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To cite this version:

Kaouthar Kessabi, Ali Annabi, Aziza Ibn Hadj Hassine, Ingrid Bazin, Wissem Mnif, et al.. Possible chemical causes of skeletal deformities in natural populations of Aphanius fasciatus collected from the Tunisian coast. Chemosphere, 2013, 90 (11), pp.2683-2689. 10.1016/j.chemosphere.2012.11.047 . hal-04184210

HAL Id: hal-04184210

https://hal.science/hal-04184210v1

Submitted on 4 Dec 2023

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Possible chemical causes of skeletal deformities in natural populations of *Aphanius fasciatus* collected from the Tunisian coast

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HIGHLIGHTS

- ▶ Patterns of skeletal deformities were detected in natural population of *Aphanius fasciatus*.
- ▶ The high incidence of spinal deformities was observed in the most polluted site.
- ▶ The most polluted site is contaminated mainly by heavy metals and organic compounds.
- ▶ Relation between the observed deformities and chemical pollution was suggested.

ABSTRACT

This study attempt to quantify and identify skeletal deformities in natural populations of *Aphanius fasciatus* collected from the Tunisian coast and tends to found a possible relationship between these anomalies and several types of pollutants presents in the environment. Fish were collected from one reference area (S1: coast of Luza) and three polluted areas (S2: industrialized coast of Sfax, S3: coast of Khniss and S4: Hamdoun'Oued). Various patterns of skeletal deformities were diagnosed using double staining technics, and the levels of heavy metals (Cd, Cu and Zn), various polycyclic aromatic hydrocarbons (PAHs) and estrogenic compounds were determined in water and sediment from different sites. Spatio-temporal variation of the spinal deformities frequencies in *A. fasciatus* show that high incidence of spinal anomalies has been recorded in population collected from S2 in comparison to three other ones. Morphological results indicated that skeletal deformities of adult *A. fasciatus* were grouped into 13 categories that described abnormalities on spines, vertebrae, arcs and mandibles. A total of 1025 abnormalities were quantified. The results of chemical analysis showed that the levels of heavy metals and PAHs were significantly higher in S2 than in S1, S3 and S4. High level of estrogenic activity was observed only in S4. A possible correlation between environmental exposures to a mixture of pollutants in coastal waters in S2 and spinal deformities in *A. fasciatus* was suggested.

Keywords:
Aphanius fasciatus
Skeletal deformities
Endocrine disruption
Organic pollution
Metallic pollution
Tunisian coasts

1. Introduction

Spinal deformities have been reported in natural fish populations as well as in farmed fish (Aunsmo et al., 2008; Ayed et al., 2008; Kessabi et al., 2009; Bardon et al., 2009; Messaoudi et al., 2009a; Sun et al., 2009). However, skeletal malformations in farmed fish are very common and are a major problem in aquaculture (Koumoundourous et al., 2001a,b). Whereas, the observation of spinal deformities in wild specimens of teleost fish is relatively rare (Dahlberg, 1970; Da Chuna and Antunes, 1999).

While, skeletal malformations in aquaculture appear to be related to a complex interaction of genetic, procedural and infectious/immunological factors (Lall and Lewis-McCrea, 2007; Aunsmo et al., 2008, 2009; Bardon et al., 2009), the presence of deformities in natural populations are most likely related to pollution (Lemly, 2002; Kessabi et al., 2009; Messaoudi et al., 2009a). Indeed, it has been suggested that spinal deformities in natural populations of fish serve as a biomarkers of environmental pollution (De La Cruz-Aguero and Perezgomez-Alvarez, 2001; Antunes and Lopes Da Cunha, 2002; Xuan Yang, 2004).

It is well known that a wide variety of chemicals can cause skeletal deformities through such mechanisms by altering the development and bone formation. These include heavy metals such

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as lead (Pb) (Bengtsson and Larsson, 1986), cadmium (Cd), many organophosphorus pesticides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), toxaphene (Cheng et al., 2000; Teraoka et al., 2002) and natural estrogenic compounds such as ethinylestradiol (EE2) (Boudreau et al., 2004) and 17 beta-œstradiol (E2) (Urushitani et al., 2002).

