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Full Length Research Paper

Ruditapes decussatus embryo-larval toxicity bioassay for assessment of Tunisian coastal water contamination

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This paper aims to assess Tunisian coastal water quality using the clam *Ruditapes decussatus* embryos and larvae bioassays tests. Water samples collected from four stations (Monastir lagoon, Chebba, Mahres and Zarat), was used for chemical analysis and clam bioassay tests (embryogenesis, larval growth and metamorphosis). The results, based on chemical analysis, showed the highest metal contamination in Mahres and Zarat while the lowest ones were recorded in Chebba station. The Monastir lagoon is characterized by the highest level of mercury (18.1±2.16 µgL^{-1}). Compared to control, reduction of clam embryogenesis is up to 40, 60 and 67% respectively in Monastir, Zarat and Mahres while no reduction were observed in Chebba ($p>0.05$). Larval growth rate was significantly ($p<0.05$) reduced in all stations except Chebba this also shown when daily growth rate (DGR) is calculated for larvae reared in water collected from each station. A significant ($p<0.05$) reduction in larval survival is also shown in Monastir (74%), Zarat (70%) and Mahres (52.8%) compared to control (94.5%). Compared to control (82%), metamorphosis success is significantly reduced only on two stations (Zarat: 68% and Mahres: 64%) conversely survival in this stage was affected in three stations (Monastir: 69±6.7%; Zarat: 52±6.1% and Mahres: 44±5.2%) compared to the control (83±4.7%). This work showed that both clam embryos and larvae are sensitive to contaminants and can be used to evaluate seawater contaminations and monitoring pollution.

Key words: Bioassay, coastal water, embryogenesis, larvae, metamorphosis, *Ruditapes decussatus*.

INTRODUCTION

Estuaries are often highly productive ecosystems affected by urban development and industrial activities that strongly increase background levels of potentially harmful chemical and physical agents. The ever increasing number of xenobiotics and the effects of physicochemical parameters on their availability to marine organisms greatly complicate monitoring based on chemical analyses. Even if we had *a priori* knowledge of the kind of pollutants present, analytical chemistry allows determination of the degree and nature of pollution, but it does not provide evidence for biological consequences. Bioassays allow the detection of these effects by measuring biological responses on marine organisms, and particularly in their highly sensitive early life stages (His et al., 1999).

Criteria for the choice of target organisms for bioassays have been evaluated. The embryos and larvae of marine organisms are generally more sensitive to toxic substances than adults, and gametes and embryos of bivalve have been recognized as valuable tools in toxicological studies since Prytherch (1924) tested *Crassostrea virginica*. Toxicity bioassays are now used worldwide to help assess sea water and sediment quality because they can integrate the various complex effects of contaminants. The bivalve embryo bioassay, one of these procedures, has been shown to be reliable, sensitive, and ecologically relevant (Pamela and O'Halloran, 2001;

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Figure 1. Tunisian map, showing the location of seawater sampling stations for bioassay and chemical analysis.

Beiras, 2002; Nendza, 2002; Volpi et al., 2005; Novelli et al., 2006). During the past decades, numerous studies have been published on the use of bivalve embryos, either concerning the effects of individual contaminants, industrial effluents, and sediments or the assessment of sea and brackish water quality (Losso et al., 2004; Dalmazzone et al., 2004; His et al., 1999; Quiniou et al., 2005, 2007; Stronkhorst et al., 2004). Because of its sensitivity, we considered this test as the most suitable for toxicity testing to better understand environmental disturbances affecting the Tunisian coasts.

Although these methods are used globally, the potential of such toxicity tests has not been adequately explored for Tunisian coastal waters. This study describes bioassay utilizing *R. decussatus* embryos and larvae to monitor coastal water quality and is based on the sensitivity these life stages to different concentrations of water samples. Four stations were selected and water samples were chosen according to their potential effects on sensitive biological components (embryos and larvae) that could be exposed to pollutants. The aims were: a) to evaluate the replicability of all procedures; b) to rank sites (make a classification among sites) for toxicity. This work seeks to form the methodological basis for providing reliable toxicological data to contribute to seawater quality assessment and monitoring in transitional environments.

