Responses of primary cultured haemocytes derived from the marine gastropod *Haliotis tuberculata* to an industrial effluent exposure

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Received: 1 July 2016 / Accepted: 22 November 2016 / Published online: 12 December 2016
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Abstract This study assessed the responses of primary cultured haemocytes from the marine gastropod *Haliotis tuberculata* exposed to the increasing concentrations of industrial effluent (0, 0.5, 1, 10, 15 and 20%) discharged into the Tunisian coastal area. Analyses showed the presence of metals such as cadmium (Cd), chromium (Cr), copper (Cu), manganese (Mn), zinc (Zn), nickel (Ni) and lead (Pb) in the effluent. The effects of this mixture of pollutants on abalone haemocyte parameters were reflected by a significant decrease of cell viability, phagocytotic activity and reactive oxygen species (ROS) production as well as morphological and lysosomal membrane alterations. Thus, these results indicated that our primary culture system represents a suitable in vitro model for monitoring of anthropogenic contaminants in aquatic environments.

Keywords Haemocytes · *Haliotis tuberculata* · Immune parameters · Industrial effluent · In vitro · Primary culture

Introduction

Several studies only consider the toxicity of chemicals in isolation whereas in the environment, organisms are exposed to a large number of different chemicals at the same time. The assumption of mixture toxicity based on the individual component data can lead to a significant under- or over-estimation of the potential risk that a mixture may present (Heys et al. 2016). Such mixture is found in industrial effluents which are continuously discharged into marine environments and cause instability and disorder of ecosystems (Cong et al. 2012; Ladhar-Chaabouni et al. 2012). Several studies showed that exposure to effluents causes a variety of stress-related changes in aquatic organism health (Salo et al. 2007; Gagne et al. 2008; Kamel et al. 2012). Among aquatic organisms, the European abalone *Haliotis tuberculata*, which is a marine benthic gastropod living in coastal areas along the eastern Atlantic to the west coast of Africa, was used as sensitive indicator species for coastal pollutions (Gorski and Nugegoda 2006; Zhu et al. 2011). Primary cultures derived from abalone tissues have been successfully used for cytotoxicity tests

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(Ladhar-Chaabouni et al. 2014; Gaume et al. 2012; Latire et al. 2012; Mottin et al. 2010). Since haemocytes play a key role in digestion, metabolite transport, shell repair (Mount et al. 2004) and immune system (Adema et al. 1991; Gopalakrishnan et al. 2009), they provide a suitable model to study in vitro toxicity of pollutants (Marigomez et al. 1990; Soto et al. 1996; McIntosh and Robinson 1999). In the present study an in vitro approach was chosen, and represents an alternative experimental method to whole animal testing, due to the reduced use of animals, capability for standardization, low cost and rapid performance associated with this method, in addition to ethical considerations (Schirmer 2006; Shuilleabhain et al. 2006). This approach could provide preliminary information to perform in vivo experiment. Thus, the objective of the present study was to investigate the in vitro effects of a mixture of pollutants contained in the effluent of a phosphate treatment plant using haemocyte primary culture from *H. tuberculata*. The toxicity of the effluent was assessed on the viability, the cell morphology, the phagocytic activity, the ROS production and the stability of lysosomal membranes.

Materials and methods

Metal concentrations

The effluent used in this study was discharged by a phosphate treatment plant (SIAPE) localized near Skhira in the gulf of Gabès (Tunisia). Heavy metal analyses (Cd, Cr, Cu, Mn, Ni, Pb and Zn) were carried out on effluent waters using an inductively coupled plasma optical emission spectrometer (ICP-AOS, Thermo Scientific iCAP 6300 DUO).

Specimens

Adult abalones of the species *H. tuberculata* were collected by France Haliotis (Plouguerneau, France). The animals were maintained in natural and continuously aerated seawater at 17 °C and regularly fed with a mixed algal diet (*Laminaria sp.* and *Palmaria sp.*) at the Centre de Recherche en Environnement Côtier (C.R.E.C., Luc-sur-Mer, Basse-Normandie, France). The abalones were acclimated at least 2 weeks before the experiments began.

