for 20 min. The supernatant was added to an equal volume of buffered phenol/chloroform/isoamyl alcohol (PCI) (25:24:1, v/v, pH 8.0) and gently mixed. The sample was allowed to settle for 5 min before 12 000 g centrifugation at 4 °C for 5 min. The transferred aqueous layer was then digested using 5 μL of ribonuclease A (10 mg mL^{-1}) for 30 min at 37 °C and extracted successively using equal volumes of PCI. The DNA was precipitated by adding cold absolute ethanol and sodium acetate buffered to pH 5.2. The sample was centrifuged at 12 000 g for 15 min; and the pellet was rinsed with 500 L of 70% ethanol, air dried, and dissolved in 400 L of TE buffer (10 mM Tris, 1 mM EDTA). The DNA sample was separated into two equal portions for fluorescence determination of double-strand DNA (dsDNA) and single-strand DNA (ssDNA). The fluorescence of dsDNA and ssDNA was measured using a spectrofluorometer with an excitation wavelength of 360 nm and an emission wavelength of 450 nm. The DNA strand break was calculated as F values, which was determined by dividing the double-strand value by the double-plus-single-strand value in the sample.

2.4.3 TPH uptake analyses

The gill tissues were transferred into a glass beaker. Homogenization was performed without any solvent addition using an UltraTurrax (IKA-Maschinenbau, Staufen, Germany). The rod and blades were rinsed successively with cyclohexane and distilled water between samples. The homogenate was then transferred to vials sealed with a Teflon lid and stored at −80 °C until analysis. TPH in the tissues were analyzed by ultraviolet spectrophotometry (Neff and Anderson 1975). This method was tested with 1 mL of TPH standard spiked in unpolluted clam tissues, obtaining a recovery up to 83%.

2.5 Statistical analysis

Data were subjected to ANOVA to determine the differences in enzyme activity before and after exposure, as well as within the groups. Each value was quoted by mean standard deviation (S.D.) and analyzed using one-way ANOVA. The significant difference between the control and the exposed clams was tested with Dunnet's test. Pearson test was employed to test correlative analysis. Statistical significance was defined as $p < 0.05$ throughout. Data were tested using SPSS 16.0 for Windows.

3 Results

3.1 EROD activities

The EROD responses of WAF and CEWAF are shown in Figure 1. The results showed that the EROD activities for the 0.01 mg L^{-1} and 0.04 mg L^{-1} CEWAF treatment groups increased and became significantly different from control values by day 3 ($p < 0.05$). In addition, the EROD activities remained high and significantly different from those of the control until the end of the experiment (day 15). However, significant effects on EROD activity were found only in the 0.04 mg L^{-1} WAF treatment group at day 3 and day 15.

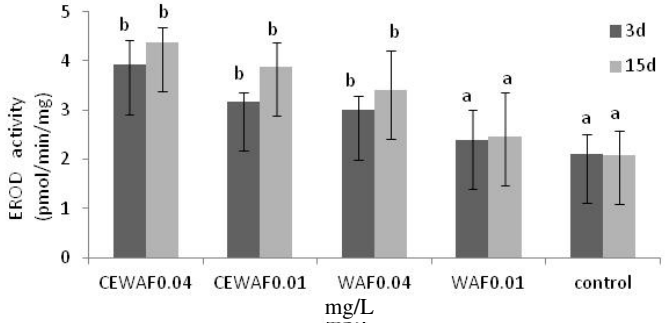

Fig. 1. Activity of hepatopancreas ethoxyresorufin-O-deethylase (EROD) of *Sinonovacula constricta* clams 3d and 15d exposed to clean water and different WAF and CEWAF concentrations. control, clean water. Different letters (a and b) denote significant difference ($p < 0.05$).