The Mediterranean killifish, *Aphanius fasciatus* (Valenciennes, 1821) is a small cyprinodont fish inhabiting brackish waters in coastal habitats of the central and eastern Mediterranean Sea. It tolerates a wide range of physico-chemical parameters, such as temperature (5–39 °C) and salinity (0–180 g L^{-1}). We have recently described, in natural populations of this species collected from the Gulf of Gabes in Tunisia, a complex spinal deformities consisting of a consecutive repetition of scoliosis, lordosis, and kyphosis from the head to the caudal fin. Deformed fish were eight times more frequent in the industrialized coast of Sfax than in the coast of Luza which is unaffected by human activities, possibly indicating a relationship between pollution levels and spinal deformities (Messaoudi et al., 2009b). However, a direct relationship between observed spinal deformities and pollution was not determined in this species.

Therefore, this study attempt to quantify and identify skeletal deformities in *A. fasciatus* populations which is naturally exposed to several types of pollutants. For this purpose fish were collected from three polluted localities: S2, S3 and S4, and from a reference site S1. In addition, heavy metals (Cd, Cu and Zn), PAHs, estrogenic activities and estrogenic compounds were also determined in water and sediment of these sites in order to establish a relationship between these pollutants and the high incidence of spinal deformities detected in *A. fasciatus*.

2. Material and methods

2.1. Study sites and sampling

2.1.1. Sites characterization

Sampling sites were selected in the Tunisian coast (Fig. 1), three polluted sites and a reference one. The S2, surrounded by important industrial activities, mainly crude phosphate treatments and chemical industries, was chosen as a most polluted site. This site is contaminated with heavy metals, essentially Cd. The S1, located 50 km north of S2 apparently unaffected by human activities, was used as reference sample site (Barhoumi et al., 2009; Messaoudi et al., 2009b). Pollution in S3 caused by human activities is related to the proximity of the industries in Monastir South, aquaculture activities, the penetration of the water weir South Bay loaded Khniss of pollutants from the village and neighboring areas of culture, and, particularly through the wadi and the activity of washing the wool. The discharge of hot water by Power station via S4 used for overflow sewage, contributes to a sizeable rise in water temperature, this temperature can reach 36 °C. It also contributes to environmental enrichment in nutrients especially ammonium which is an indicator of urban pollution.

2.1.2. Sample collection

Fish were caught with a hand nets. Caught fish were counted and the percentage of deformed fish was calculated from 2007 to 2010. 75 normal specimens (mean total length 3.14 ± 0.36 cm; mean total weight 0.54 ± 0.17 g) and 77 deformed specimens (mean total length 2.79 ± 0.34 cm; mean total weight 0.45 ± 0.15 g) were collected for the morphological study.

Water samples were collected from all localities at a depth of 0.5 m in clean bottles from the different areas. The upper 5 cm of sediments were also collected. Sampling bottles were previously cleaned by soaking with 10% nitric acid (HNO_3) and rinsed with ultrapure water.

2.2. Analytical procedures

2.2.1. Skeletal deformities analysis

The double staining method was used to evaluate anomalies observed in specimens. Normal fish and fish showing a morphological deformity were fixed in buffered 10% formalin and stained using the "Alcian Blue-Alizarin Red" double staining method (Park and Kim, 1984). Alcian Blue 8 GX (Sigma) was used to stain cartilage in blue followed by Alizarin RedS (Sigma) to stain calcified