MATERIALS AND METHODS

Sites description and sampling procedure

Four sampling sites located along Tunisian coasts were chosen (Figure 1). Sampling sites were chosen on the basis of population abundance of the most important local bivalve, the clam (*R. decussatus*). Zarat is an important site used to grow bivalve juveniles (Tunisian-japaneese project for growing clams), Mahres is also one of the most important site. Our laboratory and the nursery were located at the site of Monastir. Chebba is considered as the most distant site, all sites (Zarat, Mahres, Monastir and Chebba) have potential for clam juveniles' culture.

Triplicate of surface water sample, serving for bioassay, were taken in polypropylene flasks from each site and stored at room temperature (22°C). For chemical analysis, water was sampled in 250 ml polypropylene flasks and stored at 4°C. All flasks used were previously already washed with ultrapure acids. The hydrographic conditions (temperature, salinity, pH and dissolved oxygen) were analyzed in situ by means of Orion and Hanna electrodes.

Metal analysis

The background metal concentrations in seawater were determined for the entire samples collected from the four stations are given in Table 1.

The metal concentrations were determined in seawater collected from the four stations. After filtration, the metal samples were extracted by using ammonium pyrrolidine dithiocarbamate (APDC) and methyl isobutyl ketone (MIBK) and the heavy metals in seawater were estimated according to the method of Tewari et al. (2001). The samples were examined for Cu, Pb, Cd, Zn, and Ni by using an air-acetylene flame atomic absorption spectrophotometer (AAS; Spectra AA-10 Varian). Mercury was analyzed by automated cold vapor AAS, according to Weltz and Schubert-Jacobs (1991). To avoid contamination, all glassware and equipment used were acid-washed. To check for contamination, procedural blanks were analyzed once for every five samples.

R. decussatus embryotoxicity bioassay

Clams (*R. decussatus*) used in this work were conditioned in our laboratory for at least 5 month and fed on the microalgae *Isochrysis galbana* and *Chaetoceros calcitrans*. Handling conditions of adult stock were 20- 22°C temperature, 34.5–36.0 ppt salinity, 6.2– 6.6 mg/L O2, and 7.5– 8.4 pH.. The embryo toxicity test was performed according to the method proposed by His et al. (1997). Adults were induced to spawn by thermal stimulation (temperature cycles at 20 and 28°C). Gametes of good quality derived from the best males and females were selected (sperm with high motility and eggs with homogeneous dimensions and regular shape) and filtered at 30 µm (sperm) and 100 µm (eggs) to remove impurities. Eggs (1000 ml) were fertilized by injecting 10 ml of sperm; fecundation was verified by microscope, controlling the presence of the fertilization membrane and the number of sperm cells (10–20) around each egg (His et al., 1997). Egg density was determined by counting four subsamples of known volume. Fertilized eggs were delivered at a density of 60 eggs/ml into experimental vials containing seawater collected from studied sites and artificial seawater (control), reconstituted according to ASTM (1998) at 34 ppt salinity, and were incubated for 24 h at 23°C. The assay was terminated after 24 h using 10% formalin solution when D-shape larvae were observed in control. One hundred larvae were counted, distinguishing between normal larvae (D-shape) and abnormalities (malformed larvae and prelarval stages). The acceptability of test results was based on negative control for a percentage of normal D-shape larvae ≥ 80% (His et al., 1999).

Growth and survival experiment

After 24 h, swimming veliger larvae (D-larvae stage) were re-

Table 1. P values registered after analysis of variances using one-way ANOVA comparison test between Control and studied sites.

	Chebba	Monatir	Zarat	Mahres
Embryogenesis	1	0.035	0.0026	0.0021
Larval growth	0.92	0.03	0.036	0.006
Larval mortality	0.95	0.039	0.031	0.007
metamorphosis	1	0.97	0.048	0.045

Table 2. Heavy metals contents (µg/L) in water collected from studied stations (Mean ± SD).