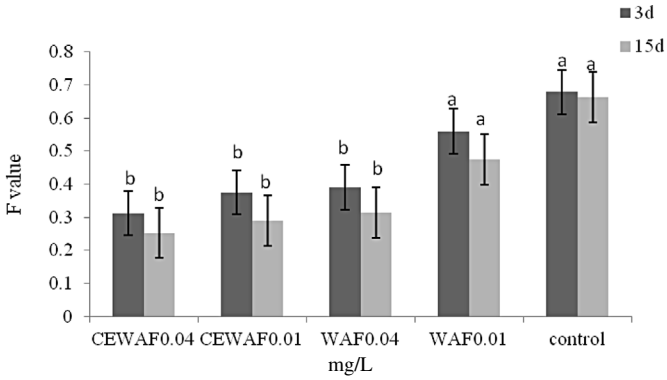

Fig. 2. *F*-value of DNA damage of *Sinonovacula constricta* clams 3d and 15d exposed to clean water and different WAF and CEWAF concentrations. control, clean water. Different letters (a and b) denote significant difference ($p < 0.05$).

3.2 DNA damage

After exposure to WAF and CEWAF, the *F*-value decreased during the experimental period (Fig. 2), whereas significant inhibition ($p < 0.05$) occurred in groups of 0.04 mg L^{-1} after exposure to WAF. However, a significant decrease ($p < 0.05$) in all CEWAF-treated groups was observed throughout the exposure period.

3.3 Relationship between EROD and DNA damage levels

The correlation between the induction of *F*-value at the protein and enzyme activity (EROD) level differed between CEWAF and WAF (Fig. 3). With CEWAF, the EROD activities and *F*-values of proteins were consistent in the case of clams; Pearson product-moment was $r = 0.953$ for EROD ($r^2 = 0.909$) activity. For WAF, the correlation between EROD activity and protein *F*-value was generally lower than that of CEWAF; Pearson-product moment was $r = 0.802$ for EROD ($r^2 = 0.644$) activity.

Table 1. Mean (±SD) TPH tissue content of *Sinonovacula constricta* 3d and 15d exposed to different WAF and CEWAF conditions (mg L^{-1}).

Groups	TPH (μg g wet tissue^{-1})	
	3d	15d
WAF0.04	6.96 ± 0.45^{b}	7.23 ± 0.68^{b}
WAF0.01	5.89 ± 0.31^{b}	6.51 ± 0.93^{b}
CEWAF0.04	6.98 ± 1.12^{b}	11.05 ± 1.36^{d}
CEWAF0.01	6.19 ± 0.56^{b}	9.13 ± 1.03^{c}
control	3.82 ± 0.74^{a}	3.96 ± 0.58^{a}

* Different letters denoted significant differences $p < 0.05$.

3.4 TPH accumulation

Results of the *S. constricta* TPH uptake analysis are shown in Table 1. The TPH uptake by the water control group was lower than that of the WAF and CEWAF groups. However, a significant difference ($p < 0.05$) was observed between the TPH uptakes of the 0.04 mg L^{-1} WAF and 0.01 mg L^{-1} CEWAF groups. The uptake by CEWAF-exposed groups was higher than the uptake by WAF-exposed groups. The uptakes at CEWAF and WAF concentrations of 0.04 mg L^{-1} groups were significantly different between these two groups and were significantly different from those of the controls at day 15.

3.5 Relationship between TPH accumulation and EROD and DNA damage levels

Linear regressions indicated an extremely high correlation between tissue TPH uptake and gill EROD enzyme activity, as well as between tissue TPH uptake and the *F*-value of protein levels (Figs. 4 and 5). Pearson-product moment was $r = 0.80$ for EROD ($r^2 = 0.638$) activity of the WAF-exposed group, both for the CEWAF-exposed group, and $r = 0.90$ ($r^2 = 0.813$) as function of EROD activity (Fig. 4). The *F*-value of protein levels showed good correlation (Fig. 4), but the correlation was poorer in the WAF-exposed group ($r = 0.85$, $r^2 = 0.826$) than in the CEWAF-exposed group ($r = 0.93$, $r^2 = 0.861$).