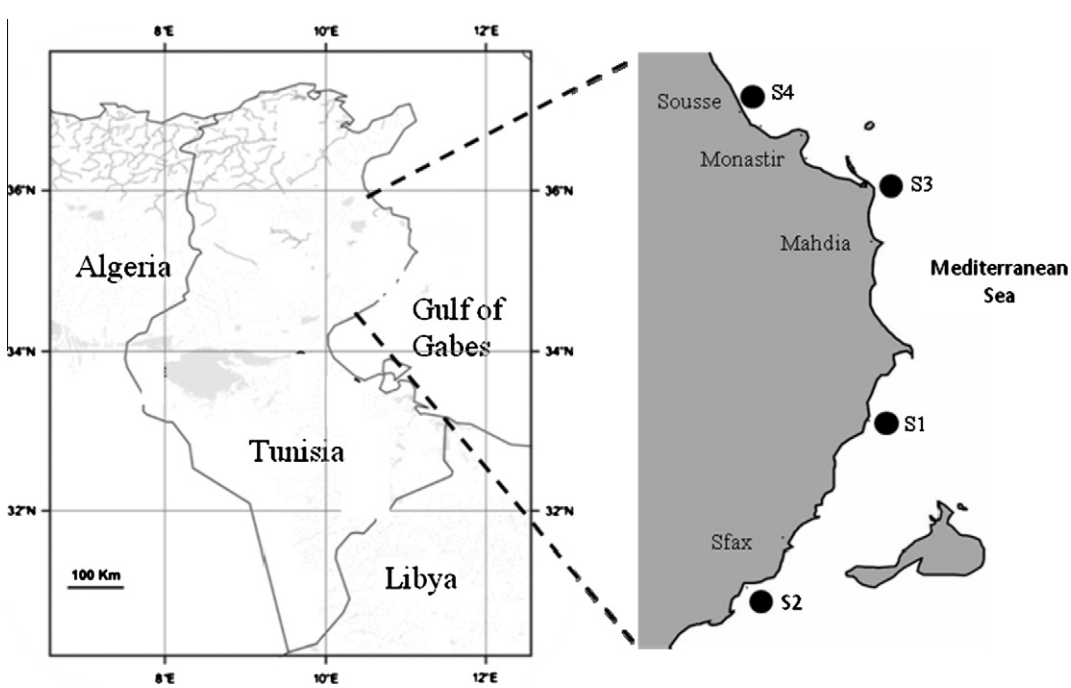

Fig. 1. Location of the sampling sites. S1: coast of Luza: S2: industrial coast of Sfax; S3: Khniss; S4: Hamdoun'Oued.

structures in red. The combination of KOH and peroxide treatment tends to increase the transparency of soft tissues and allow clear observation of skeletal structure.

2.2.2. Metal analysis

Water samples were stabilized at pH 2 with 1 M nitric acid prior to direct determination of total metal concentrations. Sediment samples were oven-dried for 48 h at 100 °C and 100 mg of each sample was mineralized at 250 °C with a set of acids composed of 1 ml of nitric acid, 2 ml of fluorhydric acid and 0.5 ml of perchloric acid and then adjusted to 10 ml with deionised water. Samples were analyzed to determine Cd, Cu and Zn concentrations using a graphite furnace AAS technique (ZEEnit 700). Samples were analyzed in triplicate. The variation coefficient was usually less than 10%. Metal concentrations are reported as $\mu g\, g^{-1}$ of dry weight for sediments and as $\mu g\, L^{-1}$ for water samples.

2.2.3. PAHs (polycyclic aromatic hydrocarbons) determination

The assays performed were carried out according to the protocol of CEAEQ (www.ceaeq.gouv.qc.ca). PAHs levels in sediment were determined by gas chromatography coupled with mass spectrometry. The following compounds were analyzed: acenaphthylene, acenaphthene, naphthalene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a) anthracene, chrysene, benzo(b) fluoranthene, benzo (e, a)pyrene, benzo(g, h, i) perylene, and dibenzo [a, h] anthracene. Data are reported as μg of PAHs/ g dry weight.

2.2.4. YES assay

Both agonist and antagonist activities of chemical dyes were examined using the Yeast strain BY4741 (García-Reyero et al., 2001). This recombinant yeast strain carries the β-galactosidase reporter gene under the control of the estrogen responsive element ERE. The test consisted on the measurement of the β-galactosidase activity (fluorescence at 460 nm after excitation at 355 nm; Fluoroskan, Twinkle LB 970, BERTHOLD Technologies) after 6 h of exposure to the solutions to be analyzed.

Within each experiment, an internal control was set up for the reference estrogen, 17β-estradiol on one plate. The upper part of each plate was filled with the solvent control (methanol), nine concentrations of E2 (1.0×10^{-12}, 1.0×10^{-11}, 1.0×10^{-10}, 1.0×10^{-9}, 1.0×10^{-8}, 1.0×10^{-7}, 1.0×10^{-6}, 1.0×10^{-5} and 1.0×10^{-4} M).

For the effluent samples, dilution series (1–1000) of concentrated extract were prepared in distillated water. Each test for effluent samples was evaluated at least three times by independent experiments. Agonist activities of effluent samples were measured as % of maximal fluorescence intensity produced by 17β-estradiol.

2.2.5. Estrogens detection

The derived estrogens were separated on a waters 2695 HPLC machine, coupled with UV detector DAD996. The injection of 40 μl of samples was performed at 25 °C. The column was a C18 Ascentis C18, 5 cm × 4.6 mm I.D., 5 μm; the mobile phase was a mixture of water: acetonitrile (55:45); and the flow rate was set at 0.4 ml min^{-1}. A UV detector was used to detect estrogens at 220 nm.