Metals stations	Cd	Pb	Cu	Ni	Zn	Hg
Mahres	15.48±1.73	31.7±2.67	78.9±5.4	4.4±0.21	124±6.97	8.5±0.14
Monastir	7.24±1.54	19.5±2.60	15.7±3.56	3.8±0.37	48.7±3.95	18.1±2.16
Zarat	11.40±1.45	25.8±1.49	108.5±6.67	1.8±0.25	94±4.79	4.6±0.15
Chebba	3.05±0.21	4.3±0.44	4.3±2.17	0.6±0. 1	18.4±1.50	2.8±0.07

suspended in a 2 L glass beakers, approximately 2×10^4, containing seawater collected from all studied sites and artificial seawater for the control preparation (3 replicates per treatment) which was replaced daily. Larvae were fed daily with phytoplankton mixture (*Isochrysis Tahiti* and *Chaetoceros calcitrans*). The mean length of the larvae (30 individuals per treatment) was recorded after 2, 4, 6, 8, 10, 12 and 14 d of rearing. Larval survival was assessed at the end of the experiment by counting under microscope dead and live larvae in the first 100 larvae encountered.

Metamorphosis experiments

This experiment was carried out when pediveligers were competent to metamorphose, as indicated by a larval size (180 µm) and appearance of feat. The number of pediveligers was calculated first, and placed in 10 ml (4-6 ind. ml^{-1}) Petri dishes (3 replicates) filled with different water samples and ASW (control). The number of postlarvae (metamorphosed larvae) was recorded 72 h later using microscopy. This experiment was conducted at 23°C and salinity was adjusted at 37 ppt in all preparations.

Statistical analysis

Responses to each treatment (percentage of abnormality in embryo development) were corrected for effects in control tests by applying Abbott's formula (ASTM, 1998). Comparisons among sites and the control were conducted for embryogenesis, larval survival, growth and metamorphosis using one-way analysis of variance and Dunnett's multiple comparison tests (Table 1). Differences were considered significant at $p \leq 0.05$. All statistical analysis were done using SPSS 14.0 for Windows software.

RESULTS

Metal analysis

Heavy metal concentrations of surface water sampled from studied stations are shown in Table 2. Mahres showed maximum values for all pollutants in water except for mercury (maximum in Monastir) and copper (maximum in Zarat). The concentrations of metals found in water collected from Mahres, Monastir and Zarat are considered relatively high compared to concentrations found in Chebba water. We note that concentrations of copper and zinc in Mahres and Zarat were higher than EC50 recorded for *R. decussatus* (Fathallah et al., 2010) and As Chebba site showed the least heavy metals amounts, it can be considered as reference site.

Embryotoxicity bioassay

Percentages of embryos successfully developing to normal 'D' stage after 24 h incubation in water collected from the four selected stations and control water are shown in Figure 2. Analysis of variance revealed a significant reduction of embryogenesis in three stations among the four selected ones. Mahres and Zarat waters showed the highest toxicity effect on *R. decussatus* embryos, they reduced significantly ($p < 0.01$) embryogenesis success to up to 50% comparing to control. Reduction of embryogenesis is also significant ($p < 0.05$) in Monastir station but lower than reduction observed in the two previous stations (Mahres and Zarat); we estimate that this reduction is due to high level of mercury found in this station. Results showed that number of normal 'D' stage larvae is not significantly ($p > 0.05$) different in Chebba water comparing to control. These founding are in concordance with water metal concentrations in studied stations (Table 2).