4 Discussion

The petroleum industry causes environmental pollution problems worldwide, and pollutants can produce multiple changes at various biological levels. Therefore, the most frequently used biomarkers serve as the earliest warning signals of disturbance measured at the molecular and biochemical levels (Dabrowska et al. 2014). DNA damage and EROD activity are some of the early warning biomarkers recommended for assessing contaminant effects in marine environments (Ramachandran et al. 2004).

EROD activity is a reliable and repeatable measure of exposure to hydrocarbons from crude oil (Ramachandran et al. 2004). EROD is involved in the first phase of metabolism through oxidation, reduction, or hydrolysis (Goeptar et al. 1995), and its induction is a clear sign of the enzymatic activity of CYP1A1 and CYP1A2 because of the presence of

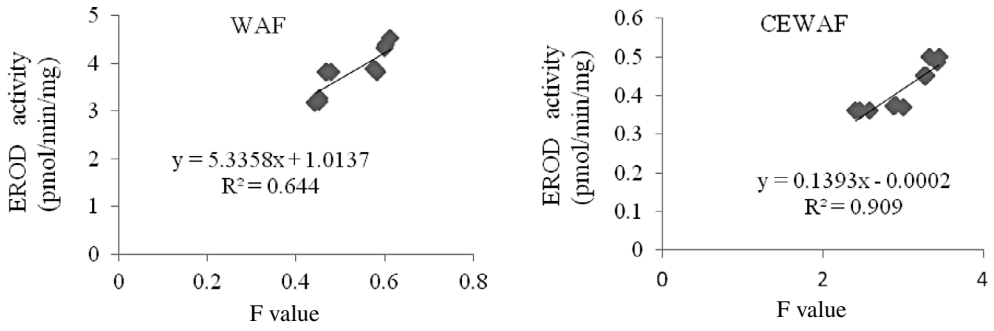

Fig. 3. Correlation of EROD and *F*-value of *Sinonovacula constricta* clams from WAF and CEWAF exposure respectively.

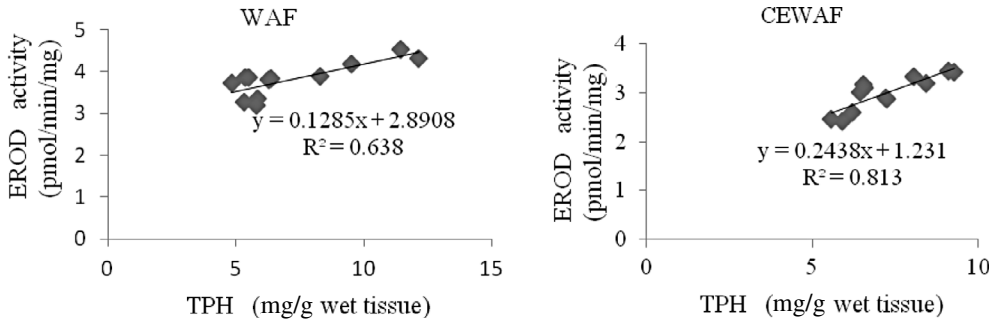

Fig. 4. Correlation of EROD and TPH tissue content of *Sinonovacula constricta* clams from WAF and CEWAF exposure respectively.