Primary cell cultures

Haemocytes were cultured as previously described (Lebel et al. 1996; Serpentine et al. 2000; Mottin et al. 2010). Briefly, after making a medio-lateral incision in the abalone foot, haemolymph of two adult abalones was collected (20–25 mL per animal) using 20 mL syringe fitted with a 25-gauge hypodermic needle. Haemolymph was transferred to a sterile tube and diluted 1:4 in cooled sterile anticoagulant modified Alsever's solution (115 mM glucose; 27 mM sodium citrate; 11.5 mM EDTA; 382 mM NaCl) (Bachère et al. 1988). Haemocytes were counted with a hemocytometer and rapidly plated at a density of 300,000 cells per well in 24-well plates (neutral red assay) or 500,000 cells per well in 12-well plates (MTT, flow cytometry analysis), into which three volumes of sterile artificial seawater (436 mM NaCl, 53 mM MgSO₄, 20 mM Hepes, 10 mM CaCl₂, 10 mM KCl, final pH 7.4) were added. The cultures were maintained at 17 °C in a CO₂ free incubator. After 90 min of incubation, the cells were covered with Hank's sterile 199 medium modified by the addition of 250 mM NaCl, 10 mM KCl, 25 mM MgSO₄, 2.5 mM CaCl₂ and 10 mM Hepes (final pH of 7.4). The medium was supplemented with 2 mM L-glutamine, 100 µg mL⁻¹ streptomycin, 60 µg mL⁻¹ penicillin G and 2 mM concanavalin A (Sigma-Aldrich, Saint-Quentin Fallavier, France). The cells were then maintained at 17 °C for 24 h before beginning the experiments (Lebel et al. 1996; Serpentine et al. 2000; Mottin et al. 2010).

Effluent exposure

After incubation time, the medium was aspirated and replaced by the effluent contaminated medium. Effluent was diluted in Hank's sterile medium to final concentrations of 0.5, 1, 10, 15 and 20%. In each contaminated medium as well in control medium, the same volume of distilled water was added to check the potential effect of diluted culture medium. Cells were exposed to different concentrations of effluent during 24 h (classically used period) at 17 °C. At the end of the experiments, cells observations were carried out for morphological status using a Leica DM IRB inverted microscope equipped with a computer assisted microscopic image analysis system, Leica Application suite software version 4.1. All experiments were performed in quadruplet.

MTT assay

Cell viability was assessed using the MTT assay as previously described by Mosmann (1983). This assay is based on the reduction of MTT into purple formazan crystals by the succinate dehydrogenase enzyme in the mitochondrial respiratory chain. This test was adapted to molluscan cell cultures by Domart-Coulon et al. (2000). Briefly, 10% (v/v) of the MTT stock solution (5 mg MTT mL⁻¹ of PBS) (Sigma-Aldrich, Saint-Quentin Fallavier, France) was added to the cultured haemocytes. After 24 h of incubation, an equal volume of isopropanol containing 0.04 N HCl was added to each culture to dissolve the converted formazan. The absorbance was then measured at a wavelength of 570 nm with a 630 nm reference.

Neutral red assay

Neutral red (NR) dye accumulates in the lysosome compartment of cells (Lowe and Pipe 1994). It spreads into cells by membrane diffusion or pinocytosis and an alteration in its uptake may reflect damage to the plasma membrane and consequent changes in the volume of the lysosome (Lowe and Pipe 1994). Dead cells or cells with membrane damage cannot accumulate the dye. A reduction in lysosome membrane stability has been reported in mussels and oysters exposed to heavy metals, and has been proposed as an indicator of cell damage (Moore et al. 2006). Briefly, 10% (v/v) of the neutral red stock solution (0.5% neutral red in PBS 1X) (Sigma-Aldrich, Saint-Quentin Fallavier, France) was added to the cultured haemocytes: the medium containing effluent was aspirated and replaced by neutral red medium and the plates were further incubated for 3 h at 17 °C. The following step consisted of rapidly washing wells with MPS. Next, the neutral red dye incorporated in lysosomes of viable cells was released into the medium with 300 µL desorb solution (1% acetic acid–50% ethanol). Absorbance was estimated photometrically at 540 nm and 650 nm with a microtiter plate spectrophotometer.

Flow cytometry analysis of abalone haemocytes

Haemocyte analysis was performed using Gallios flow cytometer (Beckman Coulter®), and 10,000 events were counted for each sample. The results were expressed as cell cytograms, indicating the size (FSC

value), the complexity (SSC value) and the level of fluorescence using the FL1 channel as described previously (Mottin et al. 2010).

