SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 35, No. 9, pp. 2270–2280, 2016
© 2016 SETAC
Printed in the USA

ASSESSING THE TOXICITY OF SEDIMENTS USING THE MEDAKA EMBRYO–LARVAL ASSAY AND 2 OTHER BIOASSAYS

BADREDDINE BARHOUMI,† ‡ CHRISTELLE CLÉRANDEAU,‡ LAURE LANDI,‡ ANAÏK PICHON,‡ FLORANE LE BIHANIC,‡ DOMINIQUE POIRIER,‡ PIERRE ANSCHUTZ,‡ HÉLÈNE BUDZINSKI,‡ MOHAMED RIDHA DRISS,† and JÉRÔME CACHOT*‡

†Laboratory of Heteroatom Organic Chemistry, Department of Chemistry, Faculty of Sciences of Bizerte, University of Carthage, Jarzouna, Tunisia

‡Laboratory EPOC, UMR CNRS 5805, University of Bordeaux, Talence Cedex, France

(Submitted 27 August 2015; Returned for Revision 16 October 2015; Accepted 27 January 2016)

Abstract: Sediments are sinks for aquatic pollutants, and analyzing toxicity in such complex matrices is still challenging. To evaluate the toxicity of bioavailable pollutants accumulated in sediments from the Bizerte lagoon (Tunisia), a novel assay, the medaka embryo–larval assay by sediment contact, was applied. Japanese medaka (*Oryzias latipes*) embryos were incubated in direct contact with sediment samples up to hatching. Lethal and sublethal adverse effects were recorded in embryos and larvae up to 20 d postfertilization. Results from medaka embryo–larval assay were compared with cytotoxicity (Microtox®), genotoxicity (SOS chromotest), and pollutant content of sediments. The results highlight differences in the contamination profile and toxicity pattern between the different studied sediments. A significant correlation was shown between medaka embryo–larval assay by sediment contact and SOS chromotest responses and concentrations of most organic pollutants studied. No correlation was shown between pollutant levels and Microtox. According to the number of sediment samples detected as toxic, medaka embryo–larval assay by sediment contact was more sensitive than Microtox, which in turn was more sensitive than the SOS chromotest; and medaka embryo–larval assay by sediment contact allowed sediment toxicity assessment of moderately polluted sediments without pollutant extraction and using an ecologically realistic exposure scenario. Although medaka embryo–larval assay by sediment contact should be tested on a larger sample set, the results show that it is sensitive and convenient enough to monitor the toxicity of natural sediments. *Environ Toxicol Chem* 2016;35:2270–2280. © 2016 SETAC

Keywords: Sediment of the Bizerte lagoon   Fish embryo–larval assay   Microtox   SOS chromotest   Metal   Organic pollutant

INTRODUCTION

Most chemicals of anthropogenic origin can bind to particulate matter and accumulate overtime in the sediment [1]. Contaminated sediments can be directly toxic to benthic organisms [2] and can also be a threat for pelagic ones via direct contact or bioaccumulation through the food web [3]. Several chemical, ecotoxicological, or integrated approaches have been developed to evaluate the hazards of contaminated sediments [4–8].

In the aquatic environment, benthic species are directly exposed to contaminants adsorbed on the particle phase but also to those dissolved in the interstitial water and in the water layer at the water–sediment interface. Thus, the evaluation of sediment quality implies the study of these different phases. The use of elutriates (aqueous extracts) can provide information on the potential toxic effects of leached contaminants released from dredged material [9,10] but is unlikely to provide information on the possible effects of contaminants retained in the solid phase and deposited on the seabed. Organic solvent extraction allows extraction of most organic pollutants adsorbed onto particulate matter. Furthermore, a major issue of assaying sediment toxicity is that the toxicity of the extracts may not be the result of anthropogenic contamination alone. Common natural compounds such as ammonia and sulfur can also accumulate in the sediment and cause toxic effects to living organisms when they are bioavailable [11,12]. The testing of whole sediment, as opposed to the use of aqueous-phase and solvent extracts, is more environmentally relevant because it takes into account the dissolved contaminants and those adsorbed to sediment particles [13,14]. After consideration of each test phase, it appears that testing all exposure routes is necessary for a more comprehensive assessment of sediment toxicity. In addition, the use of a battery of test species, from various phyla and trophic levels, has been advocated by a number of researchers [15–17] because testing a single species may not detect contaminants with a specific mode of action.

In the present study, we employed a multiassay approach to characterize the toxicity of Bizerte lagoon (Jarzouna, Tunisia) sediment samples. Considering their key role in ecosystem functioning as decomposers, and/or a food source, and their wide distribution in aquatic ecosystems in particular in the sediment compartment, heterotrophic bacteria are widely used for the assessment of sediment toxicity. In addition, the use of fish embryos has been recommended as an early warning indicator of ecosystem stress because of their high sensitivity to pollutants and their ecological importance in population renewal and food webs [1,18]. The sediments are putative spawning ground for many pelagic and benthic organisms including fish. Therefore, the use of fish early life stages to evaluate sediment toxicity is ecologically relevant [1]. Three bioassays were employed in the present study: Microtox® was applied to measure the acute toxicity of sediment organic extracts and on aqueous sediment elutriates. The SOS-chromotest [19] was used to measure the genotoxic potency of sediment organic extracts. The medaka embryo–larval assay in sediment contact [20,21] was used for whole-sediment toxicity testing. In the medaka embryo–larval assay in sediment contact assay, embryos of the Japanese medaka (*Oryzias latipes*) are incubated at the surface of sediment for the whole duration of the embryonic phase. This species offers many

This article includes online-only Supplemental Data.

* Address correspondence to jerome.cachot@u-bordeaux.fr

Published online 29 January 2016 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/etc.3388

advantages for developmental toxicity testing: high fecundity, short maturation time, year-round spawning, well-described stages of development, transparent chorion and skin of embryos and prolarvae, ease of handling, low maintenance costs, high tolerance to a wide range of temperatures and salinities, low baseline embryo and larval mortality, high hatching rate, and high sensitivity to toxicants [20–27].

The Bizerte lagoon is the second largest coastal brackish water area in Tunisia. Its ecological importance comes from the high number of aquatic and avian species feeding and reproducing in the area. Unfortunately, this lagoon is also highly impacted by numerous human activities including expanding urbanization, agriculture, fishing, aquaculture, shipping, and various industries (cement works, metallurgy, boatyards, tire production). As a consequence, a large panel of pollutants (organochlorine pesticides [OCPs], polychlorinated biphenyls [PCBs], polycyclic aromatic hydrocarbons [PAHs], and heavy metals) has been detected in sediments of the Bizerte lagoon [28–32], but to date no data on sediment toxicity are available.

The objectives of the present study were 1) to evaluate the toxicity of 5 natural sediments from the Bizerte lagoon using the medaka embryo–larval assay by sediment contact, 2) to compare toxicity data between different assays, and 3) to analyze the toxicity resulting from the contamination and physicochemical properties of the studied sediments.

MATERIALS AND METHODS

Sampling locations

The Bizerte lagoon is a large Mediterranean brackish water area of 128 km^2 located in the north part of Tunisia. The lagoon is linked to the Mediterranean Sea by a 7-km-long canal, and is linked to Ichkeul Lake by the Tinja River. Five sampling stations were selected in the present study (Figure 1), based on concentrations of major classes of organic pollutants determined in previous studies [31,32]. These stations were also selected according to the location of the point source of pollution: cement works, metallurgical industry, boatyards, tire production factories, agriculture, and so forth. A description of these 5 stations is provided in the Supplemental Data.

Sample collection

The superficial layer (0–10 cm) of sediment from the 5 selected stations was collected in March 2011 using a small Birge-Ekman grab sampler. After collection, samples were packed into aluminum jars and transported cool to the laboratory, where they were immediately stored in a freezer at –20 °C. All sediment samples were freeze-dried, then sieved using a 2-mm mesh to eliminate debris, homogenized, and stored at 4 °C prior to being characterized with bioassays and chemical analysis.

Physicochemical characterization of sediments

The finer grain size fraction (<63 μm) of each sediment sample was determined by wet sieving according to Savinov et al. [33]. Total organic carbon (TOC) was quantified using the LECO CS 125 analyzer by the method described in Etcheber et al. [34]. Ammonium content (NH_4) was measured using a colorimetric procedure [35]. Organochlorinated pollutants (PCBs, OCPs) were measured by high-resolution gas chromatography and PAHs, by high-performance liquid chromatography according to protocols described in Barhoumi et al. [31,32].