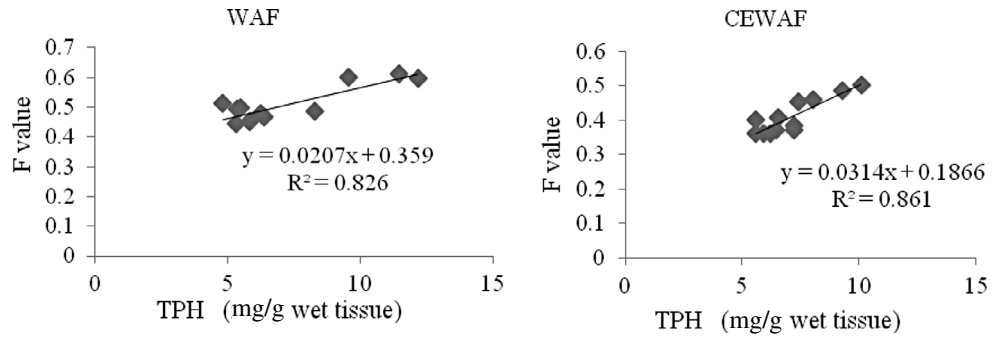

Fig. 5. Correlation of *F*-value of DNA damage and TPH tissue content of *Sinonovacula constricta* clams from WAF and CEWAF exposure respectively.

xenobiotics. In fact, increases in EROD activity have been reported in bivalves after exposure to organic pollutants (Pérez-Cadahía et al. 2004). Our experimental results demonstrated that EROD activity in the gills of *S. constricta* increased after 3 and 15 days of exposure to 0.04 mg L^{-1} and 0.01 mg L^{-1} crude oil (Fig. 1). After the absolute levels of EROD activities between WAF and CEWAF were compared, the highest absolute values of EROD activity were observed at the higher concentration (0.45 mg L^{-1}) followed by the lower concentration (0.01 mg L^{-1}) in CEWAF. The exposure response histogram for all five groups clearly denoted a greater EROD response to CEWAF than to WAF (Fig. 1). Similar observations of higher EROD activity in fish exposed to CEWAF versus WAF have been reported (Cohen et al. 2001; Gagnon and Holdway 2000; Ramachandran et al. 2004).

Considering these findings, we are interested in investigating the *S. constricta* EROD responses to lower WAF and CEWAF concentration ranges. The components in oil that have been associated with environmental risk are PAHs because of their carcinogenic and mutagenic properties (Varanasi 1989). EROD assay is currently considered as a useful tool for identifying the presence of hydrocarbons in the environment, ranging from severe pollution to low-level contamination (Pacheco and Santos 2002). When PAHs enter the system of an organism, a series of oxidative processes mediated by the cytochrome P450 system occurs (phase I); CYP1A1 isozyme is predominant, which can be assessed by measuring EROD (Da Silva Rocha et al. 2012). In our study, we observed a significant increase in the enzyme activity of EROD in response to a higher dose of crude oil compared with controls (Fig. 1).

The results of this investigation are similar to those of previous studies; that is, CYP1A1 mRNA expression was modulated by crude oil stress and enzymes of EROD may play an important role in the detoxification of xenobiotics (Gagnon and Holdway 1998).

Lyons et al. (2011) found that dispersing oil markedly increases hydrocarbon concentrations in test solutions based on measured concentrations of PAH. The increase in hydrocarbons may have been caused by the presence of oil droplets in emulsion or by the increased dissolution of hydrocarbons from the surfaces of numerous droplets (surface area effects) (Cohen and Nugegoda 2000). Therefore, dispersed WAF oil (CEWAF) had the highest concentration of TPHs compared with WAF crude oil. The EROD response of clams in this experiment suggested that Jiefeite 001 solubilizes PAHs more effectively from CEWAF than from WAF.

PAHs in oil may also damage DNA through the formation of ROS, especially highly reactive OH radicals (Nahrgang et al. 2010; Shugart 1998). Delunardo (2015) demonstrated that the amount of DNA damage was linked to increased concentrations of crude oil. In the present study, results from the alkaline unwinding assay revealed an increase in DNA damage in both doses of assayed crude oil compared with that in the control group (Fig. 2). However, no significant difference was found in the 0.01 mg L^{-1} WAF treatments over the entire exposure period compared with the control (Fig. 2). The implication is that crude oil poses no threat to clams in this process. Genotoxicity results from metabolism of B[a]P to electrophilic intermediates by phase I mixed function oxidases, followed by binding of metabolites to nucleophilic sites within DNA (Newbold and Brookes 1976) Significant research on biomarkers based on shellfish has focused on oxidative damage to lipids, proteins, and DNA, showing that the toxicity effect indices are closely associated with the concentration of environmental contaminants (Binelli et al. 2010). Singer (2000) found that the hydrocarbon concentrations in dispersed Central Gulf oil were four to five times higher than those in WAF with naphthalenes as the dominant fraction. This observation is probably associated with the mild degree of PAH bioaccumulation at the low WAF concentration exposure.