Phagocytic activity

Phagocytosis was measured by ingestion of Fluorospheres® carboxylate-modified beads (1 µm diameter, Molecular Probes) as previously described (Sauvé et al. 2002a; Hégaret et al. 2003; Auffret et al. 2006). Briefly, 7 µL of bead solution (Molecular Probes, Fisher Scientific, Illkirch, France) was added to each well. Cells were incubated for 60 min at 17 °C in the dark. Following the incubation, the wells were scraped gently, and the samples were centrifuged at 500 × g for 10 min at 4 °C. The resulting pellet was mixed with 300 µL of 3% paraformaldehyde (PFA). Samples were stored at 4 °C until analysis. Phagocytosis was measured as the proportion of cells that gave a fluorescent signal equal to or greater than the fluorescence of three beads. Thus, data were expressed in percentage of haemocytes that engulfed three beads or more (data were given directly by the cytometer).

Reactive oxygen species (ROS) production

ROS production was evaluated using the 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, Saint-Quentin Fallavier, France) method (Bass et al. 1983), as adapted to mollusc cells by Lambert et al. (2003). DCFH-DA is a cell-permeable non-fluorescent probe which is de-esterified intracellularly and turns to highly fluorescent 2',7'-dichlorofluorescein upon oxidation. Haemocytes were incubated for 60 min at 17 °C in the dark with DCFH-DA to a final concentration of 100 µM. Following the incubation, the wells were scraped gently, and the samples were centrifuged at 500 × g for 10 min at 4 °C. The resulting pellet was mixed with 300 µL of 3% paraformaldehyde (PFA). Samples were stored at 4 °C until analysis. The results were expressed as the percentage of cells exhibiting fluorescence.

Data analysis

Results were expressed as mean ± S.D. The means were calculated from quadruplets for each experiment. The significance of the differences between mean values was estimated using one-way ANOVA followed by a

post hoc test (Tukey test). After statistical analysis the mean optical density (MTT and NR assays) in controls was assigned a value of 100%. For the phagocytosis and ROS production, the mean percentage in controls was assigned a value of 100%. Significance was set at $p < 0.05$ using the program SPSS software.

Results

Metal concentrations in effluent

Results obtained from the effluent analysis are presented in Table 1. It shows the presence of Cd, Cr, Cu, Mn, Zn, Ni and Pb with different concentrations. The most abundant metals are Cd, Cr and Zn whose concentrations exceed the French norms. Nevertheless, only Cd concentration exceeds the Tunisian norms.

Effects of effluent on haemocyte viability

Haemocytes were exposed for 24 h to 0, 0.5, 1, 10, 15 and 20% of industrial effluent and cell viability was assessed colorimetrically by using the MTT assay. The results are presented in Fig. 1. A tendency of a decrease of cell viability was observed with the effluent concentration increase. However, no significant difference from the control was found for industrial effluent concentrations lower than 15%. Haemocyte viability decreased significantly ($p < 0.05$) beginning at a concentration of 15% when using MTT assay. The highest concentration (20% of effluent) decreased the cell viability by 76% compared to 100% control.

Effect of effluent on haemocyte lysosomal membrane stability

After 24 h of exposure to different concentrations of an industrial effluent, variations of haemocyte

Fig. 1 Effect of industrial effluent on haemocyte viability as determined by the MTT reduction assay. Cells were exposed to 0, 0.5, 1, 10, 15 and 20% of industrial effluent for 24 h. Experiment was made in quadruplet (significant differences at $p < 0.05$)

lysosomal membrane stability were determined using NR assay (Fig. 2). Compared to controls, a significant increase ($p < 0.05$) of NR staining of lysosomes was observed after an exposure to 10% of effluent. This increase was 32% compared to the 100% control. A significant decrease ($p < 0.05$) was then observed when using 15 and 20% of effluent. This decrease was 66.8 and 46.88% compared to the 100% control, respectively.

Effects of effluent on haemocyte morphological parameters

Light microscopy showed that haemocyte morphology changed in response to effluent treatment. Cells cultured for 24 h in the absence of effluent presented an elongated shape with large pseudopods and were mostly interconnected (Fig. 3a). The same shapes were observed at concentrations of 0.5, 1 and 10% of effluent (Fig. 3b–d). When they were exposed to 15% of effluent, shrunk cells with no extensions became abundant and a reduction of contact with neighbouring cells was observed (Fig. 3e, f).

