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Research

Effect of *in vivo* contamination by effluent from phosphate treatment industry on the clam *Ruditapes decussatus*

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Abstract
This study focused on industrial effluent characterization and determination of its effects on the clam *Ruditapes decussatus*. The analysis results revealed that the effluent having acid pH and high concentrations of some chemicals, comparing to the norms, such as $SO4^{2-}$, NO_3^-, K^+, Mg^{2+}, F and Cd. The effect of the effluent on the clam *R. decussatus* was reflected by the LT50 determined after an exposition to different doses (5%, 10%, 20%, 30%) during 25 days and by the concentration/activity of two biomarkers: malondialdehyde (MDA) and acetylcholinesterase (AChE) determined after a contamination by effluent (1%) during 16 days. The stress on stress results showed that animals were stressed even with the lowest effluent concentration. An increase of MDA concentrations was observed in the digestive glands of exposed clams. Nevertheless, no significant variation of AChE activity was observed.

KEY WORDS: Biomarkers, contamination, effluent, *Ruditapes decussatus*

INTRODUCTION

The phosphatic field holds an important position within the Tunisian economy both in labour level and in trade balance worldwide. The Tunisian phosphate industry is fifth among the international operators in the field (Brahim and Ghorbel, 2012). Despite the economical importance of the phosphate treatment plants in Tunisia, the impact on the environment is becoming more and more concerning. Effluent from these plants can be regarded as ''hot spots'' of discharge releasing large amounts of pollutants into the aquatic environment (serbaji, 2000). As the chemical composition and toxicity of effluent are complex, the bioactive constituents responsible for disruptions in physiological function are often un-known. Recent evidence suggests that heavy metals may be a significant source of toxicity for aquatic organisms living in polluted environments and may be partially responsible for disruptions in physiological function. Biochemical changes have been demonstrated in the clam *Ruditapes decussatus* (Smaoui- Damak et al. 2006; 2009) and the cockles *Cerastoderma glaucum* (Machreki-Ajmi et al. 2007) exposed to *in situ* contamination by effluent from the phosphate treatment plant using biomarkers which are suitable tools for the early and sensitive detection of chemical exposure and may have a potential prediction of biological effects at higher biological organization level (population level) (Lopez-Barea, 1995). Nevertheless, in field situations there are many more parameters, which may be interacting at any one time. Each stressor and their interaction altered some aspect of metabolism and in combination with the effects on physiology of aquatic organisms it becomes rather complex (Paul pont, 2010) for this aim we are interested, in the present study, to determine the effect of these effluents in *R. decussatus* under laboratory conditions using the variation of LT50 and the concentration /activity of some biomarkers (MDA, AChE). But above all, effluent was analyzed and the concentrations of some chemical species (Na^+, K^+, Cl^-, NO_3^-, SO_4^{2-} ...) were determined in order to have recent data about effluent composition.

MATERIALS AND METHODS

Effluent analyses

Effluent of phosphate treatment plant (Fig. 1) was analysed. The color was noted by visual observation. The pH and the redox potential were recorded with the help of ultra basic Denver instrument pH meter. Turbidity and COD were determined by a Hach DR/4000U spectrophotometer. Taking into account the industrial origin of the effluent, some heavy metals (Cd, Ni, As and Hg) were measured, following digestion of the samples with concentrated nitric

acid, using an Perkin-Elmer Analyst 800 Atomic Absorption Spectrometer (Perkin Elmer, Norwalk, USA) equipped with Zeeman background correction and an AS-800 auto sampler by graphite furnace and graphite tubes with integrated platform (Perkin Elmer) belonging to the Department of legal medicine and toxicology, University of Granada, school of Medicine, Spain. As was determined using a hydride generation-Quartz furnace atomic absorption spectrometry belonging to the same laboratory. Fluorine was also investigated with the help of a potentiometric floride sensor marquee . Cl^-, $SO4^{2-}$ were measured by anionic HPLC Beckman 166 Detector using Super-sep Metrohm column , NO_3^+, K^+, Na^+ and Ca^{2+} were measured by cationic HPLC Metrohm 790 using Metrosep cation 1-2 . COD, Turbidity and ion measurements were conducted in the Radio Analysis and Environment laboratory LRAE-University of Sfax.