A wide range of biomarkers can be used to evaluate the exposure and effects of PAH in marine organisms; for example, the induction of phase I P450 enzyme measured as an increase in EROD activity (Rewitz et al. 2006) has been widely considered. These phase I enzyme-activated metabolites can bind to DNA and cause genotoxicity by changing its molecular structure (Da Silva Rocha et al. 2012). In the present study, both EROD activity and alkaline unwinding assay were successfully applied to assess the biochemistry and genotoxic effects of crude oil on the gill EROD activity and DNA damage of *S. constricta*. DNA damage was significantly correlated with EROD activity at CEWAF and WAF exposure (r^2 = 0.909 and r^2 = 0.644, respectively) (Fig. 3). The genotoxicity observed on the gill tissues of *S. constricta* seemed to result from crude oil reactive metabolites produced by the activation of phase I metabolism as previously discussed.

In our study, TPH tissue content increased significantly with nominal concentrations. The small amounts of TPH observed in the control groups were significantly different between all groups after 3 and 15 days of exposure, but both were significantly lower than those of the crude-oil-treated groups (Table 1). The four crude oil tissue concentration results showed that each was rapidly taken up and bioaccumulated in the clams. The results also showed that TPH accumulation in the tissues increases in parallel with exposure dose, which is in accordance with the results from previous TPH accumulation studies in fish (Ramachandran et al. 2004). According to our results, the accumulation of CEWAF in clams at 0.45 mg L^{-1} levels was significantly greater than that of WAF in the same conditions at day 3 (6.98 ± 1.12 and 6.96 ± 0.45, respectively) and day 15 (11.05 ± 1.36 and 7.23 ± 0.68, respectively), implying that CEWAF was taken up more easily by the clams. Moreover, the highest TPH tissue content was found after 15 days in the group with exposure to 0.04 mg L^{-1} CEWAF, which was significantly different from the other groups. A correlation coefficient (Pearson r value) of 0.90 was calculated between EROD activity and TPH fraction of the CEWAF treatment of crude oil, as well as between EROD activity and TPH fraction of WAF-treated oil (0.80) (Fig. 4). Dispersant effectiveness is a major controlling factor in the bioavailability of hydrocarbons (Ramachandran et al. 2004). Fingas (1995) reported that higher hydrocarbon ratios in CEWAF versus WAF for Scotian Light compared with Terra Nova indicate that lighter oils would disperse more effectively as bulk oil droplets than heavier ones. The results of the alkaline unwinding assay indicate the close correlation between DNA damage and tissue TPH uptake at WAF and CEWAF exposure (r^2 = 0.826 and r^2 = 0.861, respectively) as shown in Figure 5. The PAH concentration was increased by the dispersant, which was significantly different between CEWAF and WAF. Therefore, this contamination may have resulted from the significantly higher PAH tissue content of CEWAF-treated groups than WAF-treated groups.

5 Conclusion

In the present study, both EROD activity and alkaline unwinding assay were successfully applied to assess biochemistry and genotoxic effects of crude oil on the gill EROD activity and DNA damage of gill cells on the *S. constricta* clam. These biomarkers were significantly correlated between themselves, as well as with the amount of TPH taken up by the clams. The close correlation between tissue TPH uptake and EROD activity, as well as DNA damage and tissue TPH uptake (Figs. 4 and 5), indicate that EROD activity and DNA damage are more ecotoxicologically effective as biomarkers. Exposure assessment established the relationship between environmental toxicant concentrations and organism accumulation while accounting for environmental and biological factors.