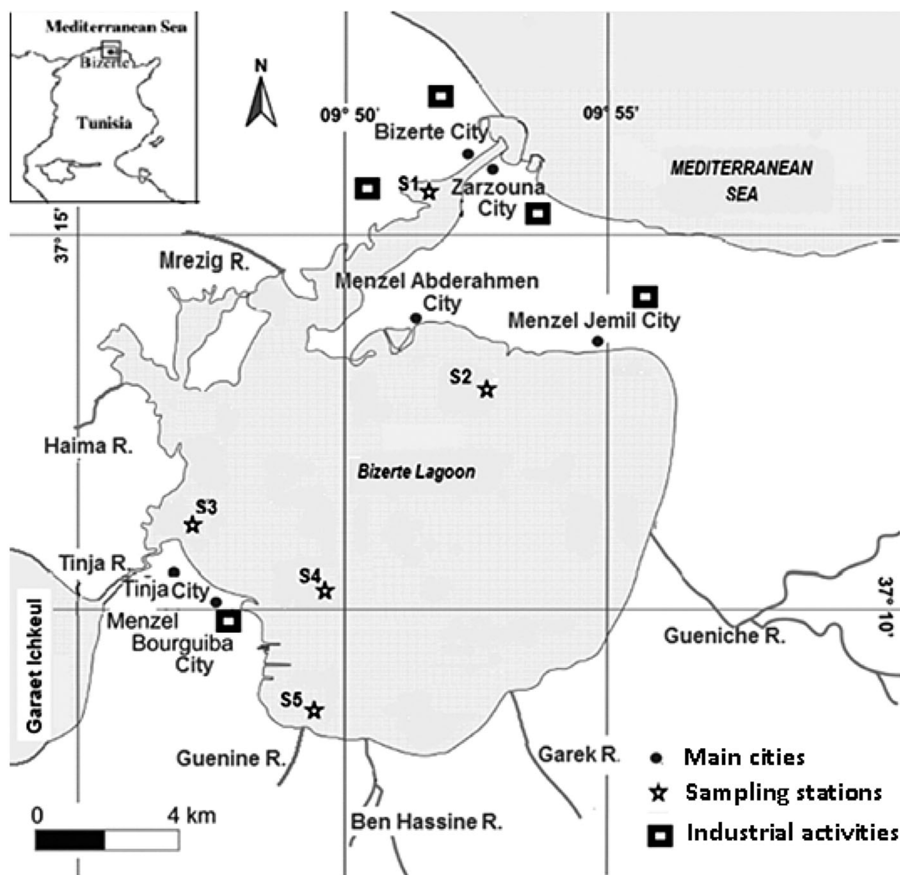

Figure 1. Map of the studied area with location of sampling stations.

To determine heavy metal content, 0.5 g of dried and powdered sediment was treated with 70% nitric acid at 100 °C for 2 h. After digestion, the solution was diluted to 25 mL with ultrapure water. Trace metal concentrations (Ag, Co, Ni, As, Cd, Pb, Cu, and Zn) of the digested solutions were determined by external calibration using inductively coupled plasma mass spectrometry (HP 4500 Series; Agilent Technologies). Standard solutions were prepared from $1\,g\,L^{-1}$ multielemental solutions. To evaluate the effectiveness of the digestion procedure, sediment certified reference materials (marine sediment PACS2; National Research Council of Canada) were analyzed in a manner identical to that used for the samples. Results showed good agreement between obtained and certified values for the metals analyzed (Cd, Cr, Cu, Ni, Pb, Zn, Ni, Fe, and Mn) with <10% deviation.

Sample preparation for toxicity measurement

Toxicity was measured on 3 different fractions of sediment (elutriate, organic extract, and solid phase) using Microtox, the SOS chromotest, and medaka embryo–larval assay by sediment contact (Figure S1, Supplemental Data).

The protocol applied for sediment elutriate preparation (water-extractable phase) was a derivation of that of Environment Canada [36]. In brief, 6 g of freeze-dried sediment was mixed with 30 mL 2% NaCl, shaken vigorously for 15 min, and then centrifuged at 300 *g* for 10 min at 15 °C. The supernatant was decanted and filtered through a 0.45-μm filter, and the pH was measured using an electronic pH meter (Consort P902; Scientific Instruments). The aqueous elutriates were stored at 4 °C in the dark prior to toxicity testing (Microtox Acute Toxicity 100% Test) within 24 h.

Organic-phase extracts were prepared by extraction of 5 g freeze-dried sediment with dichloromethane (Fluka) using an ASE 200 accelerated solvent extractor (Dionex). The operating conditions were as follows: 100 °C oven temperature, 8 min static time, pressure 100 bars, 5 min heating-up time, 60% flush volume, and 1 min purge time. Dichloromethane-dissolved extracts were dried using a RapidVap evaporator (Labconco) and a nitrogen stream and then resuspended in 200 μL of dimethyl sulfoxide (Sigma). A blank of extraction was prepared by the same procedure but without sediment.

For the solid-phase sediment assay with medaka embryo–larval assay by sediment contact, 35-mm-diameter plastic Petri dishes (3/treatment) were filled with 3 g of freeze-dried sediment. Sediment was then moistened with 2 mL egg rearing solution (NaCl 85.6 mM, KCl 0.4 mM, $CaCl_2$ 0.4 mM, and $MgSO_4$ 1.4 mM, pH 7) and maintained at 26 °C overnight before the beginning of the experiment for equilibration. A Nitex mesh (mesh opening 1000 μm; Sefar Filtration) was laid onto the sediment to avoid egg sinking.

Bioassay conditions

Two Microtox protocols were applied for sediment acute toxicity testing: the 100% test protocol on sediment aqueous extract (elutriate) and the basic test procedure on sediment organic extracts. In both cases a standard procedure [37,38] was applied, and the model 500 analyzer (Kontron Instruments) and lyophilized bacteria cultures (R-Biopharm) were used. The Microtox data acquisition software was used to calculate 50% effective concentration (EC50) values, expressed as milligrams of dry weight sediment.

For detection of direct and indirect acting genotoxicants in sediments, the SOS chromotest [39] was performed using *Escherichia coli* PQ37 strain with and without addition of an S9 microsomal fraction obtained from the liver of β-naphthoflavone and phenobarbital-treated Sprague-Dawley rats (Trionova-Biochem). Responses were expressed as mean induction factor (3 replicates) for 25 mg equivalent dry weight sediment per assay. Samples with a significant SOS induction factor compared to control solvent were considered genotoxic when the ratio exceeded 2, moderately genotoxic when the ratio was in the range 1.5 to 2.0, and slightly genotoxic when the ratio was 1.0 to 1.5 [40].

Finally, the toxicity of the studied sediments was evaluated by medaka embryo–larval assay by sediment contact [20,21]. Japanese medaka (*O. latipes*) embryos of the CAB strain were supplied by UMS Amagen at 24 h postfertilization. An artificial reference sediment was used as a negative control. The preparation and composition of this artificial sediment are reported in Le Bihanic et al. [41]. Twenty-five embryos were laid onto the grid at the sediment surface in each Petri dish (3 replicates/treatment). Embryos were covered with a thin layer of egg rearing solution (2 mL), which was renewed every day. Exposures were performed in a climate chamber (Snijders Scientific) at 26 ± 0.3 °C under 5000 lux lighting and a 12:12-h light:dark cycle. Each embryo was examined daily under a dissecting microscope for stage of development, appearance of visible lesions, and death. In addition, dissolved oxygen was measured daily at the sediment–water interface for the whole duration of the assay using a PA2000 oximeter (Unisense). Embryo exposure was stopped at the hatching peak of the control condition (e.g., at 12 d postfertilization [dpf]), and unhatched embryos from all treatments were transferred in clean egg rearing solution. Newly hatched larvae were recorded daily up to the end of the experiment (20 dpf) and placed in 40 mL dechlorinated water (1/2 v/v tap water and osmosed water, 26 °C, pH 7.5, conductivity 180 μS, hardness 53 mg/L $CaCO_3$) and observed daily until complete resorption of the vitellus (in the present study, an additional 3–4 d). Several acute and subacute endpoints were measured on embryos or prolarvae including viability, cardiac activity, morphological abnormalities, time to hatch, and hatching success. Embryonic and larval viability were checked daily. Dead embryos (whitish opaque appearance or absence of cardiac contraction after 3 dpf) were counted and removed. Cardiac activity was assessed at day 10 postfertilization in 5 randomly selected embryos per replicate as described by Barjhoux et al. [21]. Time to hatch and hatching success were checked daily from the day of first hatching to the end of the experiment at day 24 pf. Only embryos able to fully exit the chorion were considered "hatched"; otherwise, they were counted as "not hatched." Only successfully hatched larvae were considered for time to hatch determination. Developmental abnormalities were examined every day on each newly hatched larva using a Leica MZ75 stereomicroscope and photographed by a charge-coupled device camera (Leica DFP420C). Five different categories of abnormalities were recorded including edemas (pericardial, perivitelline, cranial), axial skeletal deformities (body axis, scoliosis, lordosis), craniofacial deformities (skull and jaw), cardiovascular anomalies (hemorrhage, anemia), and eye anomalies (dystrophia, pigmentation alteration, absence of eye).