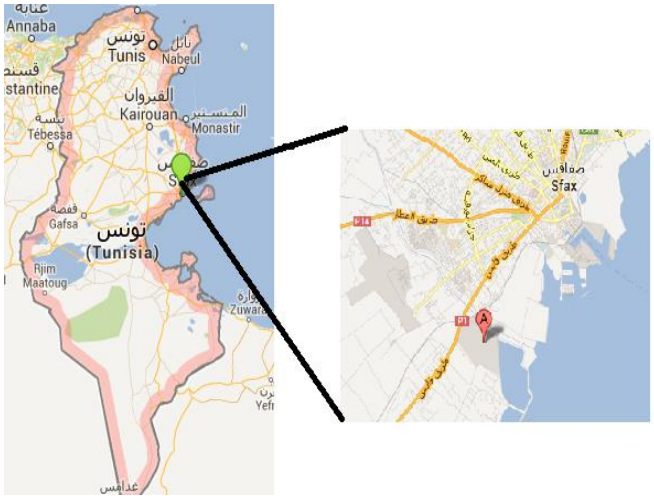

Figure 1. Location of the crude phosphate treatment plant.

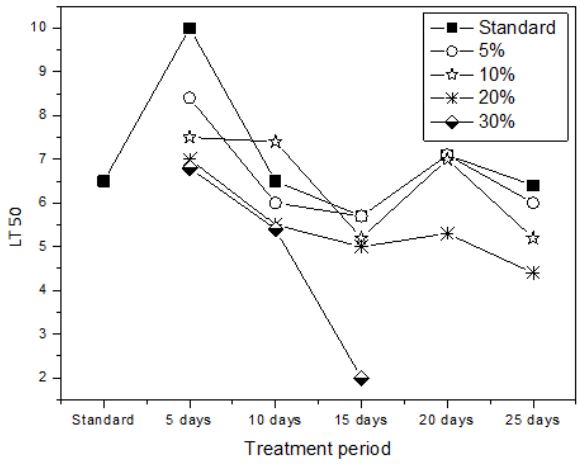

Figure 2. The LT50 variation of *R. decussatus* after 5, 10, 15 , 20 and 25 days of contamination with 5, 10, 20 and 30% of effluent from phosphate treatment plant

Stress on stress test

Ruditapes decussatus were collected from Sidi Mansour (site located 12 km to the north of Sfax) in June. 850 clams were distributed in 5 tanks, each one containing 170 samples. Animals were held in 7 L of sea water and exposed to different doses of effluent (5%,10%, 20% and 30% of final concentration) for 25 days. During the experiment, the sea water and effluent were renewed twice a week. Control animals were held in the same conditions. After contaminant exposure, animals were exposed to anoxia by air exposure. Survival was assessed daily. Death symptoms were considered to be open valves and absence of muscular activity. Lethal time corresponding to 50% of dead animals (LT50) in each group was calculated.

Contamination experiment

Based on stress on stress results a second contamination was realized. 60 clams of 30 mm ± 3mm of length were held in 7 L of sea water and contaminated with 1 % of the same effluent during 16 days . The Clams were randomly sampled on days : 0,4,8 and 16 for the measurement of MDA concentration and AChE activity.

Malondialdehyde analysis

A cold KCl solution (150 mM) was used for the extraction of malondialdehyde (MDA) in digestive glands. 1,1,3,3-Tetraethoxypropane was used for the standard and the reaction was developed with the addition of 2-thiobarbituric acid (Sunderman et al. 1985).

Acetylcholinesterase analysis

For acetylcholinesterase measurements, gills were homogenized in phosphate buffer (0.1 M, pH 7.4) at a ratio of 3 ml of buffer for 1 g of tissues. The homogenate obtained was then centrifuged at 9000 g for 20 min at 48°C. An aliquot of the supernatant was used for measuring AChE according to the method of Ellman et al. (1961), modified for microplate reading by Bocquené and Galgani, (1998). The extracts were incubated in the presence of acetylthiocholine iodide as substrate and 5.5'-dithiobis-2-dinitrobenzoic acid (DTNB). The reaction was carried out at 25°C and the absorption was measured by a spectrophotometer at 412 nm. The enzymatic reaction rate was quantified against a blank without substrate for each activity measurement. A second blank was performed without sample to subtract the

spontaneous hydrolysis of the substrate. AChE activity is expressed as nmol of the product developed per minute and per mg of proteins. The quantity of protein present in the homogenate was determined according to the methods of Bradford, (1976) at 595 nm, using bovine serum albumin (BSA) as a reference standard.