Growth and survival experiment

Growth

Larval growth (shell height increase), for all studied

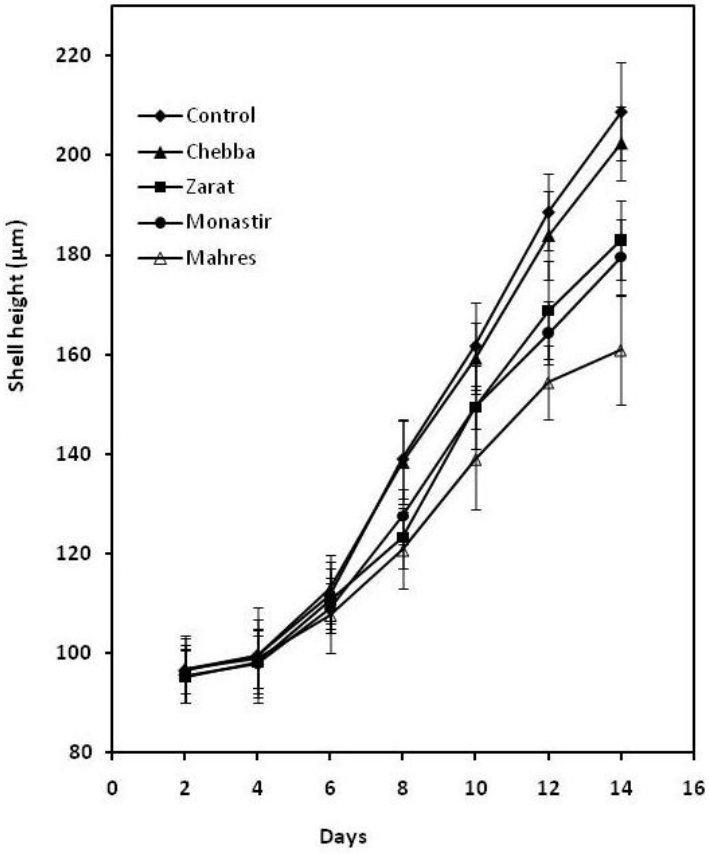

Figure 3. *Ruditapes decussatus* height increase in larvae reared from D-shaped stage to pediveliger stage in sediment elutriates of different studied sites.

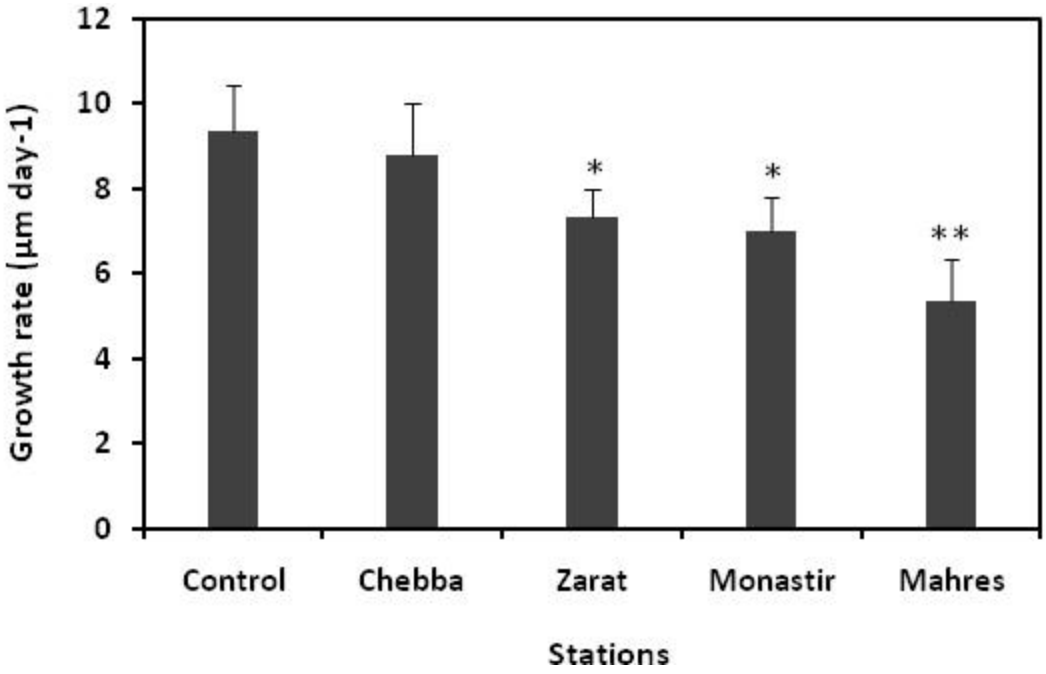

Figure 4. Mean growth rates (µm day^{-1}) of *R. decussatus* larvae exposed to sediment elutriate of different studied sites for 14 days. * indicates treatments significantly less than the control ($p<0.05$).

