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Full Length Research Paper

Validation of *Orchestia gammarellus* enzymatic activities in several sites of Tangier's bay (Morocco)

Hikmat Douhri* and Fouad Sayah

PER-CEEM, Laboratoire de Biologie Appliquée and Sciences de l'Environnement, Faculté des Sciences et Techniques, Université Abdelmalek Essaâdi, BP 416, Tangier, Morocco.

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Marine biodiversity is increasingly at risk because of coastal contamination. Biomarkers of pollutant exposure can be very useful for marine biodiversity conservation. The aim of this research was to validate the enzymatic activities (catalase, esterase, α-amylase and acetylcholinesterase) of *Orchestia gammarellus* in several sites of Tangier's bay to improve predictive performance of responses to coastal environmental pollutants. Our results showed a significant increase of catalase (e.g. there was an augment from 0.6 ± 0.2 µmol/min/µg of proteins in individuals sampled from Mnar to 5.0 ± 0.7 µmol/min/µg of proteins in those taken from Port). Esterase activity was significantly decreased (e.g. there was a decrease from 8.2 ± 1.5 nmol of formed β-naphthol/min/mg of proteins in individuals sampled from Mnar to 2.6 ± 0.9 nmol of formed β-naphthol/min/mg of proteins in those taken from Port). Acetylcholinesterase activity's level exhibited a significant difference between individuals taken from Mnar (7.6 ± 1.4 µmol/min/mg of proteins) and those taken from Port (3.1 ± 0.6 µmol/min/mg of proteins). There was a significant difference in the proteins level between individuals taken from Mnar (1.2 ± 0.3 µg/mg of fresh weight) and those collected from Port (0.3 ± 0.1 µg/mg of fresh weight). The level of consumed starch showed a significant difference between individuals taken from Mnar (9.8 ± 0.4 µg/min/fresh weight) and those taken from Port (8.5 ± 0.4 µg/min/fresh weight).

Key words: Acetylcholinesterase, biodiversity conservation, catalase, esterase, *Orchestia gammarellus*, α-amylase.

INTRODUCTION

The marine ecosystem plays an important socio-economical role in Morocco (Banaoui et al., 2004). Unfortunately, Moroccan marine ecosystems are continually endangered by contamination due to human activities. In fact, the Mediterranean coastline of Tangier receives many pollutants resulting from urban and industrial wastewaters that cause environmental perturbbation (El Hatimi et al., 2002). A significant degradation of marine biodiversity and a depletion of the most sensitive species may be caused by this pollution. Biomarkers are biological responses allowing the detection of deleterious effects of contaminants on the biota at low levels of organization (Huschek and Hansen, 2005) before the perturbation of the whole community. In fact, they can give an insight into ecosystem health being very useful to preserve marine biodiversity. Biochemical and molecular responses are famous by their rapid activation (Moreira and Guilhermino, 2005).

Marine invertebrates represent an integrant part of aquatic ecosystem and for this reason they are essential keys to evaluate its health (Rickwood and Galloway, 2004). Indeed, these organisms are protected against oxidative stress by several defense mechanisms with antioxidant enzymes such as catalase (Legeay et al., 2005). In fact, catalase involves in the defense against oxidative damage (Pellerin-Massicotte, 1997). Besides, esterase involves in the metabolism of insecticides (Haubruge and Amichot, 1998) and acetylcholinesterase is essential in nerve impulse transmission (Dellali et al., 2001). In marine invertebrates, the carbohydrate metabolism may be disturbed by xenobiotics (De Coen et al., 2001; Jayaprada et al., 1991). Because of this, the study of biomarkers related to the carbohydrates

*Corresponding author. E-mail: hdouhri@gmail.com. Tel: +212 66 06 56 95. Fax: +212 39 99 45 00.