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References

Aas E., Baussant T., Balk L., Liewenborg B., Andersen O.K., 2000, PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod. Aquat. Toxicol. 51, 241–258.

Binelli A., Cogni D., Parolini M., Provini A., 2010, Multi-biomarker approach to investigate the state of contamination of the R. Lambro/R. Po confluence (Italy) by zebra mussel (Dreissena polymorpha). Chemosphere 79, 518–528.

Bonacci S., Corsi I., Chiea R., Regoli F., Focardi S., 2003, Induction of EROD activity in European eel (*Anguilla anguilla*) experimentally exposed to benzo[a]pyrene and β-naphthoflavone. Environment International 8, 467–473.

Cappello T., Maisano M., D'Agata A., Natalotto A., Mauceri A., Fasulo S., 2013, Effects of environmental pollution in caged mussels Mytilus galloprovincialis. Mar. Environ. Res. 91, 52–60.

Carr R.S., Linden O., 1984, Bioenergetic responses of Gammarus salinus and Mytilus edulis to oil and oil dispersants in a model ecosystem. Mar. Ecol. Prog. Ser. 19, 285–291.

Ching E.W.K., Siu W.H.L., Lam P.K.S., Lihong Xu, Yongyuan Zhang, Bruce J.R., Rudolf S., Wu S., 2001, DNA adducts formation and DNA strand breaks in green-lipped mussels (*Perna viridis*) exposed to benzo[a]pyrene: dose-and time-dependent relationships. Mar. Poll. Bull. 42, 603–610.

Cohen A.M., Nugegoda D., 2000, Toxicity of three oil spill remediation techniques to the Australian bass Macquaria novemaculeata. Ecotoxicol. Environ. Safety 47, 178–185.

Cohen A.M., Nugegoda D., Gagnon M.M., 2001, The effect of different oil spill remediation techniques on petroleum hydrocarbon elimination in Australian bass (Macquaria novemaculeata). Arch. Environ. Contam. Toxicol. 40, 264–270.

D'Agata A., Cappello T., Maisano M., Parrino V., Giannetto A., Brundo M.V., Ferrante M., Mauceri A., 2014, Cellular biomarkers in the mussel Mytilus galloprovincialis (Bivalvia: Mytilidae) from Lake Faro (Sicily, Italy). Ital. J. Zool. 81, 43–54.

Da Silva Rocha A.J., Gomes V., Rocha M.J.D.A., Hasue F.M., Santos T.C.A., Bícego M.C., Taniguchi S., Van Ngan P., 2012a, EROD activity and genotoxicity in the seabob shrimp Xiphopenaeus kroyeri exposed to benzo[a] pyrene (BaP) concentrations. Environ. Toxicol. Pharmacol. 34, 995–1003.

Dabrowska H., Kopko O., Góra A., Waszak I., Walkusz-Miotk J., 2014, DNA damage, EROD activity, condition indices, their linkages with contaminants in female flounder (Platichthys flesus) from the southern Baltic Sea. Sci. Total Environ. 496, 488–498.

Delunardo F.A.C., de Carvalho L.R., Da Silva B.F., Galão M., Val A.L., Chippari-Gomes A.R., 2015, Seahorse (*Hippocampus reidi*) as a bioindicator of crude oil exposure. Ecotoxicol. Environ. Safety 117, 28–33.

Fenech M., Chang W.P., Kirsch-Volders M., Holland N., Bonassi S., Zeiger E., 2003. HUMN project:Detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutation Res. 534, 65–75.

Fingas M., 1995, oil spills and their clenup. Chemistry and Industry 24, 1005–1008.

Gagnon M.M., Holdway D.A., 1998, MFO induction in Atlantic salmon (*Salmo salar*) during and after exposure to Bass Strait crude oil. Australas. J. Ecotoxicol. 4, 29–35.

Gagnon M.M., Holdway D.A., 2000, EROD induction and biliary metabolite excretion following exposure to the water accommodated fraction of crude oil and to chemically dispersed crude oil. Arch. Environ. Eontam. Toxicol. 38, 70–77.