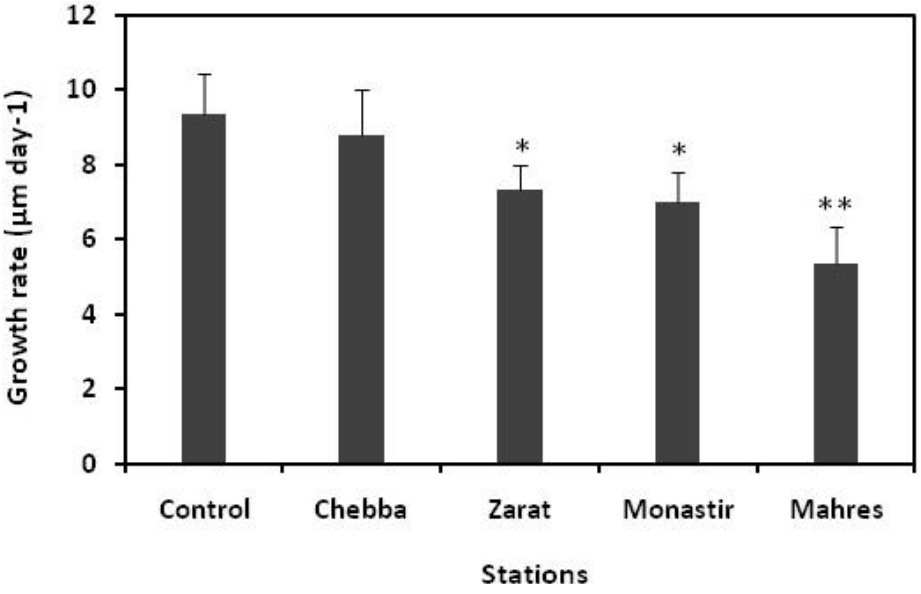

Figure 4. Mean growth rates (µm day^{-1}) of *R. decussatus* larvae exposed to sediment elutriate of different studied sites for 14 days. * indicates treatments significantly less than the control ($p<0.05$).

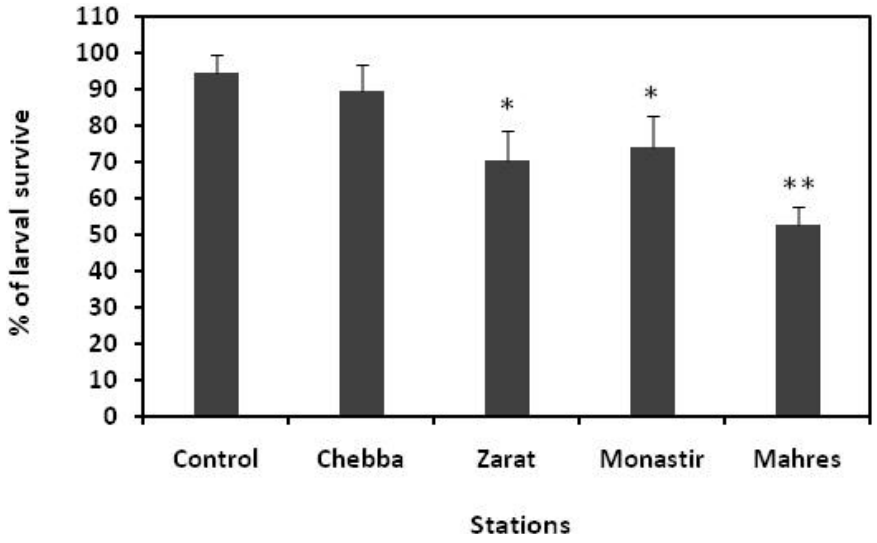

Figure 5. Survival at the end of larval stage (14 days) of *R. decussatus* larvae exposed to sediments elutriates of differents studied sites. Asterisks indicate the samples which significantly differ from the controls, *$p<0.05$ while ** at $p<0.01$.