Table 1 Trace metal concentrations in effluent collected at the exit of a phosphate treatment plant

	Cd	Cr	Cu	Mn	Zn	Ni	Pb
Effluent	0.953	0.994	0.381	0.445	2.757	0.469	0.174
Tunisian norms	0.005	2	1.5	1	10	2	0.5
French norms	0.2	0.5	0.5	1	2	0.5	0.5

Results are expressed as mg L⁻¹ of effluent

Fig. 2 Effect of industrial effluent on haemocyte lysosomal membrane stability using the neutral red assay. Cells were exposed to 0, 0.5, 1, 10, 15 and 20% of industrial effluent for 24 h. Experiment was made in quadruplet (significant differences at $p < 0.05$)

Effects of effluent on immune parameters

The effects of effluent on two cellular activities were assessed by flow cytometry after 24 h of exposure. Under our experimental conditions, the percentage of haemocytes that engulfed three beads or more was $10.07 \pm 2.65\%$ for the control. The phagocytic activity was significantly inhibited ($p < 0.05$) when cells were exposed to concentrations of 10% and higher of effluent (Fig. 4). This decrease was 77.16% compared to the 100% control.

Similarly, a significant decrease of ROS production ($p < 0.05$) in cells exposed to 10% of effluent and higher was observed (Fig. 5). This decrease was 30.04, 40.64 and 26.51% compared to the 100% of control when cells were exposed to 10, 15 and 20% of effluent, respectively.

Discussion

In the field, exposure to single contaminant is not realistic. In fact, pharmaceuticals, nonylphenols, metals as well as other substances, are present in complex mixtures and non-target organisms would consequently never be exposed to a single compound but rather to a complex array of pollutants (Farcy et al. 2011). To mimic environmental conditions, we decided to study the in vitro effects of industrial effluent (mixture of pollutants) using haemocyte primary culture from the European abalone *H. tuberculata*.

The chemical analysis of the industrial effluent showed the presence of metals such as Cd, Cr, Cu, Mn, Zn, Ni and Pb. Knowing that it was a global effluent produced by three different units of production (sulfuric acid, phosphoric acid, and fertilizers) of the SIAPE factory, it was not surprising to find high metal concentration in the studied effluent especially Cd concentration which exceeds Tunisian and French norms (T.N. 106.002 and French Ministerial Decree of 22 February 1998, respectively). Since the exact composition of effluent was unknown, only the effect of metals was widely discussed. Thus, a short term exposure to the effluent, showed a significant decrease of cell viability beginning at a concentration of 15%. Such decrease was not detected when *H. tuberculata* haemocytes were exposed only to CdCl_2 (1 and $100 \mu\text{g mL}^{-1}$) during 24 h (Ladhar-Chaabouni et al. 2014). It appears that a mixture of pollutants including metals had a stronger toxicity than CdCl_2 in *H. tuberculata* haemocytes after a short term exposure. Nevertheless, after a long term exposure (10 days) to different concentrations of CdCl_2 a significant decrease of cell viability beginning at a concentration of $500 \mu\text{g L}^{-1}$ was observed (Latire et al. 2012). In addition to Cd, the effect of Zn on *H. tuberculata* haemocytes was determined (Mottin et al. 2010). A decrease of haemocyte viability was observed beginning at ZnCl_2 concentration of 100 mM ($13,600 \text{ mg L}^{-1}$) which is much higher than that detected in the effluent. Once again, it appears that a mixture of metals had a stronger toxicity than ZnCl_2 in *H. tuberculata* haemocytes after a short term exposure. In addition to the cell viability, the effects of metals such as Cd and Zn on *H. tuberculata* haemocyte morphology were determined. Results showed that in controls, cells were interconnected and elongated with large pseudopods. In the presence of metals, haemocytes became isolated and more rounded (dependently of metal concentrations). Similar results were observed in the present study. Under light microscopy, the presence of spreading cells with interconnected pseudopods (Fig. 3a) indicates the good metabolic status of our cells. Nevertheless, after effluent exposure (15 and 20% of effluent) cells with round shape and no extensions of abalone haemocytes became abundant (Fig. 3e, f). Haemocytes morphological changes were also observed in *M. galloprovincialis* exposed to increased concentrations of CdCl_2 of $100 \mu\text{M}$ ($18,332 \mu\text{g mL}^{-1}$) and greater (Olabarrieta et al.