Statistical analysis

Data for all bioassays except Microtox are presented as mean ± standard deviation. Statistica 7.1 software (StatSoft) was used for all statistical analyses. Data were initially checked for normality (Shapiro-Wilk test, 1% risk) and homogeneity of variances (Levene test, 5% risk). Then, an analysis of variance

(ANOVA) followed by the post hoc Tukey test was performed to determine differences between treatments. If data transformation was not sufficient to perform parametric analysis, the nonparametric Kruskal-Wallis ANOVA and Bonferroni-Dunn test were used ($p < 0.05$). Pearson's correlation coefficient was calculated to investigate possible relationships between parameters. In addition, principal component analysis was applied to obtain an overview of the spatial distribution of the studied parameters and to differentiate the different studied sites.

RESULTS

Sediment chemistry

The physicochemical characteristics of studied sediments are presented in Table 1. Concentrations of TOC were relatively uniform between stations (0.8–1.9%) except station S1, which showed the highest content of TOC (3.9%). The percentage of the finest fraction (<63 μm) and the concentration of ammonium (NH_4) were both highest at station S4 and lowest at S5.

To interpret the observed toxicity, chemical characterization of sediments was carried out (Table 1). The highest concentrations of organic pollutants (∑PAHs, ∑PCBs, and ∑OCPs) were measured at the S1 station. The S1 station was also shown to have the highest concentrations of As, Cd, Cu, and Zn. In contrast, levels of Ag, Co, Ni, and Pb were found to be relatively comparable over the different stations.

Concentrations of chemicals were compared with the effects range low and effects range median criteria established by Long et al. [42] for marine and estuarine sediments. In the present study, no chemical level exceeded effects range median criteria (except Ag), but the heavy metal analytes As, Cu, Pb, and Zn were higher than effects range low levels. Also, acenaphthene and fluorene at station S1 were above effects range low levels. For organochlorines, the concentrations of ∑dichlorodiphenyltrichloroethanes (DDTs) and *p,p*′-DDT were above effects range low and effects range median levels, respectively (data not shown). The ΣPCB concentration was lower than the sediment quality guidelines (Table 1). According to the micro pollutant content of sediments of the Bizerte lagoon, adverse effects may potentially be expected in aquatic organisms.

Sediment extract acute toxicity

According to Microtox results, all sediment organic extracts from the 5 sampling stations were highly toxic (data not shown). In contrast, aqueous extracts of sediment exhibited site-specific toxicity patterns (Table 2). The most toxic sediment elutriate was collected from station S3, with a 30-min EC50 value of 80.6 mg dry weight sediment mL^{-1}. The least toxic samples were collected from stations S2 and S5. Exposure to S1 and S4 sediment aqueous extracts resulted in a maximal bioluminescence inhibition of 52.9% and 53.2% after 30-min exposure, respectively. Based on toxicity of elutriates, sediments can be ranked in the following order: S3 > S4 = S1 > S5 = S2.

Sediment extract genotoxic potency

Results of the SOS chromotest showed a significant genotoxicity of S1 organic extract in comparison to control solvent ($p < 0.05$) but only after S9 microsomal fraction addition. The genotoxicity of sediment extracts was not significantly different between stations, with S9 addition (Figure 2) or not (data not shown). Sediment extract from station S1 tended to be more genotoxic (moderately genotoxic) than that from other stations, with an induction factor of 1.95 ± 0.12. According to Mersch-Sundermann et al. [40], sediment extract from S1 was moderately genotoxic, whereas extracts from S2, S3, and S4 were only slightly genotoxic and

Table 1. Physicochemical and chemical characteristics of the fine (<2 mm) sediment fraction for the 5 sampled stations in the Bizerte lagoon

	S1	S2	S3	S4	S5	ERL[a]	ERM[a]
Fine particle (<63 μm) (%)	52.2	50.1	69.3	91.5	32.1		
TOC (%)	3.9	0.8	1.9	1.3	1.0		
NH_4 (μM)	72.0	108.0	113.0	162.0	20.0		
Metals ($\mu g\ g^{-1}$ dry wt)							
Ag	10.1	12.2	14.0	21.6	16.8	1.0	3.7
As	21.2	11.2	12.1	11.7	9.9	8.2	70.0
Cd	0.7	0.2	0.5	0.4	0.3	1.2	9.6
Co	4.7	5.6	8.0	8.4	3.7	—	—
Cu	35.5	10.9	21.0	18.5	11.9	34.0	270.0
Ni	16.4	10.3	18.7	18.9	6.5	20.9	51.6
Pb	55.2	26.3	43.9	56.8	36.4	46.7	218.0
Zn	182.3	85.7	142.2	169.1	151.0	150.0	410.0
∑Metals	326.0	162.3	261.0	305.5	236.5		
Organic contaminants ($ng\ g^{-1}$ dry wt)							
∑14-PAHs[b]	394.1	60.8	39.7	79.4	55.8	4022	44,792
∑9-$PAHs_{mut}$[c]	276.1	43.7	29.9	65.9	47.8		
∑5-$PAHs_{car}$[d]	120.8	21.1	12.0	28.1	22.0		
∑12-PCBs[e]	14.6	0.8	3.9	3.8	5.2	22.7	180
∑4-OCPs[f]	14.0	2.2	3.2	2.0	1.0		

[a]Values taken from Long et al. [42].
[b]∑ = naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenzo[*a,h*]anthracene, and benzo[*g,h,i*]perylene. Values taken from Barhoumi et al. [32].
[c]Sum of mutagenic polycyclic aromatic hydrocarbons: phenanthrene, fluoranthene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenzo[*a,h*]anthracene, and benzo[*g,h,i*]perylene. Values taken from Barhoumi et al. [32].
[d]Sum of carcinogenic polycyclic aromatic hydrocarbons: benzo[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, and dibenzo[*a,h*]anthracene. Values taken from Barhoumi et al. [32].
[e]∑ = congeners 28+31, 52, 44, 101, 149, 118, 153, 138, 180, 194, and 209. Values taken from Barhoumi et al. [31].
[f]∑ = hexachlorobenzene, *p,p*′-dichlorodiphenyltrichloroethane, *p,p*′-dichlorodiphenyldichloroethylene, *p,p*′-dichlorodiphenyldichlorethane. Values taken from Barhoumi et al. [31].
ERL = effects range low; ERM = effects range medium; PAH = polycyclic aromatic hydrocarbon; TOC = total organic carbon

Table 2. Toxicity of elutriates of sediments collected from 5 stations of the Bizerte lagoon and measured with the Microtox® assay

Station	Exposure time (min)	Inhibition of bioluminescence (%)[a]	EC50[b] (mg mL^{-1})
S1	5	24.6	>200
	15	14.3	>200
	30	52.9	179.7 (146.6–220.1)
S2	5	2.9	>200
	15	0.5	>200
	30	0.7	>200
S3	5	54.6	>200
	15	67.0	>200
	30	76.7	80.6 (63.5–102.3)
S4	5	31.3	>200
	15	42.8	>200
	30	53.2	186.9 (162.1–215.4)
S5	5	2.3	>200
	15	0.5	>200
	30	9.7	>200

[a]At the highest tested concentration of 200 mg equivalent dry sediment per milliliter.
[b]Corresponding 95% confidence intervals in parentheses.
EC50 = 50% effect concentration.

extract from S5 was not genotoxic. The genotoxicity of S1 extract is likely related to progenotoxicants because genotoxic response was obtained only after addition of S9.

Whole-sediment embryo–larval toxicity assay

Dissolved oxygen saturation was measured daily for the whole duration of embryo exposure to sediments (10 d). Maximum oxygen saturation at 26 °C was approximately 8.09 mg L^{-1}. Dissolved oxygen varied slightly from 74.5 ± 1.3% oxygen saturation at S3 to 79.8 ± 1.5% at S2 (Figure S2, Supplemental Data). Baseline embryonic and larval viability in the control group were 71.9 ± 14.2% and 97.8 ± 3.8%, respectively (Figure 3). Viability for sediment-exposed fish ranged from 86.1 ± 6.6% (station S5) to 92.0 ± 8.0% (stations S3 and S4) at the embryonic stage (Figure 3A) and from 90.2 ± 13.4% (station S5) to 100% (station S2) at the larval stage (Figure 3B). No significant difference was detected between stations. The cumulative embryo–larval viability at 20 dpf for the different sediment exposure conditions varied from 67.8 ± 12.1% (station S1) to 92.0 ± 8.0% (station S4) and showed no significant difference in comparison to the control group (Figure 3C). However, the cumulative viability of medaka for S2 and S4 conditions was significantly higher than for S1. Hatching rate at 20 dpf was not significantly different conditions; it varied between 60.9 ± 15.1% at S1 and 82.7 ± 14.0% at S3 (Figure 4A,B). Embryonic developmental time varied from 14.1 ± 1.0 d (station S2) to 17.7 ± 0.4 d (station S3). It was significantly higher for all stations than for the control group (10.4 ± 0.4 d; Figure 4C). Mean time to hatch was also significantly higher for S3, S4, and S5 in comparison to S2 and for S3 in comparison to S1 and S2. No significant difference was found in the averaged cardiac activity at 10 dpf between the control group and embryos exposed to sediments of the Bizerte lagoon (Figure 5). However, a significant difference was observed between stations S1 and S3 with marked bradycardia for embryos exposed to sediments of the former station (87.3 beats/min and 104.6 beats/min on average, respectively; Figure 5).