RESULTS AND DISCUSSION

Effluent analysis

Results obtained from the effluent analysis were presented in table 1. Table showed high concentrations of dissolved salts and consequently a high salinity of effluent, low pH and high concentrations of some inorganic pollutants (NO_3-,$SO4^{2-}$, Cl^-, Na^+, K^+, Mg^{2+}, Ca^{2+}, F^+, Cd, Ni, As and Hg). Salinity is an important abiotic factor that affects the growth and survival of marine organisms. The continuous introduction of effluent may change the salinity of the surrounding sea water, thereby causing negative effects on bivalves. All bivalves are osmo conformers (Gosling, 2003) possessing little if any capability for osmotic regulation of their extracellular fluid. Adjustment of cell volume is brought about by regulating the amount of intracellular free amino acids (Griffiths and Griffiths, 1987). Variation of salinity is known to influence metabolic and physiological parameters in bivalves including heart rate (Bakhmet et al. 2005), respiration (Stickle and Sabourin, 1979), energy acquisition (Gardner and Thompson, 2001) and growth rate (Westerbom et al. 2002). Metabolic and physiological parameters could be also influenced by the variation of pH. The bivalvian exoskeleton, composed largely of calcium carbonate ($CaCO_3$) (Pechenik, 2009), can dissolve under conditions of reduced pH (Greenaway, 1971; Chapman et al. 1982; Maeda-Martínez, 1987; Michaelidis et al. 2005), posing potentially grave problems for developing embryos with their thin calcified shells (Maeda-Martínez, 1987; Cancino et al. 2000; Montory et al. 2009). On the other hand, dissolving calcium from the shell can provide acid–base regulation (Silverman et al. 1987), reducing the rate or even reversing the direction of pH change (Chaparro et al. 2009 ; Montory et al. 2009). At reduced pH, Bamber, (1990) showed growth suppression, tissue weight loss, reduced shell size, shell dissolution and suppressed feeding activity occurred and abnormal behavior analogous to narcosis (excessive shell gaping, torpor) in three species of commercial bivalve mollusk (*Ostrea edulis, Crassostrea gigas* and *Mytilus edulis)*. The author concludes that seawater at pH <7 is intolerable to bivalve mollusks. Excess environmental K^+ may cause changes in membrane polarization which can interfere with Na^+/K^+ ATP'ases. A reduction in ATP'ase activity would result in loss of energy necessary to sustain ciliary beating and, eventually, to a loss of that activity. The second effect associated with K^+ intoxication in gill tissue was cellular swelling (Fisher et al., 1991). Wildrige et al. (1998) showed that 200 mmol/L of K^+ caused inhibition of valve closure and a decrease of filtration rates in *Dreissena polymorpha*. Except for Hg, the amount of Cd, As and Ni exceeding the norms, especially Cd. Cd, As and Ni, are toxic metals that have no known vital or beneficial effect on clams. Their accumulation over time in the bodies of the animal can cause serious damage on cellular, enzymatic and genome skills. Monitoring and prevention of heavy metal pollution is one of the hot topics in environmental researchers. In the biomonitoring of aquatic heavy metal, different methods or techniques can be adopted like analysis of biomarkers such as MDA and AChE activity.

Parameters	Measures	Tunisian norms (1989) mg/L
Particular matter	2,1 mg/L	-
Turbidity	110 FAU	-
Conductivity (T=25°C)	58 mS/ cm	-
pH	2,5	6.5<pH<8.5
redox Potential E°	275 mV	-
COD	0	-
Cl^-	34096,5 mg/ml	-
NO_3^-	0 mg/ml	90
SO_4^{2-}	9202 mg/ml	1000
Na^+	19519 mg/ml	-
K^+	660 mg/ml	1000
Mg^{2+}	2330 mg/ml	2000
Ca^{2+}	2353,5 mg/ml	-
F	17 mg/ml	5
Cd	0.011 mg/l	0.005
Ni	0.0048 mg/l	2
As	0.0095 mg/l	0.1
Hg	0.0047 10^{-3} mg/l	0,001

Table 1. The physico-chemical characteristics of effluent from phosphate treatment plant and compare with standards permissible limits.