Getter C.D., Baca B.J., 1984, A laboratory approach for determining the effects of oils and dispersants on mangroves: Oil Spill Chemical Dispersants. Research, Experience, Recommendations, STP 840, 5–13.

Goeptar A.R., Scheerens H., Vermeulen N.P., 1995, Oxygen and xenobiotic reductase activities of cytochrome P450. CRC Crit. Rev. Toxicol. 25, 25–65.

Hamoutene D., Payne J.F., Rahimtula A., Lee K., 2002, Use of the Comet assay to assess DNA damage in hemocytes and digestive gland cells of mussels and clams exposed to water contaminated with petroleum hydrocarbons. Mar. Environ. Res. 54, 471–474.

Harikrishnan Thilagam S.G.H.Q., 2010, 17β estradiol induced ROS generation, DNA damage and enzymatic responses in the hepatic tissue of Japanese sea bass. Ecotoxicology 19, 1258–1267.

Hodson P.V., Kloepper-Sams P.J., Munkittrick K.R., Lockhart W.L., Metner D.A., Luxon L., Smith I.R., Gagnon M.M., Servos M., 1991, Protocols for measuring mixed function oxygenases of fish liver. Can. J. Fish. Aquatic Sci. 1829, 1–51.

Khan R.A., Payne J.F., 2005, Influence of a crude oil dispersant, Corexit 9527, and dispersed oil on capelin (*Mallotus villosus*), Atlantic cod (*Gadus morhua*), longhorn sculpin (*Myoxocephalus octodecemspinosus*), and cunner (*Tautogolabrus adspersus*). Bull. Environ. Contam. Toxicol. 75, 50–56.

Lewis A., Aurand D., 1997, Putting dispersants to work: overcoming obstacles: International Oil Spill Conference Technical Report IOSC, pp. 157–164.

Livingstone D.R., Lemaire P., Matthews A., Peters L.D., Porte C., Fitzpatrick P.J., Förlin L., Nasci C., Fossato V., Wootton N., 1995, Assessment of the impact of organic pollutants on goby (*Zosterisessor ophiocephalus*) and mussel (*Mytilus galloprovincialis*) from the Venice Lagoon, Italy. biochemical studies. Mar. Environ. Res. 39, 235–240.

Lyons M.C., Wong D., Mulder I., Lee K., Burridge L.E., 2011, The influence of water temperature on induced liver EROD activity in Atlantic cod (*Gadus morhua*) exposed to crude oil and oil dispersants. Ecotoxicol. Environ. Safety 74, 904–910.

Nahrgang J., Camus L., Carls M.G., Gonzalez P., Jönsson M., Taban I.C., Bechmann R.K., Christiansen J.S., Hop H., 2010, Biomarker responses in polar cod (*Boreogadus saida*) exposed to the water soluble fraction of crude oil. Aquatic Toxicol. 97, 234–242.

Neff J.M., Anderson J.W., 1975, An ultraviolet spectrophotometric method for the determination of naphthalene and alkylnaphthalenes in the tissues of oil-contaminated marine animals. Bull. Environ. Contam. Toxicol. 14, 122–128.

Newbold R.F., Brookes P., 1976, Exceptional mutagenicity of benzo(a)pyrene diol epoxide in cultured mammalian cells. Nature 261, 52–54.

Nogueira P., Lourenço J., Rodriguez E., Pacheco M., Santos C., Rotchell J.M., Mendo S., 2009, Transcript profiling and DNA damage in the European eel (*Anguilla anguilla L.*) exposed to 7, 12-dimethyl benz[a]anthracene. Aquat. Toxicol. 94, 123–130.

Pacheco M., Santos M.A., 1998, Induction of Liver EROD and Erythrocytic Nuclear Abnormalities by Cyclophosphamide and PAHs in Anguilla anguilla. Ecotoxicol. Environ. Safety 40, 71–76.