stations, is illustrated in Figure 3. Growth was significantly ($p<0.05$) reduced, compared to control, in water collected from Mahres, Monastir and Zarat from Day 6 to Day 14. Conversely, larvae reared in water collected from Chebba didn't show a significant height reduction ($p>0.05$). The calculation of the daily growth rates (DGR) of larvae confirmed those results (Figure 4). In effect, compared to the control, larval DGR is significantly ($p<0.05$) lower in Mahres, Monastir and Zarat waters.

Survival

Larval survival recorded after 14 days of rearing (pediveliger stage reached) is shown in Figure 5. Mean *R. decussatus* larval survival ranged from 52.9 to 89.8%. Significant reduction in survival, relative to the control,

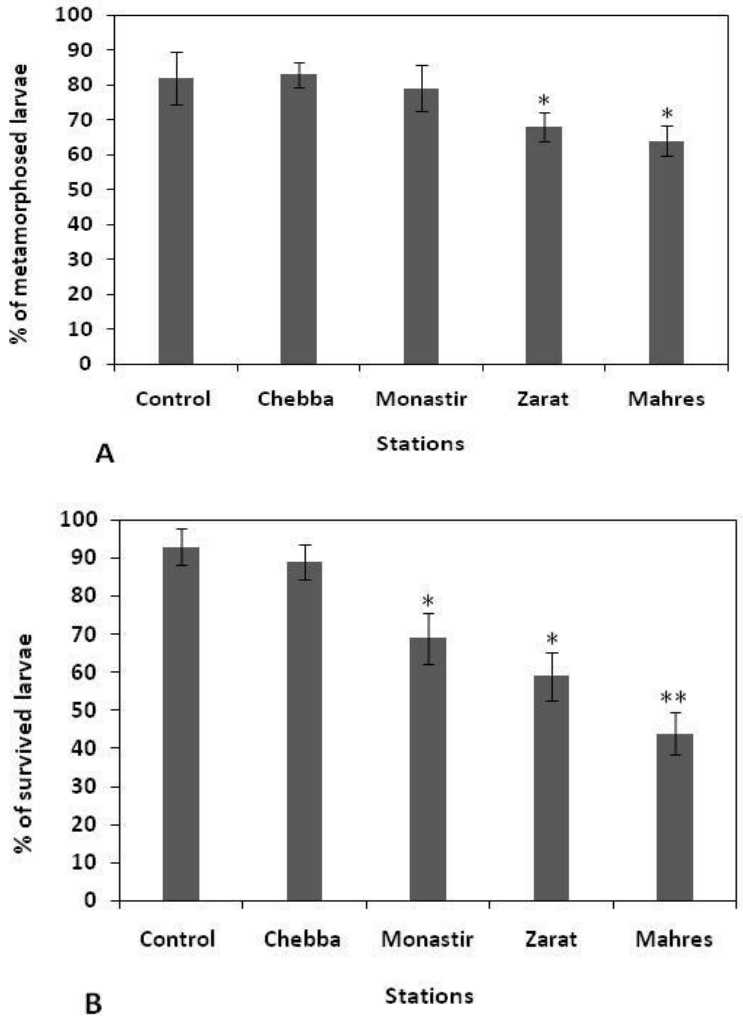

Figure 6. Percentage of metamorphosed individuals (A) and survival (B) at the end of metamorphosis stage of *R. decussatus* pediveligers exposed to surface water collected from different studied stations. Asterisks indicate the samples which significantly differ from the controls, **p*<0.05 while ** at *p*<0.01.

was exhibited in water collected from three stations Monastir (74.1%), Zarat (70.7%) and Mahres (52.8%) (Dunnett's tests, p < 0.05). Conversely no significant statistical differences in survival were shown between the control and the fourth site (Chebba: 89.8%).