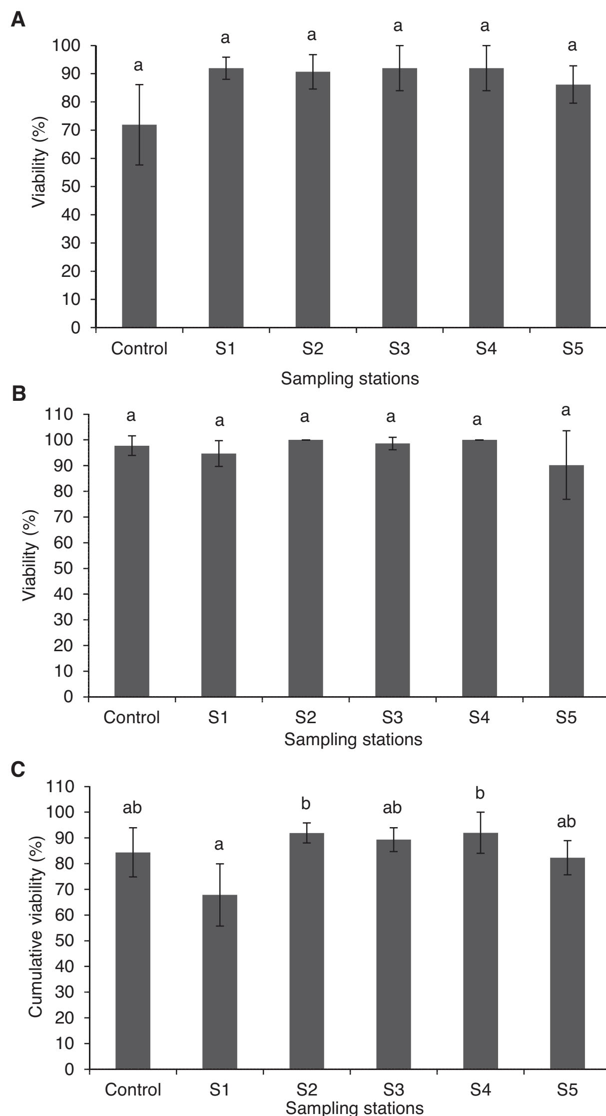

Figure 3. Embryonic (**A**), larval (**B**), and cumulative (**C**) viabilities after exposure of medaka embryos to 5 superficial sediments of the Bizerte lagoon for 20 d. The data shown correspond to the mean percentage of 3 independent replicates ± standard deviation. Conditions with different letters are significantly different ($p < 0.05$).

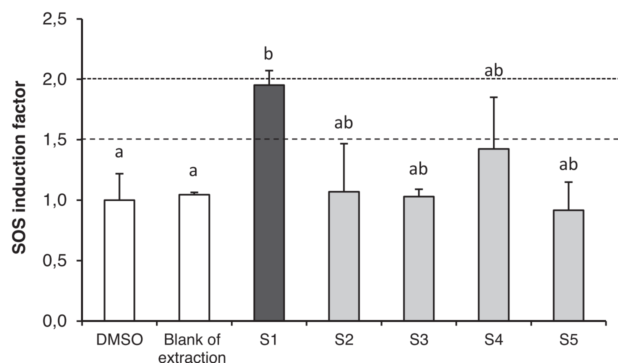

Figure 2. Genotoxicity of organic extracts of sediments collected in the Bizerte lagoon. The SOS chromotest was performed with and without S9 microsomal fraction, but only results with S9 are presented. The amount of organic extract was 0.025 g dry weight equivalent sediment per assay. Values are mean induction factor ± standard deviation for 3 replicates. DMSO = dimethyl sulfoxide. Conditions with different letters are significantly different ($p < 0.05$).

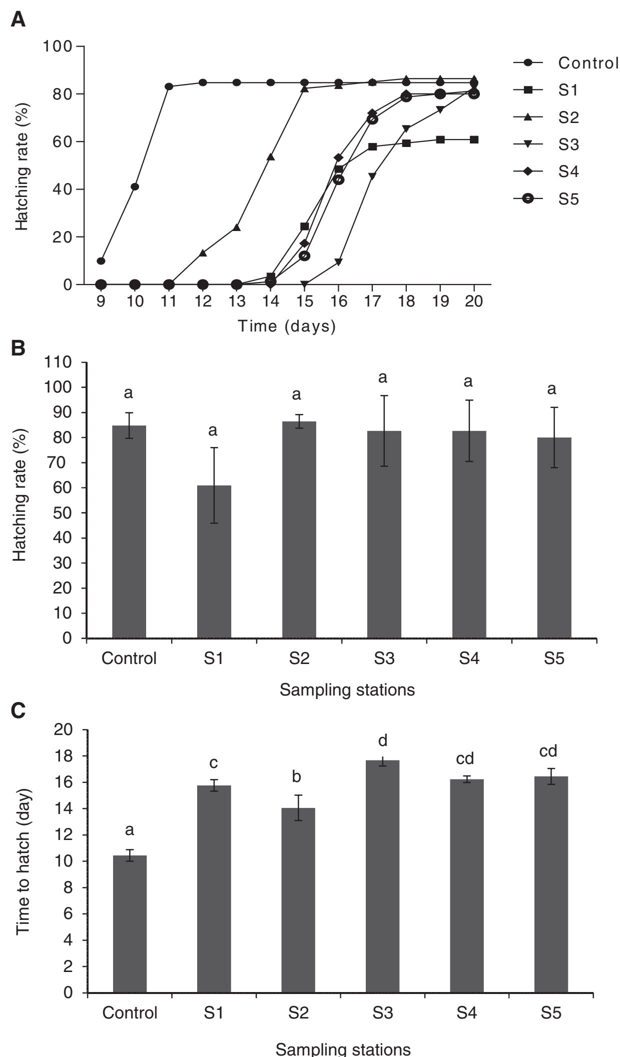

Figure 4. Hatching rate (**A**, **B**) and time to hatch (**C**) of medaka embryos exposed for 20 d to superficial sediments from 5 stations of the Bizerte lagoon in comparison to a control sediment. Data correspond to the mean percentage of 3 independent replicates ± standard deviation. Conditions with different letters are significantly different ($p < 0.05$).

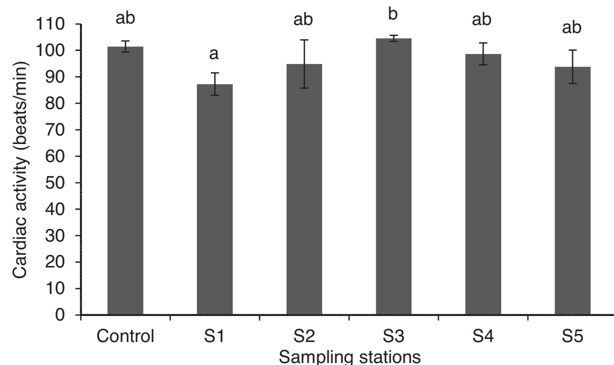

Figure 5. Cardiac activity measured in 10 dpf medaka embryos following exposure to superficial sediments from 5 stations of the Bizerte lagoon. Values represent the mean response (± standard deviation) from 3 replicates. Conditions with different letters are significantly different ($p < 0.05$).

Morphological abnormalities in medaka larvae were recorded at hatching for each individual and are summarized in Table 3. The percentage of malformed larvae in the control group was approximately 17%. After sediment exposure, larval deformities at stations S1 and S5 were statistically higher than the control group ($p < 0.05$). If we compare each type of malformation at all stations, morphological abnormalities (excluding spinal deformities) were generally of a low occurrence in the control group but tended to increase (albeit nonsignificantly) with sediment exposure. The most frequently observed abnormalities were axial skeletal deformities and craniofacial deformities, with percentages ranging from $51.1 \pm 9.4\%$ to $67.4 \pm 16.7\%$ and from $19.6 \pm 2.3\%$ to $27.6 \pm 8.6\%$ of the deformities observed, respectively. Edemata, cardiovascular anomalies, and eye anomalies were also detected but at much lower frequencies. If we compare malformation profiles between sites, spinal deformities were significantly more frequent than edemata and eye anomalies at S1 ($p < 0.01$), S4 ($p < 0.001$), and S5 ($p < 0.05$). No other differences in the malformation profile were observed between stations (Table 3). Some representative larval abnormalities are illustrated in Supplemental Data, Figure S3.