Stress on stress test

The stress on stress experiment was performed on animals previously exposed to effluents. Lethal time

for 50% of individuals (LT50) was determined and the effect of contaminant exposure on anoxic survival time in clams was shown in Figure 4. A decrease of the LT50 with the increase of effluent concentrations and the time of exposure was observed. The 30% and the 20% effluent concentrations have a remarkable negative effect on the survival of the clams. Those tow last concentrations lead to a faster death which explains the lowest TL50 values . Nevertheless, even 5% effluent caused a decrease of the LT50 from the 5th day. That is why clams were contaminated by only 1% effluent in order to determine MDA concentration and AChE activity. The reduction of survival in air, or stress on stress response, was measured as an index of a general stress syndrome in *R. decussatus* and showed that the studied clams have suffered from joint stress effects caused by both cadmium contamination and oxygen deficiency. The same result was observed with the same species (Hamza-Chaffai et al. 1998), *M. galloprovincialis* (Viarengo et al. 1995) and *M. trossulus* (Veldhuisen-Tsoerken et al. 1991).

Biomarker responses

The measurement of MDA and AChE activity was performed, respectively, in two different tissues (digestive glands and gills) of *R. decussatus* and results were represented in Fig 3 and 4. In Figure 3, the effect of effluent on MDA concentration of *Ruditapes decussatus* was observed. Compared to controls, MDA concentration increased significantly since the 4th day of exposure. Figure 4 showed that AChE activity measured in gills was unaffected across effluent contamination. The ROS, which results from the Cd exposure, alter the structure of the cell membranes by stimulating the lipid peroxidation process (Harris, 1992; Stohs et al. 2000). A radical attack on lipids leads to the formation of lipidhydroperoxides (lipid-OOH) (Leibovitz and Siegel, 1980; Storey, 1996), which can decompose to yield alkanes, alkenes, ketones and aldehydes (Zielinski and Portner, 2000). The most important aldehyde produced is malonedialdehyde (MDA). The presented work demonstrate that exposure to effluent stimulate lipid peroxidation in digestive glands of *Ruditapes decussatus*, reflected by the increase of MDA concentration in this organ of exposed animals. Increased levels of MDA following metal exposure have been reported in some other bivalves like *Pyganodon grandis* (Couillard et al. 1995; Giguère et al. 2003), *Mytilus edulis* (Gérét et al. 2002), *Bathymodiolus azoricus* (Bebianno et al. 2005), *Unio tumidus* (Cossu et al. 2000). Nevertheless, this type of response cannot be generalized since other researchers have failed to detect such increases (Viarengo et al. 1990; Thomas and Wofford, 1993; Bonneris et al. 2005). In the present study, the MDA levels were elevated only in the digestive glands, indicating a bioaccumulation of contaminants. Indeed, the gills have been noticed as a short-term storage organ, whereas absorption through the digestive gland leads to an accumulation of toxic metals for a longer time (Amiard et al. 1989).

Monitoring studies of neurotoxic compounds are mostly based on the inhibition of AChE activity, the majority relating this response to a contamination by organophosphorous, carbamates and heavy metals (Najimi et al. 1997; Fulton and Key, 2001; Bonacci et al. 2006; Machreki-Ajmi et al. 2007; Ladhar-Chaabouni et al. 2009). Interestingly, in the present work, AChE activity measured in gills, was unaffected during the study period indicating that compounds present in effluent had negligible effects in gill AChE of *R. decussatus* despite the high concentrations of heavy metals determined in the effluent. Ochoa et al. (2013) explained the absence of AChE inhibition in gills of oysters exposed to pesticide by the presence of insensitive acetylcholinesterases in gills of the common oyster. Bocquené et al. (1997) described the presence of two AChE in the common oyster, one ('A' acetylcholinesterase) anchored to the membrane via glycolipid, not glycosylated and sensitive to organophosphate and carbamate inhibitors and the other ('B' acetylcholinesterase), hydrophilic, glycosylated and highly resistant to inhibitors. The reproductive status of bivalves may also affect enzyme activities. Radenac et al. (1998) reported that AChE activities may increase during spawning due to decreased levels of proteins in tissues. Three hypothesis could be advanced to explain the absence of AChE inhibition by effluent: i) AChE is a biomarker not specific to metal contamination ii) the presence of two forms of AChE in the clam *R. decussatus* as described in the common oyster iii) *R. decussatus* was collected in summer. At this period clams are in spawning stage as described by smaoui-Damak et al. (2006).