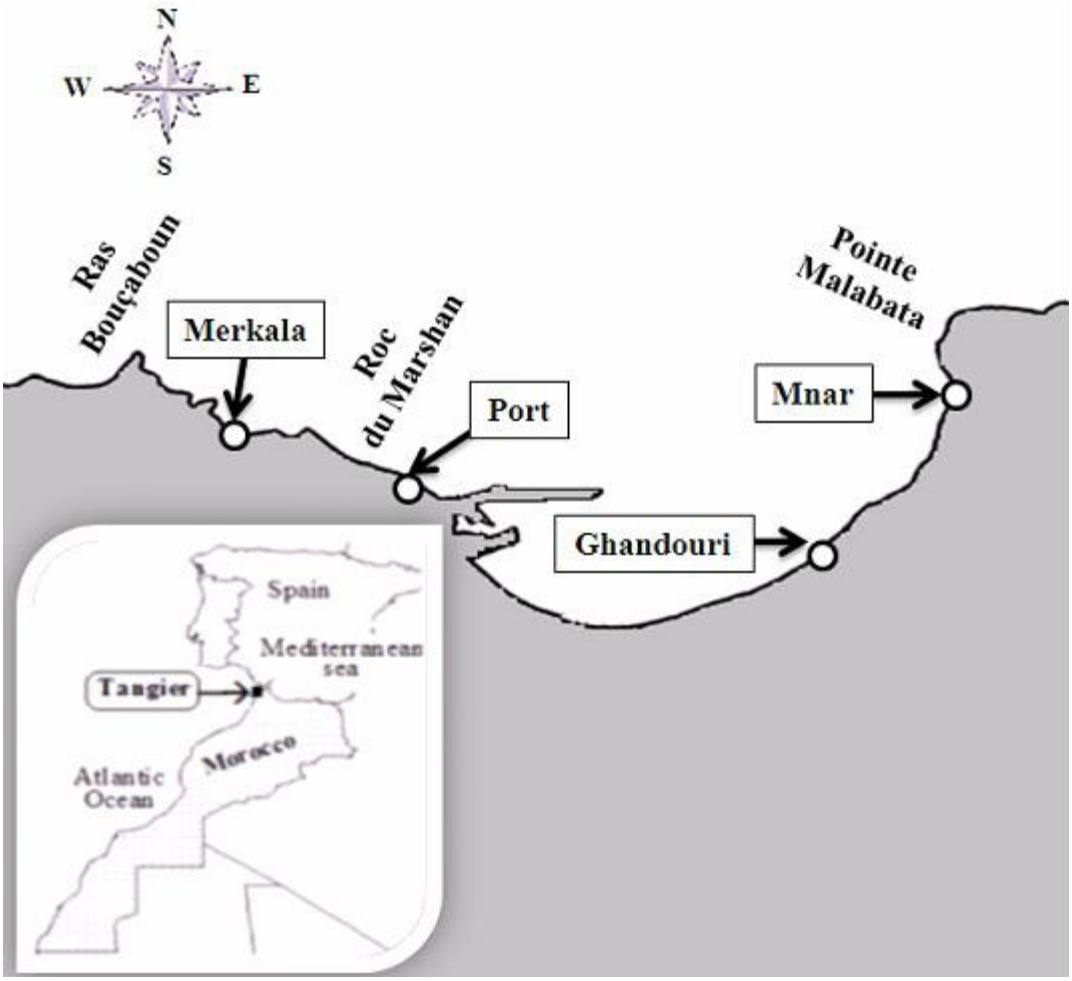

Figure 1. Localization of the sampling sites in Tangier's bay (Morocco).

metabolism is more and more required. The α-amylases (α-1, 4-glucan-4-glucanohydrolases; EC3.2.1.1) are hydrolytic enzymes that are found in microorganisms, plants and animals. These enzymes catalyze the hydrolysis of α -D-(1, 4) glucan linkage in starch and related carbohydrates (Strobl et al., 1998). Our investigation is focused on the validation of the enzymatic activities (catalase, esterase, α-amylase and acetylcholinesterase) of *Orchestia gammarellus* in several sites of Tangier's bay to improve predictive performance.

MATERIALS AND METHODS

Sampling

Our sampling stations are situated along Tangier's bay (Figure 1). The Mnar station is located far from all sources of contamination. Merkala receives sewage and industrial effluents; Ghandouri receives industrial effluent discharges and Port receives sewage effluents in addition to the high traffic and maritime activities rejections. The choice of the amphipod *Orchestia gammarellus* (Crustacea, Amphipoda, Talitridae) (Pallas, 1766) was based on their high sensitivity as bioindicators of marine pollution. Individuals of the beachflea *Orchestia gammarellus* used in this investigation were collected during May 2006, at the moment of the low tide. Living samples brought to the laboratory in cold boxes were immediately weighed and stored at -20°C until analyses.