2.3. Statistical analysis

For the skeletal deformities, results were reported as percentage of fish affected and comparisons were performed with chi-square test. For metal concentrations, data are given as means ± SE, and differences were assessed by one-way ANOVA followed by protected least significant difference Fisher's test. Values were considered statistically significant when $p < 0.01$.

3. Results

3.1. Incidence of spinal deformities in A. fasciatus

Spinal deformities were visible on the fish body immediately upon catching. The results of spatial distribution among different sites show that the percentage of deformed fish was significantly higher ($p < 0.0001$) in S2 from 2007 to 2010 compared to the three other sites (Table 1).

3.2. Skeletal anomalies: description and frequencies

Skeletal anomalies of adult *A. fasciatus* were grouped into 13 categories that described abnormalities on spines, vertebrae arcs and mandibles. A total of 1025 anomalies were observed: 460 in the vertebrae, 478 in the spines, 73 in the arcs and 14 in mandibles. The number of anomalies recorded in normal fish was 31 (3.02%) and 994 in deformed fish (96.97%). The most common abnormalities described are shown in Fig. 2. The frequencies of fish affected with different skeletal anomalies are reported in Table 2.

3.3. Chemical analysis

The water physico-chemical parameters of sampling sites are summarized in Table 3. Our results showed that the industrialized coast of Sfax (S2) appeared as the most polluted site. In fact, PAHs and heavy metals, especially Cd and Cu, were predominant in S2 compared to the three other sites (S1, S3 and S4). Physico-chemical parameters such as pH, conductivity and dissolved O_2 are similar in all studied sites, while the highest temperature was recorded in the Hamdoun river (S4).

Results presented in Fig. 3 showed that spatial variations of incidence of spinal deformities in *A. fasciatus* populations and CdLire phonétique that spinal deformities that contents in water and sediment of studied sites were very similar.

3.4. Estrogenic activities

Our results show that the high level of estrogenic activity was observed only in S4 (60.5% in water and 99.65% of relative

Table 1
Spatio-temporal distribution of spinal deformities (percentages) in *A. fasciatus* populations from studied sites.

Year	2007			2008			2009			2010		
Sites	T	D	%	T	D	%	T	D	%	T	D	%
S1	341	9	2.63	477	20	4.19	440	13	2.95	500	20	4
S2	263	46	17.49*	518	80	15.44*	394	72	18.27*	500	71	14.2*
S3	225	4	1.77	250	7	2.8	263	5	1.9	219	3	1.36
S4	220	5	2.27	268	3	1.12	228	4	1.75	264	5	1.89

T: Total number of fish examined.
D: Number of deformed fish.
S1: Luza; S2: industrialized coast of Sfax; S3: Khniss and S4: Hamdoun river.
* $p < 0.0001$: Significance from samples of the three other sites throughout the year.