Metamorphosis experiments

The percentages of metamorphosed individuals, obtained in competent pediveligers exposed to water from studied stations, and survival rates were presented respectively in Figures 6A and 6B. This experiment showed a significant (*p*<0.05) reduction of the number of metamorphosed larvae exposed to waters collected from Zarat and Mahres compared to control. In contrast, no significant differences (*p*>0.05) was recorded between metamorphosis success in control water and the rest of studied stations (Monastir and Chebba). Larval survival, recorded at the end of settlement stage, is also affected in all stations except Chebba. In effect, after 72 h of exposure to different stations water, the number of larvae is reduced significantly (*p*<0.05) compared to control (survival = 92%) and survival percentages obtained are 69, 58 and 44% respectively in Monastir, Zarat and Mahres. However, in Chebba's water, no significant (*p*>0.05) effect was shown (survival = 89%).

Discussion

Pollution in estuarine and marine environments is considered a critical environmental issue due to anthropogenic pollution and to the high variation in

several abiotic factors that impose severe restrictions to organisms living in these areas (Matthiessen and Law, 2002; Amado et al., 2006). The present work is aimed to assess the pollution statue of four sites belonging to the Tunisian coasts by exposing *R. decussatus* embryos and larvae to water samples, collected from these sites.

In this work we selected 4 sites along the Tunisian coastal areas characterized by different levels of heavy metal contamination. Sampling sites were chosen because of their geographical distribution near urban, industrial and agricultural areas and their importance for clam culture. Monastir lagoon, where located our nursery, is located in mid Tunisia and seems to be relatively polluted since the presence of textile industry as contamination source. Chebba site is also located in mid Tunisia, seems to be relatively unpolluted since no contamination source is present. The other two sites belong to South Tunisia and are represented by Zarat and Mahres. The latter is located south to Sfax that is the most important industrial site of the country, and close to a phosphogypsum plant, that can be a source of heavy metal release (Figure 1).

Heavy metals analysis clearly showed different degrees of heavy metal loads in water sampled from selected sites. Mercury content was higher at Monastir station. Chebba station showed the lowest heavy metals levels, this can be explained by the fact that is the most distant site from industry. The highest levels of heavy metals, except mercury and copper where found in water collected from Mahres station. In fact, Mahres were characterized by relative high heavy metal loads (Cd, Zn and Cu), as previously reported by several authors. Others studies performed at Mahres site demonstrated the presence of high concentrations of such heavy metals with a corresponding increase of Metallothioneins protein levels in tissues of *R. decussatus* (Hamza-Cheffai et al., 2003 and Banni et al., 2005) and the Mediterranean mussel *Mytilus galloprovincialis* (Banni et al., 2007) and *Nereis diversicolor* (Bouraoui et al., 2009). The results confirm the pollution statue of Sfax city coasted area, due essentially to the presence of continuous discharge of heavy metals and also of organic compounds from local industrial activities as described by Banni et al. (2007); Jebali et al. (2007); Smaoui-damak et al. (2004) and Banni et al. (2003).

Bivalve (clam, oyster and mussel) embryo bioassay was one of the techniques selected for monitoring and surveillance of sediment and coastal water (Chapman and Morgan, 1983; Thain, 1992; Geffard et al., 2002; Phillips et al., 2004; King et al., 2004; Volpi Ghirardini et al., 2005). The results of the present study clearly document the sublethal and lethal responses of the development of clam embryos exposed to water samples collected from different stations. Our data provide a very good demonstration of a strong relation between the level of water pollution and *R. decussatus* embryogenesis. The clams embryos that were allowed to develop in contact water collected from different stations have shown marked developmental effects.