Proteins and enzyme assays

All procedures were carried out at 4°C. As individuals provide insufficient material, analyses were done by homogenizing a pooled sample of whole animals. Catalase activity was determined by the rate of hydrogen peroxide disappearance according to Clairborne method (1985). After samples homogenising in NaCl, the homogenate obtained was added by phosphate buffer pH 7. Then, the mixture was centrifuged at 7000 g for 20 min at 4°C. The supernatant was added by phosphate buffer and the hydrogen peroxide solution. Finally, the absorbance was read every 30 s, during 6 min at 240 nm. The activity of the catalase was expressed in µmol per minute per µg of proteins. The esterase activity measurement was spectrophotometrically realized using the β-naphthalene acetate as substrate, according to the protocol described by Van Asperen (1962). After samples homogenising in NaCl, the homogenate obtained was centrifuged at 11000 g for

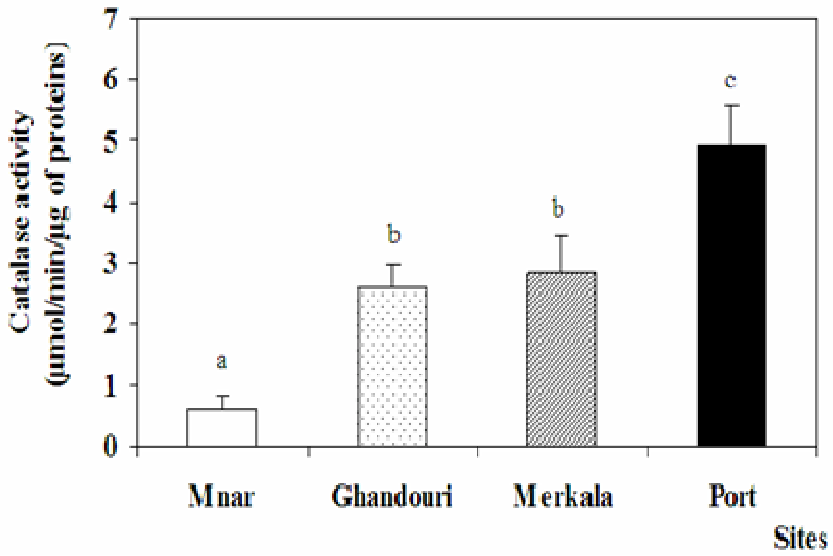

Figure 2. Catalase activity in *Orchestia gammarellus* taken from four sites with different degree of pollution. Each point represents the mean ± standard error of six replicates. Means followed by the same letters are not significantly different (P>0.05).

20 min at 4°C. The supernatant was added by sodium phosphate buffer pH 6.5 containing 0.1 mM of β-naphthyl acetate, and then, the mixture was incubated at 30°C for 15 min. Subsequently, the reaction was stopped by the addition of a colouring solution containing Fast garnet (0.3%) and Sodium Dodecyl Sulfate (3.5%) and after that the mixture was kept for 15 min at the ambient temperature. Finally, the absorbance of the complex naphthol-fast garnet was measured at 492 nm, and the esterase activity was expressed in nmol of formed β-naphthol per minute per mg of proteins.

Acetylcholinesterase activity measurement was spectrophotometrically realized according to the method of Ellman et al. (1961), by measuring the absorbance increase of the sample. After samples homogenising in NaCl, the homogenate obtained was added by phosphate buffer pH 7.4. Then, the homogenate obtained was centrifuged at 9000 g for 30 min at 4°C. The supernatant was added by phosphate buffer pH 7.4 and the Ellman reagent. Finally, the absorbance was read, every 30 s, during 6 min at 410 nm and the acetylcholinesterase activity was expressed in µmol per minute per mg of proteins. α-amylase activity was measured according to the method of Valencia et al. (2000) using iodine/iodide (0.5% I_2 and 5% KI). After samples homogenising in NaCl, the homogenate obtained was added by the citrate of Na-NaCl-$CaCl_2$ buffer 10 mM pH 5. Then, it was added by a starch solution at 0.5% and incubated for 15 min at 37°C. The iodine reagent (0.02% I_2 and 0.2% KI) was added to the mixture, and after that centrifuged at 2,000 g during 20 min. The absorbance was read at 580 nm, and the activity of the enzyme was expressed in quantity of consumed starch in µg per minute per fresh weight. α-amylase activity is proportional to the quantity of consumed starch. Protein content was quantified according to Bradford method (1976), using Bovine Serum Albumin (BSA) as standard. After samples homogenising in NaCl, the homogenate obtained was added by phosphate buffer pH 7 and centrifuged at 8000 g during 20 min at 4°C. The supernatant was added by the Bradford reagent and left during 5 min in the dark. Thereafter, the absorbance was read at 595 nm. Protein content was expressed in µg of proteins per mg of fresh weight.