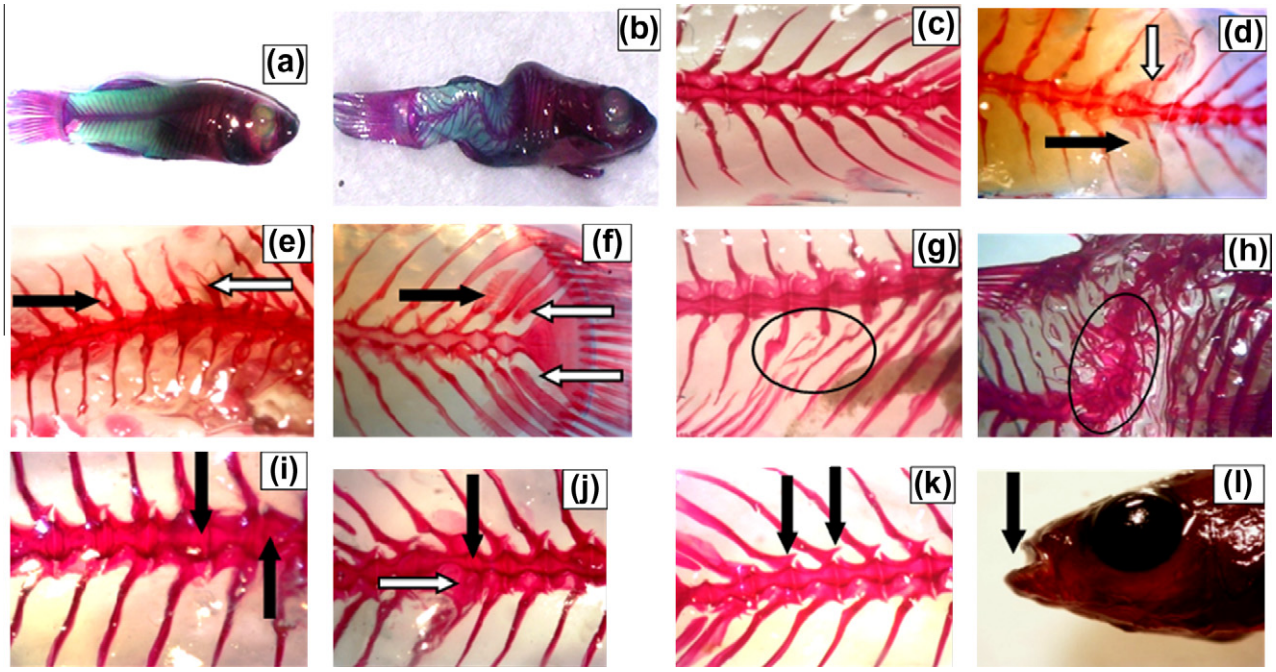

Fig. 2. Examples of skeletal anomalies in *A. fasciatus*. (a): Normal specimen, (b): deformed specimen, (c): normal vertebral column profile, (d): the black arrow points to absence of spine, the white arrow points to fused spines. (e): The black arrow points to bifurcated spine, the white arrow points to broken spine. (f): The black arrow points to short spine, the white arrows point to detachment of spines. (g): Abnormal shape of three consecutive spines, (h): fused vertebrae, (i): hypertrophied vertebrae, (j): the black arrow points to atrophied vertebrae, the white arrows point to a Hypertrophied arcs, (k): detached arcs and (l): prognathe.

Table 2
Number (*N*) and frequencies (*F*) of normal and deformed *A. fasciatus* affected with different skeletal anomalies.

	Normal ($n = 75$)		Deformed ($n = 77$)	
	N	*F*	*N*	*F*
Anomalies of spines				
Fused spines	3	0.63	46	59.74*
Bifurcation	6	8	77	100*
Detachment	8	10.66	77	100*
Broken spines	0	0	39	50.64*
Absence of spines	0	0	20	25.97*
Short spines	0	0	19	24.67*
Abnormal shape	0	0	49	63.63*
Anomalies of vertebrae				
Fused vertebrae	4	5.33	77	100*
Atrophy	3	4	50	64.93*
Hypertrophy	2	2.66	65	84.41*
Anomalies of arcs				
Arc detachment	0	0	27	35.06
Arc hypertrophy	0	0	15	19.48
Prognathe	0	0	14	18.18

n: Number of specimens examined
* $p < 0.0001$: Significance from normal fish.

estrogenic activity in suspended mater). A light difference was observed between S1, S2 and S3 (Table 4). The results related to the estrogenic compounds, detected by HPLC analysis in water and suspended mater (SM) of different areas of study; indicate the presence of estrone in water and 17β-estradiol and estriol in SM collected from S4 (Table 4).

4. Discussion

The aim of this study was to diagnose skeletal deformities and found a possible relationship between these deformities and several types of pollutants present in the environment.

Skeletal deformities, one of the most serious pathological effects in fishes, are well known for many fish species from different areas of the world (Tutman et al., 2000; Ayed et al., 2008; Sun et al., 2009). Assessing morphological deformities represent one of the most straight forward methods to study the effects of contamination on fish because of the ease of recognition and examination when compared with other types of biomarkers.

In our previous study, we have showed that CYP1A mRNA levels in deformed *A. fasciatus* collected from the industrial coast of Sfax (S2) were 10 times higher than the normal ones, suggesting a link between the extent of deformity of impacted fish and their exposure to CYP1A-inducing pollutants (dioxins, dioxin-like PCBs, PAHs) in the environment (Kessabi et al., 2010). In the present study, population from the industrialized coast of Sfax (S2) was found to have higher frequency of abnormal fishes as compared to *A. fasciatus* from the other sites. PAHs and heavy metals have been found in water and sediments of S2 with high levels as compared to other sites, suggesting a combined effect of these pollutants leading to different types of skeletal deformities observed in *A. fasciatus*. This ecological situation is closely connected with the promoted industrial development and intense human activities. This region differs greatly in many environmental aspects and observed population differences in malformation frequency might be explained by influence of environmental pollution. In fact, the pollution in this coast is due essentially to the presence of continuous discharge of pollutants from local industrial activities (Hamza-Chaffai and Pellerin, 2003) and from the phosphogypsum. Several reports have also indicated high metal concentrations in soft tissues of *Reditapes decussatus* (Hamza-Chaffai and Pellerin, 2003; Smaoui-Damak et al., 2003) and in the fish *Scorpaena porcus* (Hamza-Chaffai et al., 1995) from this coast.