Pacheco M., Santos M.A., 2002, Naphthalene and β-naphthoflavone effects on Anguilla anguilla L. hepatic metabolism and erythrocytic nuclear abnormalities. Environment International 28, 285–293.

Pérez-Cadahía B., Laffon B., Pásaro E., Méndez J., 2004, Evaluation of PAH bioaccumulation and DNA damage in mussels (*Mytilus galloprovincialis*) exposed to spilled Prestige crude oil. Comp. Biochem. Physiol. C 138, 453–460.

Pohl R.J., Fouts J.R., 1980, A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. Anal. Biochem. 107, 150–155.

Ramachandran S.D., Hodson P.V., Khan C.W., Lee K., 2004, Oil dispersant increases PAH uptake by fish exposed to crude oil. Ecotoxicol. Environ. Safety 59, 300–308.

Ramachandran S.D., Sweezey M.J., Hodson P.V., Boudreau M., Courtenay S.C., Lee K., King T., Dixon J.A., 2006, Influence of salinity and fish species on PAH uptake from dispersed crude oil. Mar. Poll. Bull. 52, 1182–1189.

Regoli F., Gorbi S., Frenzilli G., Nigro M., Corsi I., Focardi S., Winston G.W., 2002, Oxidative stress in ecotoxicology: from the analysis of individual antioxidants to a more integrated approach. Mar. Environ. Res. 54, 419–423.

Rewitz K.F., Styrishave B., Løbner-Olesen A., Andersen O., 2006, Marine invertebrate cytochrome P450: emerging insights from vertebrate and insect analogies. Comp. Biochem. Physiol. C 143, 363–381.

Rice S.D., 1985, Effects of oil on fish, Petroleum effects in the arctic environment, Engelhardt, F.R, pp. 157–182.

Shugart L.R., 1998, Structural damage to DNA in response to toxicant exposure. Genet. Ecotoxicol. 151–167.

Singer M.M., Aurand D., Bragin G.E., Clark J.R., Coelho G.M., Sowby M.L., Tjeerdema R.S., 2000, Standardization of the preparation and quantitation of water-accommodated fractions of petroleum for toxicity testing. Mar. Poll. Bull. 40, 1007–1016.

Snyder M.J., 1998, Cytochrome P450 enzymes belonging to the CYP4 family from marine invertebrates. Biochem. Biophys. Res. Commun. 249, 187–190.

Taban I.C., Bechmann R.K., Torgrimsen S., Baussant T., Sanni S., 2004, Detection of DNA damage in mussels and sea urchins exposed to crude oil using comet assay. Mar. Environ. Res. 58, 701–705.

Thilagam H., Gopalakrishnan S., Qu H.D., Bo J., Wang K.J., 2010, 17b estradiol induced ROS generation, DNA damage and enzymatic responses in the hepatic tissue of Japanese sea bass. Ecotoxicology 19, 1258–1267.

Tice R.R., Agurell E., Anderson D., Burlinson B., Hartmann A., Kobayashi H., Miyamae Y., Rojas E., Ryu J.C., Sasaki Y.F., 2000, Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 35, 206–22

Valverde M., Rojas E., 2009, Environmental and occupational biomonitoring using the Comet assay. Mutation Res. 681, 93–109.

Varanasi U., 1989, Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment, CRC Press.

Wake H., 2005, Oil refineries: a review of their ecological impacts on the aquatic environment. Estuarine, Coastal and Shelf Science, 62, 131–140.

Xiu M., Pan L., Jin Q., 2014, Bioaccumulation and oxidative damage in juvenile scallop Chlamys farreri exposed to benzo[a]pyrene, benzo fluoranthene and chrysene. Ecotoxicol. Environ. Safety 107, 103–110.

Yanxia Tao L.P.H.Z., 2013, Assessment of the toxicity of organochlorine pesticide endosulfan in clams Ruditapes philippinarum. Ecotoxicol. Environ. Safety 93, 22–30.