Statistical analysis of data

Our results were expressed as mean ± standard error of six replicates. In order to compare statistically the differences between the means obtained for reference site and those obtained for the sites with different degree of pollution, data were subjected to analysis of the variance (ANOVA) using statistica software (Statistica, 1997). The chosen level of significance was $P < 0.05$.

RESULTS

Catalase activity

The concentrations of catalase in *Orchestia gammarellus* are shown in Figure 2. Data showed that the lower level (0.6±0.2 µmol/min/µg of proteins) was recorded in individuals sampled from Mnar. Whereas an accumulation of catalase was seen in individuals taken from Ghandouri (2.6±0.4 µmol/min/µg of proteins), Merkala (2.8±0.5 µmol/min/µg of proteins) and Port (5.0±0.7 µmol/min/µg of proteins). This rise was very highly significant ($P < 0.001$).

Esterase activity

Concerning the esterase activity (Figure 3), the highest level (8.2±1.5 nmol of formed β-naphthol/min/mg of proteins) was seen in *Orchestia gammarellus* taken from Mnar. The statistical test showed that esterase activity was very highly significantly diminished ($P < 0.001$) in individuals taken from Ghandouri (4.0±0.7 nmol of formed

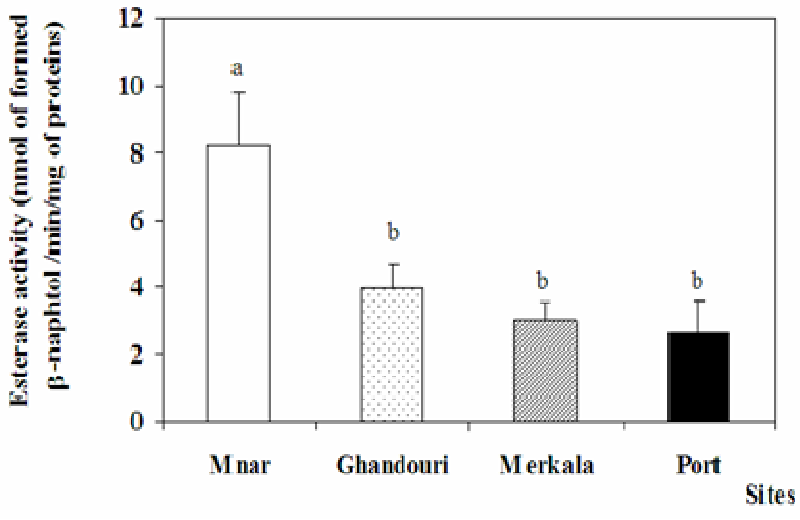

Figure 3. Esterase activity in *Orchestia gammarellus* taken from four sites with different degree of pollution. Each point represents the mean ± standard error of six replicates. Means followed by the same letters are not significantly different (P>0.05).

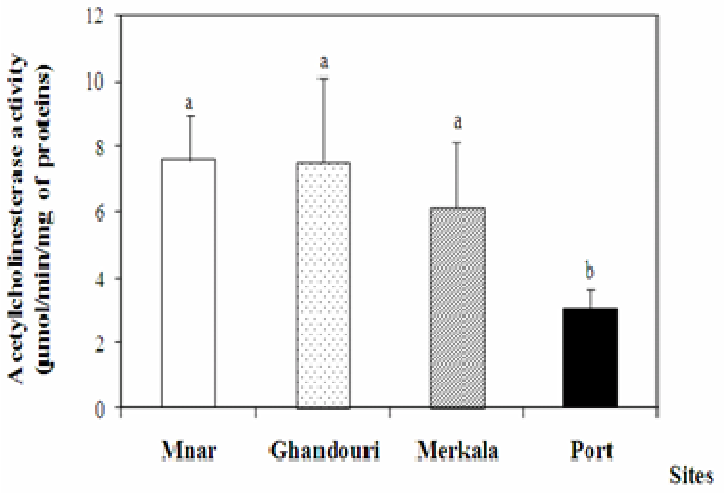

Figure 4. Acetylcholinesterase activity in *Orchestia gammarellus* taken from four sites with different degree of pollution. Each point represents the mean ± standard error of six replicates. Means followed by the same letters are not significantly different (P>0.05).