The present study showed that spatial variations of incidence of spinal deformities in *A. fasciatus* populations and CdLire phonétique that spinal deformities that contents in water and sediment of studied sites were very similar. An increase in spinal deformities rate induced by exposures of fish to Cd were reported by many

Table 3
Sites description.

Parameter	S1	S2	S3	S4
pH	8.5	8.4	8.5	8.4
Temperature (°C)	20.3	21.4	24	29.6
Conductivity (mS cm^{-1})	54.5	54.6	56	56.3
O_2 (ppm)	8.4	8.5	8.5	8.4
Heavy metals Water (μg L^{-1})				
Cd	0.03 ± 0.01	0.62 ± 0.02*	0.02 ± 0.004	0.03 ± 0.01
Cu	3.06 ± 1.54	15.29 ± 3.39*	2.73 ± 0.79	2.43 ± 0.49
Zn	12.28 ± 1.47	67,25 ± 7.96*	12.85 ± 1.5	11.62 ± 1.78
Heavy metals sediment (μg g^{-1} dry weight)				
Cd	0.05 ± 0.01	1.78 ± 0.15*	0.04 ± 0.01	0.05 ± 0.01
Cu	3.05 ± 1.19	36.84 ± 2.44*	11.98 ± 2.06	4.74 ± 1.21
Zn	32.00 ± 9.57	143.66 ± 31.08*	20.35 ± 1.86	18.38 ± 3.00
PAHs sediment (μg g^{-1} dry weight)				
Acenaphtylene	<DL	<DL	<DL	<DL
Acenaphtene	<DL	<DL	<DL	<DL
Naphthalene	<DL	0.12	0.07	0.06
Phenanthrene	<DL	0.1	0.09	0.05
Anthracene	<DL	<DL	<DL	<DL
Fluoranthene	0.05	0.29	0.21	0.18
Pyrene	0.05	0.28	0.22	0.25
Benzo(a)anthracene	0.06	0.23	0.13	0.2
Chrysene	<DL	0.25	0.09	0.15
Benzo(b)fluoranthene	<DL	0.17	0.11	0.22
Benzo(e)pyrene	<DL	0.09	0.07	0.09
Benzo(a)pyrene	<DL	0.2	0.17	0.23
Benzo(g,h,i)perylene	<DL	0.26	0.18	0.22
Dibenzo[a,h]anthracene	<DL	<DL	<DL	<DL
Σ PAHs	0.16	1.99	1.34	1.65

PAHs: total concentration of PAHs presents in each site; <DL: inferior to limit of detection; S1: Luza; S2: industrialized coast of Sfax; S3: Khniss and S4: Hamdoun river.
* $p < 0.0001$ (Significance from samples of S1, S3 and S4).

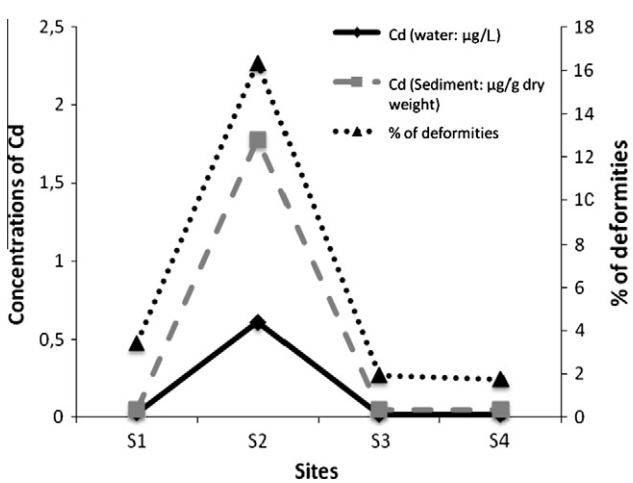

Fig. 3. Cd levels and distribution of spinal deformities frequencies in *A. fasciatus* collected from studied sites.