Multivariate analysis

Principal component analysis based on chemical (Σmetals, ΣPAHs, ΣPCBs, ΣOCPs), physicochemical (fine particles <63 μm, TOC, NH_4), and biological (Microtox, SOS chromotest, cumulative viabilities, hatching rate, cardiac activity, malformed larvae) endpoints gave a principal component (PC1) accounting for 66.2% of data variability (Figure 6A). Principal component 1 was loaded to the left by hatching rate, cumulative viability, and cardiac activity. It was loaded to the right by metals, PAHs, PCBs, OCPs, TOC content, malformed larvae, and the SOS chromotest. Principal component 2 (PC2) accounted for 25.2% of the data variability and was negatively loaded only by NH_4, fine particles <63 μm, and Microtox (Figure 6A). Variables showing no significant differences between sites, such as individual concentration of metals, embryonic viability, larval viability, edemata, axial skeletal deformities, cardiovascular anomalies, craniofacial deformities, and eye anomalies, were excluded from the principal component analysis. In addition, principal component analysis clearly differentiated 3 groups of sites (Figure 6B): S2 and S5 (first group), S3 and S4 (second group), and S1 alone (third group).

The significant relationship between variables was further studied by means of a correlation matrix (Table 4). Microtox data were not correlated with any studied pollutant. In contrast, SOS chromotest results were positively correlated with levels of ΣPAHs and ΣOCPs. Cumulative viability was inversely correlated to ΣPAHs and ΣPCBs. Hatching rate was also negatively correlated with chemical concentrations (ΣPAHs, ΣPCBs, and ΣOCPs) and TOC content. Lastly, malformed larvae were positively correlated with levels of ΣPCBs.

DISCUSSION

Sediments are very complex and heterogeneous matrices. To date, there is no universal toxicity assay that can reliably predict the potential risk of contaminated sediments. A multispecies battery of tests and a test strategy, which includes the assessment of multiple exposure phases, are advocated to reduce uncertainty in sediment quality assessment [15,43,44]. In the present study, we demonstrated the applicability of

Table 3. Malformed larvae and spectrum of malformations following exposure of medaka embryos to superficial sediments from 5 stations of the Bizerte lagoon[a]

		Spectrum of malformations (%)				
	Malformed larvae (%)	Edemata	Spinal deformities	Craniofacial deformities	Cardiovascular anomalies	Eye anomalies
Control	17.1 ± 9.0A	0.0 ± 0.0	88.9 ± 19.2	11.1 ± 19.2	0.0 ± 0.0	0.0 ± 0.0
S1	69.3 ± 13.2B*	0.0 ± 0.0a	67.4 ± 16.7b*	25.0 ± 25.0ab	7.6 ± 8.4ab	0.0 ± 0.0a
S2	33.3 ± 17.6AB	11.1 ± 19.2a	59.2 ± 22.6a	21.6 ± 4.4a	5.6 ± 9.6a	2.6 ± 4.4a
S3	49.8 ± 14.6AB	12.6 ± 11.9a	51.1 ± 9.4a	19.6 ± 2.3a	7.3 ± 3.4a	9.4 ± 11.5a
S4	37.8 ± 16.8AB	2.4 ± 4.1a	54.2 ± 3.8b*	26.6 ± 6.1ab	6.1 ± 5.6ab	10.7 ± 12.9a
S5	58.1 ± 10.7B*	3.7 ± 6.4a	60.6 ± 9.8b*	27.6 ± 8.6ab	6.3 ± 5.7a	1.9 ± 3.2a

[a]Results are expressed as percentage of impaired larvae among overall hatched individuals (data are mean ± standard deviation, $n = 3$). Capital letters indicate statistical differences between stations according to the results of one-way analysis of variance followed by Tukey's post hoc test ($p < 0.05$). Lowercase letters indicate statistical differences between developmental anomalies for the same station according to the results of Kruskal-Wallis followed by Mann and Whiney post hoc test ($p < 0.05$).
*Indicates significance.

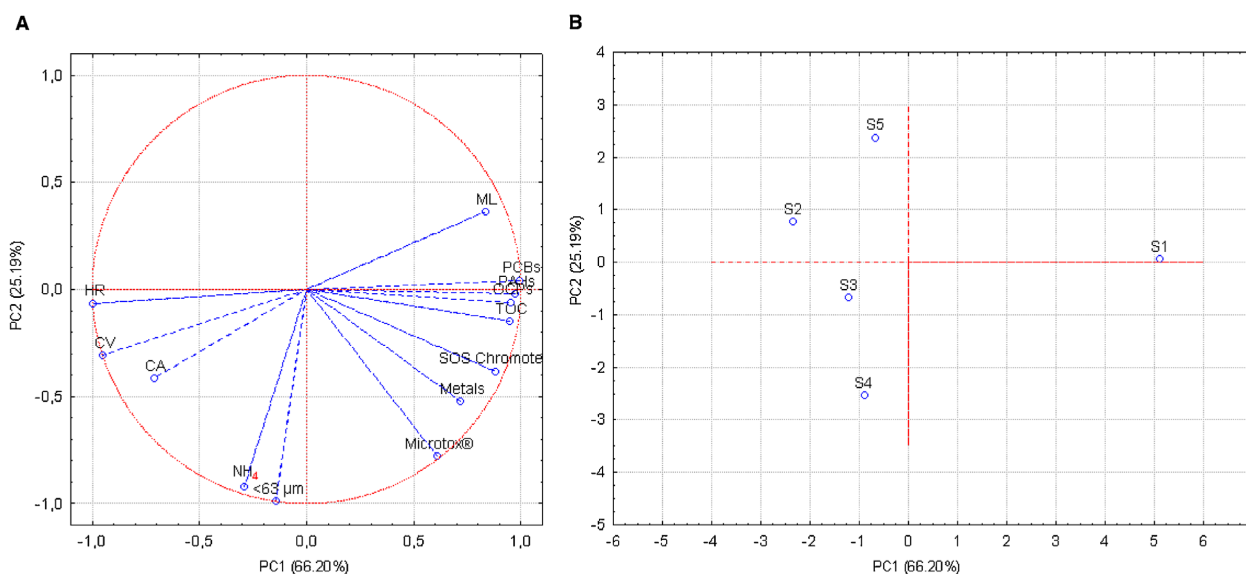

Figure 6. Principal component analysis. Results for the 2 principal components produced by chemical (Σmetals, ΣPAHs, ΣPCBs, ΣOCPs), physicochemical (fine particles <63 µm, total organic carbon, NH4), and biological (Microtox, SOS chromotest, cumulative viability, hatching rate, cardiac activity, malformed larvae) endpoints for 5 sediments collected in the Bizerte lagoon. (**A**) Plot of variable vectors. (**B**) Plot of the scores of the different studied sediments. CA = cardiac activity; CV = cumulative viability; HR = hatching rate; ML = malformed larvae; OCP = organochlorine pesticide; PAH = polycyclic aromatic hydrocarbon; PC1/PC2 = principal components 1 and 2; PCB = polychlorinated biphenyl.

medaka embryo–larval assay by sediment contact combined with the Microtox assay and SOS chromotest assay to measure the toxicity of different phases of sediments: elutriate, organic extract, and whole sediment. To interpret sediment toxicity, physicochemical and chemical analyses were combined with toxicological analysis.

Little or no toxicity of sediment elutriates from the S2 and S5 sites was detected with Microtox. Moderate toxicity was

Table 4. Pearson correlation coefficients between chemical variables, physicochemical variables, and biological endpoints

	ΣMetals	ΣPAHs	ΣPCBs	ΣOCPs	<63 µm	TOC	NH_4	Microtox[a]	SOS Chromotest
Microtox[a]	0.729	0.588	0.579	0.579	0.687	0.652	0.507	1	0.792
SOS Chromotest	0.694	0.935*	0.831	0.905*	0.199	0.855	0.134	0.792	1
Cumulative viability	–0.543	–0.905*	–0.962**	–0.877	0.436	–0.856	0.572	–0.342	–0.714
Hatching rate	–0681	–0.967**	–0.995***	–0.948**	0.208	–0.939**	0.352	0.553	–0.853
Time to hatch	0.522	–0.145	0.143	–0.058	0.270	0.189	–0.022	0.253	–0.176
Cardiac activity	–0.173	–0.773	–0.683	–0.658	0.532	–0.496	0.509	–0.129	–0.645
Abnormal larvae	0.570	0.709	0.883*	0.708	–0.454	0.768	–0.673	0.245	0.475

[a]Inhibition of bioluminescence.
*$p < 0.05$
**$p < 0.01$
***$p < 0.001$
OCP = organochlorine pesticide; PAH = polycyclic aromatic hydrocarbon; PCB = polychlorinated biphenyl; TOC = total organic carbon.

measured for S4 and S1 elutriates with approximately 50% bioluminescence inhibition at 30-min exposure. The toxicity was more pronounced for S3 elutriate, with approximately 77% inhibition. No significant correlation was observed between organic pollutants or metal concentrations except Ni and acute toxicity of elutriates. This poor correlation could have multiple origins including low pollutant bioavailability and antagonistic or synergistic interactions between pollutants in aqueous extracts and the low sensitivity of the assay to the extracted pollutants.