β-naphthol/min/mg of proteins), Merkala (3.0±0.5 nmol of formed β-naphthol/min/mg of proteins) and Port (2.6±0.9 nmol of formed β-naphthol/min/mg of proteins).

Acetylcholinesterase activity

Acetylcholinesterase activity's value in *Orchestia gammarellus* are shown in Figure 4. As for the esterase activity, the highest value of acetylcholinesterase activity (7.6±1.4 µmol/min/mg of proteins) was seen in individuals taken from Mnar. Variance analysis showed that the inhibition of acetylcholinesterase activity was very highly significant (P < 0.001) in individuals taken from Port (3.1±0.6 µmol/min/mg of proteins).

Proteins level

The proteins level in *Orchestia gammarellus* are shown in

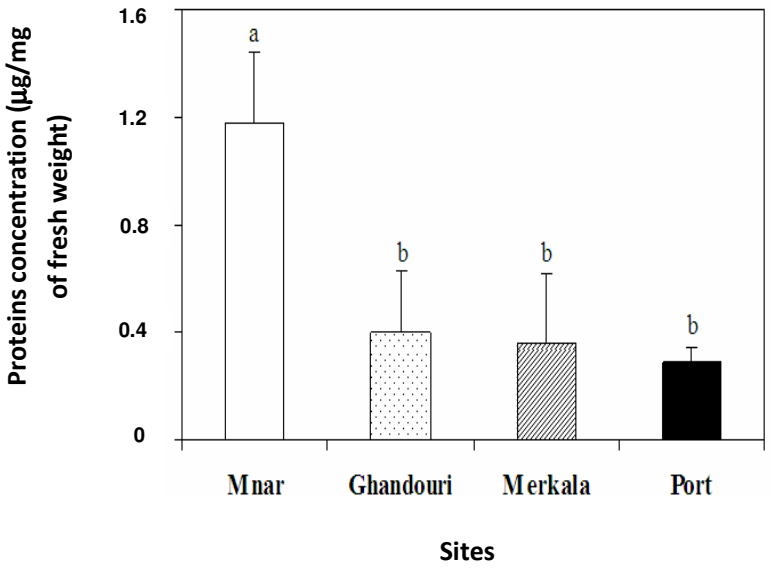

Figure 5. Proteins concentration in *Orchestia gammarellus* taken from four sites with different degree of pollution. Each point represents the mean ± standard error of six replicates. Means followed by the same letters are not significantly different (P>0.05).

Figure 5. Data show that the highest quantity of proteins (1.2±0.3 µg/mg of fresh weight) was seen in *Orchestia gammarellus* taken from Mnar. Statistical analysis showed that the decrease in the rate of proteins was very highly significant (P < 0.001) in individuals collected from Ghandouri (0.4±0.2 µg/mg of fresh weight), Merkala (0.4±0.3 µg/mg of fresh weight) and Port (0.3±0.1 µg/mg of fresh weight).

α-Amylase activity

The level of consumed starch in *Orchestia gammarellus* are shown in Figure 6. Data showed a higher level of consumed starch (higher level of α-amylase activity) in *Orchestia gammarellus* taken from Mnar (9.8±0.4 µg/min/fresh weight). Consumed starch quantity decreased in individuals collected from Ghandouri (8.8±0.4 µg/min/fresh weight), Merkala (8.7±0.4 µg/min/fresh weight) and Port (8.5±0.4 µg/min/fresh weight). This decrease was significant (P<0.05).

DISCUSSION

We previously found an alteration of abiotic parameters (increase of the temperature and a decrease of the dissolved oxygen level and the pH value) in water samples collected from Ghandouri, Merkala and Port (Douhri and Sayah, 2009). In the present study, we evaluated the validation of some enzymatic activities of the marine invertebrate *Orchestia gammarellus* in these sites. We also tested whether these biochemical responses are modified following the environmental quality. We found an increase of catalase activity in *Orchestia gammarellus* taken from the sites receiving pollutants (Ghandouri, Merkala and Port). This could be due to an induction of its biosynthesis via the Reactive Oxygen Species (ROS) generated by the metabolism of hydrocarbons (Erraioui et al., 2009). Comparable changes were seen in the worms *Laeonereis acuta* exposed to hydrogen peroxide (Da Rosa et al., 2005). However, our results are not in agreement with those of Pampanin et al. (2005) reporting a significant inhibition of catalase activity in the mussels *Mytilus galloprovincialis* transferred to polluted sites in Venice lagoon.