R. decussatus embryos development was significantly reduced in water collected for all studied stations except Chebba. This confirms chemical analysis that demonstrated Mahres as the most polluted site and that Chebba is the less contaminated one. Compared to control, reduction of embryogenesis is up to 40, 60 and 67% respectively in Monastir, Zarat and Mahres. Reduction of embryogenesis observed in Monastir is due especially to the high level of mercury recorded in the water (18.1±2.16 µg L^{-1}); this level is slightly lower than the EC50 recorded for mercury on *R. decussatus* embryos (Fathallah et al., 2010) for that reason inhibition did not reached 50 %.

The pediveliger larva of the European clam, *R. decussatus*, was explored as a bioassay organism because of nearly year round commercial availability, and common use in previous work with culture and toxicity testing. In this study clam larvae showed a normal growth in Chebba and a reduction in growth rate in Mahres, Monastir and Zarat waters comparing to the control treatment. Highest mortality is occurred in Mahres station. The present study confirm sensitivity of clam larvae to heavy metals like shown by Manahan and Crisp (1982) and can be easily used for field monitoring of water and sediment quality. Beiras and His (1994) showed a reduction in growth rate in *Crassosstrea gigas* larvae exposed to 8 and 10 µg L^{-1} of Hg from Day 6 of rearing. Wang et al. (2009) showed that *Metrix metrix* larvae exposed to 187 µg µg L^{-1} Hg grew little or died after 24 h and only grew about 20 µm when exposed to 18.5 µgL^{-1} of Hg and that larval growth was limited at a Cd concentration of 1,046 µg L^{-1} and was significantly retarded at 104 µg µg L^{-1}, they showed also that metal affect significantly larval survival. Many other works showed that heavy metals caused failure in metamorphosis (Beiras et His 1994; Hoare et al., 1995). In fact, Wang et al. (2009) showed that 104 µg l^{-1} Cd reduced larval attachment by 44.5%, while 187 µg l^{-1} Hg inhibited metamorphosis by 41.8%. 11.0 µg l^{-1} Cd and 18.5 µg l^{-1} Hg had no adverse effect on larval settlement; on the other hand, enhanced average metamorphosis was also observed at 18.5 µg L^{-1} Hg. This was in concordance with our study where metamorphosis success is affected in the most polluted stations (Mahres and Zarat), survival is also reduced at the end of this step at Mahres, Zarat and Monastir stations. But the use of larvae for bioassay is problematic because sensitivity is age dependant (Beiras and His, 1994). In our knowledge this work is this first studying contamination in Monastir lagoon, but the complex nature of the environment studied with regard to the wide range of possible sources of contamination (waste outlets, inputs from rivers, local industries, tourism and harbor activities) makes interpretation difficult. Concerning Zarat and Mahres stations, inhibition of embryos development and larval

growth and survival is due to the combined effect of the high levels of three metals (Zn, Cd and Cu) known to be toxic to bivalve embryos and larvae. Mahres is located south to Sfax that is the most important industrial site of the country, and close to a phosphogypsum plant, that can be a source of heavy metal release. This station is characterized by relative high heavy metal loads (Cd and Cu), as previously reported by several authors. In fact, many studies performed at Mahres site demonstrated the presence of high concentrations of such heavy metals and their toxic effect on adult *R. decussatus* (Hamza-Cheffai et al., 2003; Banni et al., 2005) and the Mediterranean mussel *Mytilus galloprovincialis* (Banni et al., 2007). Results obtained for all realized test and chemical analysis confirms that Mahres station is the most contaminated site and contamination found in Monastir and Zarat is lethal for embryogenesis, larval development and survival, contrarily, Chebba can be considered as a reference site because no significant differences were observed compared to control treatment for embryos and larvae bioassays.

Conclusion

It is apparent from this and other studies that embryos are preferable for acute bioassay tests due to (1) higher sensitivity to metal pollutants, (2) more rapid and simple evaluation of the lethal effects of the pollutant, and (3) more simple standardization of the bioassay, avoiding interference of variables such as larval age, larval condition and presence of algal food.

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