Fig. 3 Light microscopy images showing the morphology of *Haliotis tuberculata* haemocytes. Cells were seeded at a density of 0.5×10^6 cells per well and grown at 17 °C in culture medium for 24 h in the absence (a) or presence of 0.5% effluent

(b), 1% effluent (c), 10% effluent (d), 15% effluent (e) and 20% effluent (f). Arrow Spreading cell with pseudopod; arrow head shrunk cells with no extensions

2001; Gomez-Mendikute and Cajaraville 2003). These morphological modifications due to a metal treatment are generally associated with a disturbance of cytoskeleton organization, as demonstrated by fluorescence microscopy (Olabarrieta et al. 2001; Gomez-Mendikute and Cajaraville 2003) or proteomic analysis (Chora et al. 2009).

The NR assay is based on the fact that dye was taken up by viable cells and then concentrated in acidic organelles. It was shown that the dye is almost exclusively sequestered in lysosomes (Allison and Young 1964; De Duve et al. 1974). In the present study, uptake of neutral red was significantly increased

after exposure to 10% of effluent. A hypothesis could be advanced to explain such phenomenon: some compounds of effluent could be accumulated in lysosomes and may cause an increase in the osmotic pressure within these organelles followed by the entry of water with subsequent swelling, and fusion with other small lysosomes to form vacuoles of increasing size. The enlargement of the lysosomes results in an increase in NR uptake. The increase of NR uptake was also observed in *M. edulis* haemocytes after an exposure to $400 \mu\text{g L}^{-1}$ of cadmium during 7 days (Coles et al. 1995). At higher concentrations of effluent (15 and 20%) a significant decrease of NR

Fig. 4 Effects of industrial effluent on relative phagocytic activity compared to the 100% control. Each data point represents the mean percentage $\pm$ standard deviation of quadruplet cultures (significant differences at $p < 0.05$)

Fig. 5 Effects of industrial effluent on ROS production compared to the 100% control. Each data point represents the mean percentage $\pm$ standard deviation of quadruplet cultures (significant differences at $p < 0.05$)

retention was observed. These results reflect the impairment of membrane integrity of lysosomes of abalone haemocytes after an exposure to 15 and 20% of effluent. Similarly, Chakraborty and Ray (2009); Marchi et al. (2004) and Matozzo et al. (2001) reported increased destabilization of the lysosomal membrane of haemocytes from bivalve mollusks exposed to arsenic, mercury and cadmium, respectively. The NR test was reported as a useful indicator of cellular viability in the presence of xenobiotics (Moore et al. 1994; Lowe and Pipe 1994; Lowe et al. 1995; Lowe and Fossato 2000). In the present work, the toxicity measured by NR test was consistent with that obtained by the MTT assay, highlighting the cytotoxicity of the effluent at a concentration of 15% and higher.

Phagocytosis is the first line of cellular defence in molluscs, and measurement of phagocytosis represents a sensitive endpoint to evaluate the adverse

effects of pollutants such as metals (Sauvé et al. 2002a). Our results showed no significant influence of the effluent on phagocytic activity at concentrations lower than 10%. Nevertheless, a significant decrease was observed at 10% of effluent and higher. Such decrease was observed in *H. tuberculata* haemocytes after an exposure to high concentrations of Zn during 24 h and CdCl₂ for 10 days (Mottin et al. 2010; Latire et al. 2012). Similarly, Sauvé et al. (2002a) showed that an exposure to a concentration higher than 10⁻⁴ M of CdCl₂ lead to a significant dose related inhibition of marine and freshwater bivalve haemocyte phagocytosis. Also, Sauvé et al. (2002b) showed that Cd, Cu, Ni, and Zn caused significant immunosuppressive effects with concentrations inducing 50% inhibition ranging from 10⁻⁵ to 10⁻⁴ M in *Eisenia fetida*, *Lumbricus terrestris*, *Aporrectodea turgida*, and *Tubifex tubifex*. However, a short term exposure of *Elliptio complanata* to a mixture of xenobiotics (municipal effluents) showed an increase of the phagocytosis efficiency (Farcy et al. 2011). The same results were observed in *Mytilus edulis* exposed to untreated municipal wastewater (Akaishi et al. 2007). No significant variations of phagocytosis of abalone haemocytes after an exposure to 1 and 100 $\mu\text{g mL}^{-1}$ of CdCl₂ during 24 h was observed by Ladhari-Chaabouni et al. (2014). Similar results were obtained in *M. edulis* haemocytes exposed to 400 $\mu\text{g L}^{-1}$ of cadmium (Coles et al. 1995). Dyrinda et al. (1998) did not observe any difference in phagocytic activity from mussels originating from several sites characterized by various levels of contamination in the UK. Katsumiti et al. (2014) showed that phagocytosis was not modified in *M. galloprovincialis* haemocytes exposed to ionic Cd. Thus and according to the literature, the phagocytic response can be enhanced, depressed or not affected following exposure to toxicants. This could depend on the nature of the xenobiotic, on its concentration and on the duration of the exposure. Concerning the ROS production our results showed a significant decrease of ROS production in cells exposed to 10% of effluent and higher. Similar results were observed after the in vitro exposure of *C. virginica* haemocytes to sub-lethal concentrations (up to 15 μM) of CdCl₂ (Butler and Roesijadi 2000). The decrease of ROS production was also observed in *M. galloprovincialis* haemocytes exposed in vitro to Cu (Gomez-Mendikute and Cajaraville 2003) and in *H. tuberculata* haemocytes