In *Orchestia gammarellus* sampled from polluted sites, our study revealed a reduction of esterase activity. Our results agree with those found in the worms *Nereis* (*Hediste*) *diversicolor* exposed to the insecticide temephos in coastal marshes of Morbihan in France (Fourcy et al., 2002) and in the bivalves *Adamussium colbecki* exposed to chlorpyrifos (Bonacci et al., 2004). Our results show a higher inhibition of acetylcholine-sterase activity in *Orchestia gammarellus* sampled from the most contaminated sites. This could be done to a neurotoxic effect induced by exposure to xenobiotics such as heavy metals. Similar observations have been found in the bivalves *Perna perna* sampled from polluted sites in Moroccan coastline (Najimi et al., 1997), as well as in the bivalves *Scrobicularia plana* and the worms *Nereis diversicolor* collected from polluted sites in the

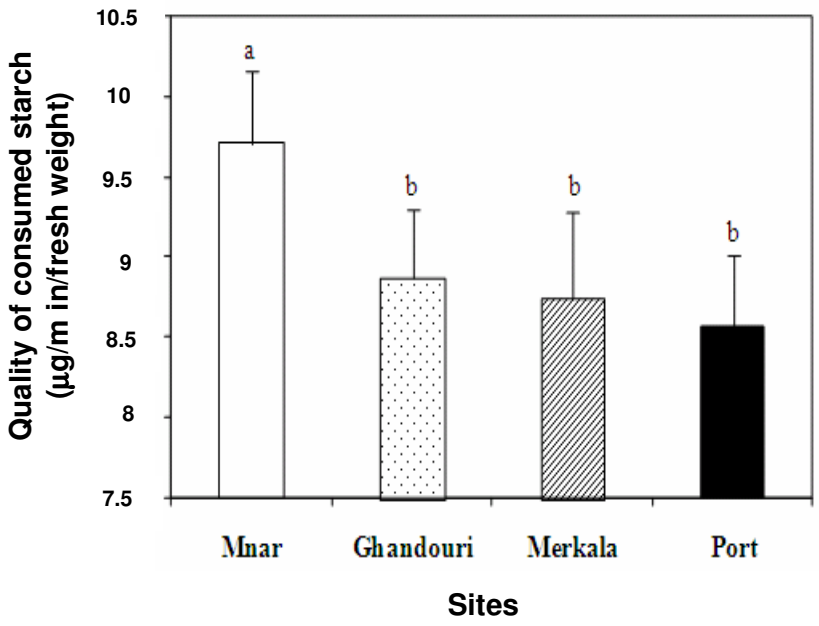

Figure 6. Consumed starch level in *Orchestia gammarellus* taken from four sites with different degree of pollution. Each point represents the mean ± standard error of six replicates. Means followed by the same letters are not significantly different (P>0.05).

Bay of Cadiz (Pérez et al., 2004). Recent work of Binelli et al. (2006) has also reported a decrease of acetylcholinesterase activity in the zebra mussels *Dreissena polymorpha* exposed to chlorpyrifos and terbutilazine. In *Orchestia gammarellus* collected from polluted sites, the proteins concentration is reduced. This could be done to a degradation of these substances or a reduction of their biosynthesis, caused by xenobiotics (Pytharopoulou et al., 2006; De Smet and Blust, 2001). Our results agree with those found in the worms *Nereis diversicolor* collected from polluted estuary of the Seine (Durou et al., 2006). Our results also showed a significant decrease of consumed starch quantity in *Orchestia gammarellus* collected from Ghandouri, Merkala and Port. This may result from a cytotoxic effect of pollution on the epithelial cells responsible for the synthesis of α-amylase. Such perturbations of carbohydrate metabolism were also reported by Moorthy et al. (1983) in freshwater mussel *Lamellidens marginalis* exposed to phosphamidon.

This work reports the use of enzymatic biomarkers of the beachflea *Orchestia gammarellus* for costal bio-monitoring. It display that there is an evident correlation between variations of the biochemical parameters studied in *Orchestia gammarellus* and the pollution's degrees of the studied sites. In conclusion, it can be stated that the selected bioindicator (*Orchestia gammarellus*) and the chosen enzymatic activities are promising tools for environmental risk assessment. They should be used for the biodiversity preservation in Tangier's bay.

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