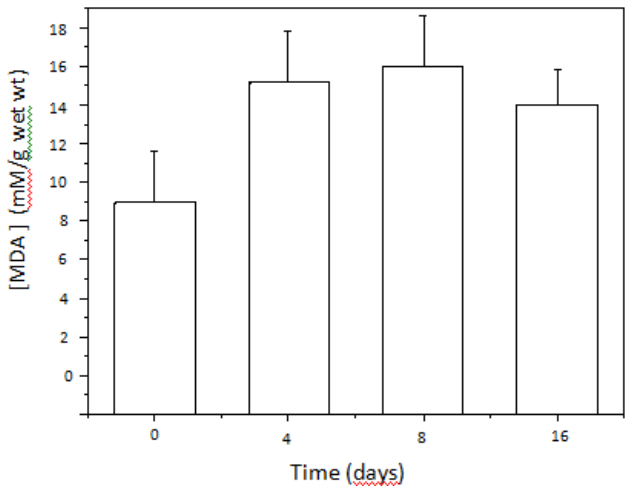

Figure 3. Variation of MDA concentrations (mM/g wet weight) in digestive gland of *Ruditapes decussatus* (n=15) exposed to effluent (1%) for 4, 8 and 16 days.

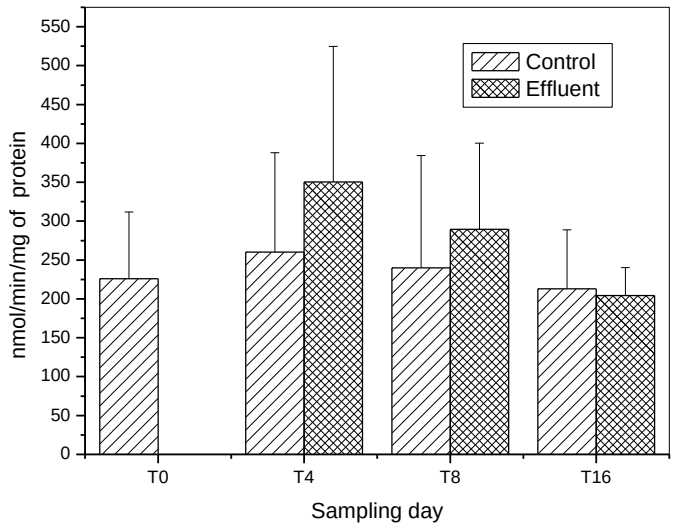

Figure 4 Variation of AChE activity (nmol/min/ mg of Prot) in gills of *Ruditapes decussatus* (n=15) exposed to effluent (1%) for 4, 8 and 16 days.

CONCLUSION

Previous studies have focused on the *in situ* effect of contamination by effluent from phosphate treatment plant. The present research appears to be the first attempt to determine the variations of LT50, MDA concentration and AChE activity in *R. decussatus* exposed to *in vivo* contamination by the industrial effluent. The results showed that since the 5th day of contamination using 5% effluent the clams were stressed. An exposure to 1% effluent showed an increase of MDA concentration in digestive glands of clams. Nevertheless, AChE activity in gills of *R. decussatus* was unaffected. A chemical characterization of effluent was also realized in order to have idea on the level of some chemicals which may have an adverse effect on the ecosystem such as heavy metals.

Results showed a high concentrations of some chemicals, comparing to the norms, such as SO_4^{2-}, NO_3^-, K^+, Mg^{2+}, F and Cd.

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