Campbell et al. [45] showed that the presence of organic matter increases the retention of metals by the formation of stable complexes between metal–clay–organic matter or metal–organic matter–clay. The observed toxicity of S4 and S1 elutriates might result from additional components and contaminants not analyzed in the present study and the possible occurrence of synergistic interactions between pollutants. For instance, organotins, which are known genotoxicants [46], were not analyzed in the present study but have been detected at high concentrations ($200\,\mu g\,kg^{-1}$) in sediments from the Bizerte lagoon [47]. The SOS chromotest demonstrated that sediments from station S1 and to a lesser extent S2, S3, and S4 contained progenotoxic organic compounds. The SOS chromotest and Ames mutagenicity test are the most frequently used assays to assess the genotoxicity of suspended matter or bottom sediments (reviewed in Chen and White [48]). The induction factor obtained for the organic extracts of Bizerte lagoon's sediments ranged from 0.92 to 1.95. Those values were of the same order of magnitude, albeit slightly lower than those reported for sediments of the Seine estuary [49]. Sediment extract from S1 was the most genotoxic among all studied samples. This genotoxicity is consistent with the organic chemical content of the sediment because S1 had the highest total concentration of organic contaminants (PAHs, PCBs, and OCPs; Table 1). The present study highlighted the genotoxic potency of pollutants accumulated in sediment from the S1 station located at the vicinity of the Bizerte harbor and the clear positive correlation between PAH and OCP concentrations and the genotoxic potency of sediment organic extracts. These results are consistent with those reported by Cachot et al. [49] and Dutka et al. [50] who showed that PAHs and probably other persistent organic pollutants accumulated in sediments are potent progenotoxicants.

The toxicity of solid phase–associated contaminants was measured with the medaka embryo–larval assay by incubating embryos in direct contact with the studied sediment [20,21]. Native whole-sediment testing using fish embryos represents a realistic scenario to simulate in situ exposure conditions [51]. Compared with the control sediment, sediments from Bizerte lagoon induced a significant reduction of embryo–larval viability and embryo cardiac activity, promoted developmental abnormalities, and postponed hatching (Figures 3–5 and Table 3). A 2-fold to 3-fold increase of embryo–larval mortality was observed at station S1 relative to other Bizerte stations. This mortality rate might be high enough to cause significant decline in the whole fish population [52]. The cumulated hatching rate at 20 dpf was clearly (but not significantly) reduced at S1 compared to the control group and the other stations. These deleterious effects in embryos and prolarvae might be explained by the high levels of organic contaminants at S1, as confirmed by the significant negative correlations between hatching rate and the concentrations of ΣPAHs, ΣPCBs, and ΣOCPs. Laboratory and field studies have shown that numerous persistent organic pollutants, such as PCBs, tributyltin, and PAHs, reduced the hatchability of eggs from brook trout [53], rainbow trout [54,55], lake trout [56], and Japanese medaka [41,57,58]. Because the Bizerte lagoon contains high levels of tributyltin and its degradation products, monobutyltin and dibutyltin [47], we can hypothesize that these compounds could be directly involved in the low hatching rates measured in the present study. Average time to hatch in the control treatment group was 10.4 dpf, with >80% of embryos hatching at 12 dpf. These results are globally consistent with the literature, which reports a hatching time between 9 dpf and 12 dpf at 25 °C to 26 °C for the Japanese medaka [59,60]. After exposure to sediments from the Bizerte lagoon, embryos exhibited significant delay in hatching (from 14.1 d to 17.7 d according to sediments tested). This may in part be the result of lower oxygen levels in the sediment samples (mean 77.2%) in comparison to the control (88.8%) because the present study's data showed significant negative correlation between time to hatch and oxygen levels. Oxygen level is a key parameter that initiates hatching in teleosts [61]. Dave and Xiu [62] suggested that delayed hatching in zebrafish embryos can result from either inhibition of the hatching enzyme chorionase or osmotic disturbances interfering with the activity of this enzyme, impairment of embryo muscular activity, and a low metabolism related to insufficient oxygenation level. The average cardiac activity (at 10 dpf) in control embryos was 101 beats/min. After exposure to S1 sediment, a moderate but not significant bradycardia was observed in exposed embryos in comparison to the control group. Bradycardia has already been reported in zebrafish embryos exposed to fluorene [63] and medaka embryos exposed to 7,12-dimethylbenz[*a*]anthracene [20]. Furthermore, exposure of medaka embryos to sediments from the 5 studied stations induced a large spectrum of developmental defects including edemata, axial skeletal deformities, craniofacial deformities, cardiovascular anomalies, and eye anomalies. This spectrum of effects is characteristic of blue sac disease, which has been largely described in fish embryos exposed to dioxin, PCBs, and PAH compounds [64,65]. Medaka developmental defects have been widely reported following exposure to various chemicals including PAHs [58,60,66,67] organophosphate insecticides such as diazinon [68], 4-nonylphenol [20,69], and antifouling agents such as triphenyltin and tributyltin [70,71], among others. It is interesting to note that the embryotoxic and teratogenic effects observed in medaka embryos and larvae are highly correlated to organic pollutants and TOC content in sediments of the Bizerte lagoon (Table 4). This result is in accordance with numerous studies reporting developmental and genotoxic effects of low or moderate concentrations of PAHs in Japanese medaka early life stages [20,58,60,66,67,72]. In addition, among the low–molecular weight PAHs detected in the present study, the concentrations of fluorene and acenaphthene in the sediment exceeded the effects range low level [42].

The medaka embryo–larval assay by sediment contact assay provides an environmentally realistic simulation of conditions of exposure with embryos incubated directly on the sediment surface. Embryos are exposed to both water-soluble chemicals that are released from the sediment into the overlying water and hydrophobic pollutants adsorbed onto particles in direct contact with the eggs. The limitation could be the chorion, which acts as a physical barrier, protecting the embryo from the penetration of some chemicals [73]. In the present study, the medaka embryo–larval assay by sediment contact was shown to be more sensitive than Microtox and the SOS chromotest for measuring toxicity of natural sediments. Indeed, 5 out of 5

sediments gave a significant positive response with the medaka embryo–larval assay by sediment contact against 3 out of 5 with the Microtox and 1 out of 5 with the SOS chromotest.

The battery of parameters applied in the present study (chemical, physicochemical, and biological endpoints) allowed discrimination of sites. The 5 stations studied exhibited contrasting toxicity patterns, ranging from the cleanest station (S5) to the most polluted station (S1). Principal component analysis confirmed that sediments from S1 were the most toxic because of the high levels of PAHs, PCBs, OCPs, and metals. The concept of integrative chemical and biological monitoring for environmental risk assessment is generally accepted. Chemical analyses provide insight into contaminant inputs, whereas biological monitoring provides indications of the toxicity of the environment and the health status of organisms. Nevertheless, in view of the present study's data, the relationship between the presence of specific contaminants, their potential toxicity, and their ability to adversely affect living organisms is very complex and still needs investigation. A point mentioned by several authors [74,75] which is implicit in the foregoing discussion is the need, in any monitoring program, for a battery of bioassays, covering several taxonomic groups and including a large panel of toxicological endpoints, to detect chemicals with different modes of action. The present study's results clearly show that different taxa and endpoints will give different responses to the multiplicity of contaminants occurring in the environment. Ideally, the test battery should include representatives of the major sediment-dwelling taxa, with a main focus on early developmental stages because they usually display the highest sensitivity to chemical contaminants.

CONCLUSION

The present study involved characterization of surface sediment in the Bizerte lagoon, using a battery of bioassays and a multiphase approach. The medaka embryo–larval assay by sediment contact provides an environmentally realistic exposure scenario and evaluates the toxicity of bioavailable pollutants from the whole sediment, for example, particles and porewater. In addition, this assay is easy to perform, sensitive, and ethically acceptable. It could prove very useful in environmental monitoring programs for sediment quality assessment. However, full validation would call for testing of a larger set of samples with contrasted physicochemical properties and types and levels of contamination. Altogether, the present study's data suggest that some sediments from the Bizerte lagoon are toxic, genotoxic, and embryotoxic and could therefore represent a significant risk for species living or feeding in sediments in this area.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3388.

Acknowledgment—The authors are grateful to the Tunisian Ministry of Higher Education and Scientific Research for the PhD grant to B. Barhoumi. The authors thank J. Emery for providing language help.

Data availability—Data, associated metadata, and calculation tools can be obtained on request to the corresponding author, Jérôme Cachot (jerome.cachot@u-bordeaux.fr).