exposed to high concentrations of Zn (Mottin et al. 2010). As suggested by Thiagarajan et al. (2006), the disturbance of the cytoskeleton could be correlated to the NADPH-oxidase complex contained in the plasma membrane. This complex was responsible for the formation of O_2^{2-} in haemocytes and could be inhibited in response to contaminants exposure. Nevertheless, Donaghy et al. (2012) showed that ROS in *Crassostrea gigas* haemocytes originate from mitochondria and not from NADPH-oxidase. Thus, further investigation should be conducted to explain the decrease of ROS production and to describe ROS sources in *H. tuberculata* haemocytes. Contrary to our results, Latire et al. (2012) showed an increase of ROS production in *H. tuberculata* haemocytes beginning at a concentration of $5 \mu\text{g L}^{-1}$ (environmental concentration reported in coastal water) of CdCl_2 . Similarly, Katsumiti et al. (2014) showed a significant increase of ROS production in *M. galloprovincialis* haemocytes after exposure to a wide range of concentrations of CdS quantum dots (QDs) ($0.001\text{--}100 \text{ mg Cd L}^{-1}$) used for treatment and diagnosis of cancer and for targeted drug delivery. A combination of two mechanisms could explain the increase of ROS production in response to contaminants exposure: contaminants act directly on the mitochondrial respiratory chain, generating overproduction of ROS, or contaminants are able to interact directly with glutathione peroxidase and catalase, decreasing their efficiency in detoxifying peroxides and H_2O_2 , with both of these processes leading to an increase in ROS level (Nzengue et al. 2008). Thus, a contaminant-induced increase or decrease in ROS production both represent a hazard to the health of individuals. Decreased production will lead to recurrent infections since invading microorganisms will not be efficiently destroyed. On the other hand, increased ROS production can lead to damages to self-tissues since ROS are totally non-specific and are able to alter a wide range of self-macromolecules such as DNA and enzymes (Coteur et al. 2005).

Conclusion

Understanding the impacts of industrial waste to the living organisms is required to design efficient treatment and to develop effective remedial methods. In the present study, in vitro exposure of abalone

haemocytes to an industrial effluent showed a decrease of cell viability, phagocytotic activity, ROS production and an alteration of morphological and lysosomal membrane of abalone haemocytes. According to Heys et al. (2016) the use of toxicity data in the form of the biological response to an entire mixture appears the best approach to represent the simultaneous exposure that organisms could encounter in the environment. A key advantage of this type of ecotoxic risk assessment is that by using the whole mixture any interactions between the component chemicals that may have been missed in a component-based approach are accounted for. In an environmental setting, it is sometimes more appropriate to look at whole mixture data if the mix in question is poorly characterized. For future investigations it will be interesting to identify all effluent's components as well as to determine the kinetic effect of the effluent.

Acknowledgements This work was supported by a grant from the Ministry of higher education and scientific research, University of Sfax, Tunisia to R. Ladhar-Chaabouni. The authors thank L. Poulain and M. Duval (Plateau Technique de Cytométrie en Flux, Université de Caen Normandie, France) for their helpful technical assistance, the technical staff of the Centre de Recherche en Environnement Côtier (Luc-sur-Mer, Normandie, France) for their assistance in animal care and F. Sdirat for English revision.

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