authors (Bervoets and Blust, 2003; Sassi et al., 2010). *Myoxocephalus quadricornis* exposed for 1 year to an artificial heavy metal-containing effluent displayed spinal curvature and increased frequency of vertebral deformities (Bengtsson and Larsson, 1986). A causal relationship between high environmental levels of heavy metals, especially Cd, and skeletal anomalies in fish has been suggested by Bengtsson et al. (1985). Buhringer et al. (1990) found highest Cd levels in the bones in rainbow trout, *Oncorhynchus mykiss* suffering from deformities compared to healthy individuals. Most of the evidence on Cd influence on induction of spinal deformities in fish is from laboratory studies. Resorption of Ca from the bone to compensate for disturbed Ca homeostasis, under Cd influence, is suggested as the main mechanism behind the observed deformities in these studies (Bengtsson and Larsson, 1986; Bervoets and Blust, 2003; Sassi et al., 2010). Cheng et al. (2000) showed that the frequency of malformations in zebrafish embryos increased with Cd concentration in culture media and demonstrated a reduced myotome formation in Cd-induced spinal deformity. These authors suggested that fish embryonic malformations induced by Cd might be mediated through ectopic expression of developmental regulatory genes.

Table 4
Estrogenic activities (%) and estrogenic compounds detected in water (W) and suspended mater (SM) of studied sites.

Sites	Estrogenic activity (%)		Estrogenic compounds	
	W	SM	W	SM
S1	17.18	6.85	E2	E2, EE2
S2	22.46	5.16	<LD or NF	E1, E3
S3	20.39	6.68	<LD or NF	E2, EE2
S4	60.5	99.65	E1	E2, E3

NF: compound not found.
<LD: inferior to limit of detection.
(E1: estrone; E2: 17β-estradiol; E3: estriol; EE2: ethynilestradiol).
S1: Luza; S2: industrialized coast of Sfax; S3: Khniss and S4: Hamdoun river.

PAHs comprise a large class of widespread environmental contaminants produced by both natural and anthropogenic sources. Due to their persistence and accumulation in soils and sediments, these compounds are now widely dispersed in the biosphere, notably in sediments of estuaries and coastal marine

areas throughout the world. It was frequently reported that spinal deformities can be experimentally induced in fish upon exposure to different organic and inorganic substances, including metals, metalloids and organic substances like PAHs, organochlorine compounds OC and dioxins (Yamauchi et al., 2006; Cachot et al., 2007; Miller et al., 2007; Holth et al., 2008; Gagnon and Rawson, 2009).

Given their high estrogenic potency and their high prevalence in the aquatic environment, the natural estrogens such as E1 (estrone), E2 (17 beta-estradiol), E3 (estriol) and the synthetic estrogens, are of particular concern in the risk assessment of endocrine disrupting compounds for wildlife (Kramer et al., 1998; Bjerselius et al., 2001; Van den Belt et al., 2001, 2002). Estrogens are potent chemicals of which the estrogenic effects can be observed in laboratory studies at 1 ng L^{-1} (Routeledge et al., 1998). In addition, length of the exposure period influenced the incidence of morphological abnormalities as abnormalities worsened as exposure progressed. This temporally cumulative effect was also observed in response to EE2 treatment, in which a 60-d exposure of *Fundulus heteroclitus* produced abnormalities at lower concentrations than a 25-d exposure (Boudreau et al., 2004). Although S3 and S4 are polluted and show high values of PAHs, and S4 shows an estrogenic activity up to 100%, the percentage of spinal deformities is low compared to S2. This confirms that estrogenic compounds or PAHs alone are not able to give a high rate of malformations in *A. fasciatus*.

The causes of the occurrence of spinal deformities in natural populations of fish are complicated and, to a large extent, unknown. They may be a response to a number of biotic factors and abiotic factors which are not related to pollution. Nevertheless, the observed association of the relatively high prevalence of spinal deformity in *A. fasciatus* and the important accumulation of heavy metals and PAHs in the industrialized coast of Sfax, seems to be very interesting and suggest that these mixture of chemical pollutants may represent a potential risk to induce spinal deformities in natural population of *A. fasciatus*. However, clear and confirmed association of skeletal abnormalities in *A. fasciatus* from the industrialized coast of Sfax with contaminants still requires additional research.

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