REFERENCES

1. Burton GA Jr. 1991. Assessing the toxicity of freshwater sediments. *Environ Toxicol Chem* 10:1585–1627.
2. Swartz RC, Ditsworth GR, Schults DW, Lamberson JO. 1985. Sediment toxicity to a marine infaunal amphipod: Cadmium and its interaction with sewage sludge. *Mar Environ Res* 18:133–153.
3. Mac MJ, Edsall CC, Hesselberg RJ, Sayers RE Jr. 1984. Flow through bioassay for measuring bioaccumulation of toxic substances from sediment. EPA-905/3- 84–007. US Environmental Protection Agency, Washington, DC.
4. Power EA, Munkittrick KP, Chapman PM. 1991. An ecological impact assessment framework for decision making related to sediment quality. In Mayes MA, Barron GM, eds, *Aquatic Toxicology and Risk Assessment*. ASTM STP 1124. ASTM International, Philadelphia, PA, USA, pp 48–64.
5. Zimmer M, Ahlf W. 1994. Erarbeitung von Kriterien zur Ableitung von Qualitatszielen fur Sedimente und Schwebstoffe—Literaturstudie. UBA-Texte 69/94. Umweltbundesamt, Berlin, Germany.
6. Anderson B, Hunt J, Phillips B, Thompson B, Lowe S, Taberski K, Scott Carr R. 2007. Patterns and trends in sediment toxicity in the San Francisco estuary. *Environ Res* 105:145–155.
7. Jiang Y-X, Liu Y-S, Ying G-G, H-W, Liang Y-Q, Chen X-W. 2015. A new tool for assessing sediment quality based on the weight of evidence approach and grey TOPSIS. *Sci Total Environ* 537:369–376.
8. Picone M, Bergamin M, Losso C, Delaney E, Arizzi Novelli A, Ghirardini AV. 2016. Assessment of sediment toxicity in the lagoon of Venice (Italy) using a multi-species set of bioassays. *Ecotoxicol Environ Saf* 123:32–44.
9. Daniels SA, Munawar M, Mayfield CI. 1989. An improved elutriation technique for the bioassessment of sediment contaminants. *Hydrobiologia* 188/189:619–631.
10. Ingersoll CG. 1995. Sediment tests. Effects, environmental fate and risk assessment. In Rand GM, ed, *Fundamentals of Aquatic Toxicology*. Taylor and Francis, New York, NY, USA, pp 231–256.
11. Gagné F, Pardos M, Blaise C, Turcotte P, Quémerais B, Fouquet A. 1999. Toxicity evaluation of organic sediment extracts resolved by size exclusion chromatography using rainbow trout hepatocytes. *Chemosphere* 39:1545–1570.
12. Pardos M, Benninghoff C, Thomas RL, Khim-Heang S. 1999. Confirmation of elemental sulfur toxicity in the Microtox assay during organic extract assessment of freshwater sediments. *Environ Toxicol Chem* 18:188–193.
13. Hollert H, Keiter S, Konig N, Rudolf M, Ulrich M, Braunbeck T. 2003. A new sediment contact assay to assess particle-bound pollutants using zebrafish (*Danio rerio*) embryos. *J Soils Sediments* 3:197–207.
14. Mages M, Bandow N, Kuster E, Brack W, Von Tumpling W. 2008. Zinc and cadmium accumulation in single zebrafish (*Danio rerio*) embryos—A total reflection X-ray fluorescence spectrometry application. *Spectrochim Acta B* 63:1443–1449.
15. Giesy JP, Hoke RA. 1989. Fresh-water sediment toxicity bioassessment—Rationale for species selection and test design. *J Great Lakes Res* 15:539–569.
16. Matthiessen P, Bifield S, Jarrett F, Kirby MF, Law RJ, McWinn WR, Sheahan DA, Thain JE, Whale GR. 1998. An assessment of sediment toxicity in the River Tyne estuary, UK by means of bioassays. *Mar Environ Res* 45:1–15.
17. Nendza M. 2002. Inventory of marine biotest methods for the evaluation of dredged material and sediments. *Chemosphere* 48:865–883.
18. Cao L, Huang W, Shan X, Xiao Z, Wang Q, Dou S. 2009. Cadmium toxicity to embryonic-larval development and survival in red sea bream *Pagrus major*. *Ecotoxicol Environ Saf* 72:1966–1974.
19. Quillardet P, Huisman O, D'Ari R, Hofnung M. 1982. SOS chromotest, a direct assay of induction of an SOS function in *Escherichia coli* K-12 to measure genotoxicity. *Proc Natl Acad Sci USA* 79:5971–5975.
20. Vicquelin L, Leray-Forget J, Peluhet L, LeMenach K, Deflandre B, Anschutz P, Etcheber H, Morin B, Budzinski H, Cachot J. 2011. A new spiked sediment assay using embryos of the Japanese medaka specifically designed for a reliable toxicity assessment of hydrophobic chemicals. *Aquat Toxicol* 105:235–245.
21. Barjhoux I, Baudrimont M, Morin B, Landi L, Gonzalez P, Cachot J. 2012. Effects of copper and cadmium spiked-sediments on embryonic development of Japanese medaka (*Oryzias latipes*). *Ecotoxicol Environ Saf* 79:272–282.
22. Gonzalez-Doncel M, De la Pena E, Barrueco C, Hinton DE. 2003. Stage sensitivity of medaka (*Oryzias latipes*) eggs and embryos to permethrin. *Aquat Toxicol* 62:255–268.
23. Iwamatsu T. 2004. Stages of normal development in the medaka *Oryzias latipes*. *Mech Dev* 121:605–618.
24. Gonzalez-Doncel M, Okihiro MS, Villalobos SA, Hinton DE, Tarazona JV. 2005. A quick reference guide to the normal development of *Oryzias latipes* (Teleostei, Adrianichthyidae). *J Appl Ichthyol* 21:39–52.

25. Carlson EA, Li Y, Zelikoff JT. 2002. Exposure of Japanese medaka (*Oryzias latipes*) to benzo[a]pyrene suppresses immune function and host resistance against bacterial challenge. *Aquat Toxicol* 56: 289–301.
26. Yao ZL, Lai QF, Zhou K, Rizalita RE, Wang H. 2010. Developmental biology of medaka fish (*Oryzias latipes*) exposed to alkalinity stress. *J Appl Ichthyol* 26:397–402.
27. Wittbrodt J, Shima A, Schartl M. 2002. Medaka—A model organism from the Far East. *Nat Rev Genet* 3:53–64.
28. Cheikh M, Derouiche A, Driss MR. 2002. Détermination par (CPG-ECD) des résidus de pesticides organochlorés dans les sédiments de la lagune de Bizerte. *Bulletin de l'Institut National des Sciences et Technologies de la Mer* 7:160–163.
29. Derouiche A, Sanda YG, Driss MR. 2004. Polychlorinated biphenyls in sediments from Bizerte lagoon, Tunisia. *Bull Environ Contam Toxicol* 73:810–817.
30. Ben Garali F A, S Ouakad M, Gueddari M. 2010. Contamination of superficial sediments by heavy metals and iron in the Bizerte lagoon, northern Tunisia. *Arabian Journal of Geosciences* 3:295–306.
31. Barhoumi B, LeMenach K, Dévier M-H., El megdiche Y, Hammami B, Ameur WB, Hassine SB, Cachot J, Budzinski H, Driss MR. 2014. Distribution and ecological risk of polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) in surface sediments from the Bizerte lagoon, Tunisia. *Environ Sci Pollut Res* 21:6290–6302.
32. Barhoumi B, LeMenach K, Dévier M-H, Ameur WB, Etcheber H, Budzinski H, Cachot J, Driss MR. 2014. Polycyclic aromatic hydrocarbons (PAHs) in surface sediments from the Bizerte lagoon, Tunisia: Levels, sources and toxicological significance. *Environ Monit Assess* 186:2653–2669.
33. Savinov VM, Savinova TN, Carroll J, Mathisov GG, Dahle S, Naes K. 2000. Polycyclic aromatic hydrocarbons (PAHs) in sediments of the White Sea, Russia. *Mar Pollut Bull* 40:807–818.
34. Etcheber H, Taillez A, Abril G, Garnier J, Servais P, Moatar F, Commarieu MV. 2007. Particulate organic carbon in the estuarine turbidity maxima of the Gironde, Loire and Seine estuaries: Origin and lability. *Hydrobiologia* 588:245–259.
35. Strickland JDH, Parsons TR. 1972. *A Practical Handbook of Seawater Analysis*. Fisheries Research Board, Ottawa, Canada.
36. Environment Canada. 1992. Méthode d'essai biologique: Essai de toxicité sur la bactérie luminescente *Photobacterium phosphoreum*. SPE 1/RM/24, no. cat. En49-24/1-24F. Ottawa, Canada.
37. AZUR Environmental. 1998. Microtox basic solid-phase test (Basic SPT). Carlsbad, CA, USA.
38. International Organization for Standardization. 1999. Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (luminescent bacteria test). Part 3: Method using freeze dried bacteria. European Standard ISO 11348-3:1998. European Committee for Standardization. Brussels, Belgium.
39. Quillardet P, Hofnung M. 1985. The SOS chromotest, a colorimetric bacterial assay for genotoxins: Procedures. *Mutat Res* 147:65–78.
40. Mersch-Sundermann V, Mochayedi S, Kevekordes S. 1992. Genotoxicity of polycyclic aromatic hydrocarbons in Escherichia coli PQ37. *Mutat Res* 278:1–9.
41. Le Bihanic F, Perrichon P, Le Menach K, Budzinski H, Cousin X, Cachot J. 2014. Development of a reference artificial sediment for chemical testing adapted to the MELA sediment contact assay. *Environ Sci Pollut Res* 21:13689–13702.
42. Long ER, MacDonald DD, Smith SL, Calder FD. 1995. Incidence of adverse biological effects within ranges of chemical concentrations in marine and estuarine sediments. *Environ Manage* 19:81–97.
43. Bombardier M, Bermingham N. 1999. The SED-TOX index: Toxicity-directed management tool to assess and rank sediments based on their hazard—Concept and application. *Environ Toxicol Chem* 18:685–698.
44. Chapman PM, Ho KT, Munns WR, Soloman K, Weinstein MP. 2002. Issues in sediment toxicity and ecological risk assessment. *Mar Pollut Bull* 44:271–278.
45. Campbell PGC, Lewis AG, Chapman PM, Crowder AA, Fletcher WK, Imber B, Luoma SN, Stokes PM, Winfrey M. 1988. Biologically available metals in sediments. no. 27694 National Research Council, Ottawa, Canada.
46. Hamasaki T, Sato T, Nagase H, Kito H. 1992. The genotoxicity of organotin compounds in SOS chromotest and rec-assay. *Mutat Res/Genet Toxicol* 280:195–203.
47. Mzoughi N, Lespes G, Bravo M, Dachraoui M, Potin-Gautier M. 2005. Organotin speciation in Bizerte lagoon (Tunisia). *Sci Total Environ* 349:211–222.
48. Chen G, White PA. 2004. The mutagenic hazards of aquatic sediments: A review. *Mutat Res* 567:151–225.
49. Cachot J, Geffard O, Augagneur S, Lacroix S, Le Menach K, Peluhet L, Couteau J, Denier X, Devier M-H, Pottier D, Budzinski H. 2006. Evidence of genotoxicity related to high PAH content of sediments in the upper part of the Seine estuary (Normandy, France). *Aquat Toxicol* 79:257–267.
50. Dutka BJ, McInnis R, Jurkovic A, Liu D. 1996. Water and sediment ecotoxicity studies in Temuco and Rapel River basin, Chile. *Environ Toxicol Water Qual* 11:237–247.
51. Kosmehl T, Hallare AV, Braunbeck T, Hollert H. 2008. DNA damage induced by genotoxicants in zebrafish (*Danio rerio*) embryos after contact exposure to freeze-dried sediment and sediment extracts from Laguna Lake (The Philippines) as measured by the comet assay. *Mutat Res* 650:1–14.
52. Hallare AV, Kosmehl T, Schulze T, Hollert H, Köhler HR, Triebskorn R. 2005. Assessing contamination levels of Laguna Lake sediments (Philippines) using a contact assay with zebrafish (*Danio rerio*) embryos. *Sci Total Environ* 347:254–271.
53. Freeman HC, Idler DR. 1975. The effect of polychlorinated biphenyl on steroidogenesis and reproduction in the brook trout (*Salvelinus fontinalis*). *Can J Biochem* 53:666–670.
54. Harris GE, Metcalfe TL, Metcalfe CD, Huestis SY. 1994. Embryotoxicity of extracts from Lake Ontario rainbow trout (*Oncorhynchus mykiss*) to Japanese medaka (*Oryzias latipes*). *Environ Toxicol Chem* 13:1393–1403.
55. Le Bihanic F, Morin B, Cousin X, Le Menach K, Budzinski H, Cachot J. 2014. Developmental toxicity of PAH mixtures in fish early life stages. Part I: Adverse effects in rainbow trout. *Environ Sci Pollut Res* 21:13720–13731.
56. Mac MJ, Edsall CC. 1991. Environmental contaminants and the reproductive success of lake trout in the Great Lakes: An epidemiological approach. *J Toxicol Environ Health* 33:375–394.
57. Nirmala K, Oshima Y, Lee R, Imada N, Honjo T, Kobayashi K. 1999. Transgenerational toxicity of tributyltin and its combined effects with polychlorinated biphenyls on reproductive processes in Japanese medaka (*Oryzias latipes*). *Environ Toxicol Chem* 18:717–721.
58. Le Bihanic F, Clérandeau C, Le Menach K, Morin B, Budzinski H, Cousin X, Cachot J. 2014. Developmental toxicity of PAH mixtures in fish early life stages. Part II: Adverse effects in Japanese medaka. *Environ Sci Pollut Res* 21:13732–13743.
59. Farwell A, Nero V, Croft M, Bal P, Dixon DG. 2006. Modified Japanese medaka embryo-larval bioassay for rapid determination of developmental abnormalities. *Arch Environ Contam Toxicol* 51:600–607.
60. Gonzalez-Doncel M, Gonzalez L, Fernandez-Torija C, Navas JM, Tarazona JV. 2008. Toxic effects of an oil spill on fish early life stages may not be exclusively associated to PAHs: Studies with Prestige oil and medaka (*Oryzias latipes*). *Aquat Toxicol* 87:280–288.
61. Rombough PJ. 1988. Respiratory gas exchange, aerobic metabolism, and effects of hypoxia during early life. In Hoar WS, Randall DJ, eds, *Fish Physiology*, Vol V 11 Academic, San Diego, CA, pp 59–161.
62. Dave G, Xiu R. 1991. Toxicity of mercury, copper, nickel, lead, and cobalt to embryos and larvae of zebrafish, *Brachydanio rerio*. *Arch Environ Contam Toxicol* 21:126–134.
63. Incardona JP, Collier TK, Scholz NL. 2004. Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. *Toxicol Appl Pharmacol* 196:191–205.
64. Wassenberg DM, Di Giulio RT. 2004. Synergistic embryotoxicity of polycyclic aromatic hydrocarbon aryl hydrocarbon receptor agonists with cytochrome P4501A inhibitors in *Fundulus heteroclitus*. *Environ Health Perspect* 112:1658–1664.
65. Elonen GE, Spehar RL, Holcombe GW, Johnson RD, Fernandez JD, Erickson RJ, Tietge JE, Cook PM. 2009. Comparative toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin to seven freshwater fish species during early life-stage development. *Environ Toxicol Chem* 17:472–483.
66. Barjhoux I, Cachot J, Gonzalez P, Budzinski H, Le Menach K, Landi L, Morin B, Baudrimont M. 2014. Transcriptional responses and embryotoxic effects induced by pyrene and methylpyrene in Japanese medaka (*Oryzias latipes*) early life stages exposed to spiked sediments. *Environ Sci Pollut Res* 21:13850–13866.
67. Le Bihanic F, Sommard V, de Lansalut P, Pichon A, Grasset J, Berrada S, Budzinski H, Cousin X, Morin B, Cachot J. 2015. Environmental concentrations of benz[*a*]anthracene induce developmental defects and DNA damage and impair photomotor response in Japanese medaka larvae. *Ecotoxicol Environ Saf* 113:321–328.
68. Hamm JT, Hinton DE. 2000. The role of development and duration of exposure to the embryotoxicity of diazinon. *Aquat Toxicol* 48:403–418.

69. Yokota H, Seki M, Maeda M, Oshima Y, Tadokoro H, Honjo T, Kabayashi K. 2001. Life-cycle toxicity of 4-nonylphenol to medaka (*Oryzias latipes*). *Environ Toxicol Chem* 20:2552–2560.
70. Nakayama K, Oshima Y, Nagafuchi K, Hano T, Shimasaki Y, Honjo T. 2005. Early-life-stage toxicity in offspring from exposed parent medaka, *Oryzias latipes*, to mixtures of tributyltin and polychlorinated biphenyls. *Environ Toxicol Chem* 24:591–596.
71. Zhang Z, Hu J, Zhen H, Wu X, Huang C. 2008. Reproductive inhibition and transgenerational toxicity of triphenyltin on medaka (*Oryzias latipes*) at environmentally relevant levels. *Environ Sci Technol* 42:8133–8139.
72. Fallahtafti S, Rantanen T, Brown RS, Snieckus V, Hodson PV. 2012. Toxicity of hydroxylated alkyl-phenanthrenes to the early life stages of Japanese medaka (*Oryzias latipes*). *Aquat Toxicol* 106–107:56–64.
73. Cameron IL, Hunter KE. 1984. Regulation of the permeability of the medaka fish embryo chorion by exogeneous sodium and calcium ions. *J Exp Zool* 231:447–454.
74. Hill IR, Matthiessen P, Heimbach F. 1994. Guidance document on sediment toxicity tests and bioassays for freshwater and marine environments. From the Workshop on Sediment Toxicity Assessment, Slot Moermond Congrescentrum, Renesse, The Netherlands, 8–10 November 1993, Society of Environmental Toxicology and Chemistry, Brussels, Belgium.
75. Roig N, Sierra J, Nadal M, Moreno-Garrido I, Nieto O, Hampel M, Gallego EP, Schuhmacher M, Blasco J. 2015. Assessment of sediment ecotoxicological status as a complementary tool for the evaluation of surface water quality: The Ebro River basin case study. *Sci Total Environ* 503–504:269–278.