Sandhopper *Talitrus saltator* (Montagu) as a Bioindicator of Contamination by Polycyclic Aromatic Hydrocarbons

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Abstract This study evaluates the use of the sandhopper *Talitrus saltator* as a bioindicator of PAHs contamination of the supralittoral zone of sandy beaches. Adult amphipods were collected at eight localities on the Tuscany shore in summer. Concentrations of 16 PAHs indicated as priority pollutants by the Environmental Protection Agency were determined. *T. saltator* is able to accumulate PAHs (total PAHs ranging from 0.75 to 62.1 ng g^{-1}), with concentrations generally greater than in the sand (total PAHs ranging from 0.04 to 38 ng g^{-1}). In particular, it accumulates Phe, Cry, and BbF. Preliminary laboratory tests indicate food as a possible route of PAHs intake by sandhoppers. Despite the need of further studies to clarify the variability of the PAH concentrations found in the substrata and in sandhoppers, *T. saltator* could be used as a bioindicator of PAHs contamination of the supralittoral zone of sandy shores.

Keywords Sandhoppers · PAHs · Bioaccumulation · Sandy beaches

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Coastal areas are subjected to a strong input of contaminants from terrestrial, fluvial and marine sources (Di Leonardo et al. 2009; Dickhut et al. 2005). Polycyclic aromatic hydrocarbons (PAHs) are a group of widespread contaminants that have been intensively studied in the various compartments of the environment (McElroy et al. 1989; Meador et al. 1995; Cincinelli et al. 2004; Cincinelli et al. 2008) because of their carcinogenic and mutagenic properties. PAHs are of concern because of their toxicity, persistence, and difficult remediation. The US Environmental Protection Agency has indicated 16 PAHs as priority pollutants. These compounds have mainly anthropogenic origins, being released into the environment via industrial processes, wood treatment facilities, household heating and road transport. Most inputs of PAHs into the marine ecosystem are linked to human activities. PAHs can also be introduced into the aquatic environment by combustion of organic matter (pyrolytic origin), petroleum hydrocarbons and short-term degradation of biogenic precursors (diagenesis). It should be underlined that the Mediterranean basin contains 82 petroleum harbours and that ships involved in the transport of oil and its by-products represent more than half of commercial maritime traffic (Legambiente 2007). Many studies have investigated the potential use of bioindicators to monitor contamination by PAHs in the marine (subtidal) environment (Meador et al. 1995; Baumard et al. 1998; Sanz-Lázaro et al. 2008). However, the supralittoral zone, characterized by a strong input of these compounds, has received little attention. The supralittoral is the zone where the major consumers of stranded organic material, i.e. talitrid amphipods and isopods, find shelter during the day, buried in the wet sand or stranded organic material. In fact, most of the energy flow deriving from the use of stranded material passes through supralittoral crustaceans (Brown and McLachlan 2006).

They are important integrators of the marine and terrestrial organic material present on the beach. Sandhoppers are a normal presence in the damp zone of Mediterranean sandy beaches. The biology and auto-ecology of talitrid amphipods are sufficiently known, and the pan-Mediterranean sandhopper *Talitrus saltator* has been the object of many studies. *T. saltator* digs temporary refuges a few centimetres below the surface of the damp belt of sandy beaches (e.g. see Wildish 1988). Environmental contamination of the supralittoral zone has received attention fairly recently, and many species of sandhoppers and beachfleas have been shown to be good biomonitors for some trace metals (see Marsden and Rainbow 2004; Ugolini and Ungherese 2012 for reviews) and POPs, such as PBDEs (Ungherese et al. 2012).

Owing to the hydrophobic character of PAHs, their concentration in water is normally very low and they are difficult to detect. Large water volumes are needed for analyses of PAHs, and their hydrophobicity/lipophilicity results in preferential partitioning into sediments. Therefore, to more accurately evaluate exposure to contaminants, it is important to detect them in organisms. The aim of this study was to assess the possibility to use the pan-Mediterranean amphipod *T. saltator* as a bioindicator of PAHs contamination of the supralittoral zone of Mediterranean sandy beaches.

Materials and Methods

The analysis of PAHs was carried out on adult and subadult individuals of *Talitrus saltator* (except for ovigerous females). Sandhoppers and sand were collected at eight localities along the Tuscany coast, Italy (FM, Morto Vecchio River mouth, Pisa, 43°44′53″N, 10°16′33″E; A, Arno River mouth, Pisa, 43°40′56″N, 10°16′53″E; C, Calambrone, Livorno, 43°34′51″N,10°17′59″E; RS, Rosignano Solvay, Livorno, 43°22′22″N, 10°26′26″E;P, Piombino, Livorno, 42°57′16″N, 10°37′08″E; O, Ombrone River mouth, Grosseto, 42°39′29″N, 11°00′52″E;AR, Albegna River mouth, Grosseto, 42°30′08″N, 11°11′34″E; AM, Albegna seashore, Grosseto, 42°30′33″N, 11°11′30″E) in summer (2008–2010). Albegna River samplings were performed in 2009 and 2010. After collection, samples were frozen, dehydrated and then freeze-dried. Analyses were carried out according to the US-EPA method 3545B (1996), an extraction procedure for semi-volatile compounds with Accelerated Solvent Extractor (Dionex mod. ASE 200). The extracts were evaporated under nitrogen flow and brought to a final volume of 1 mL by addition of acetonitrile.

PAHs were analysed by high performance liquid chromatography (HPLC). Acenaphthylene was determined with a Waters® PDA 996 photodiode series detector, while a Waters® 474 scanning fluorescence detector was used for all the other compounds (US EPA 2003).

Sixteen PAHs indicated by the US-EPA Clear Water Act as priority pollutants were quantified: Nap, naphthalene; AceP, acenaphthene; A, acenaphthylene; Fl, fluorene; Phe, phenanthrene; Ant, anthracene; Flu, fluoranthene; Py, pyrene; BaA, benzo[*a*]anthracene; Chry, chrysene; BbF, benzo[*b*]fluoranthene; BkF, benzo[*k*,]fluoranthene; BaP, benzo[*a*]pyrene; IP, indeno[1,2,3-*cd*]pyrene; DBA, dibenzo[*a,h*]anthracene; BghiP, benzo[*ghi*]perylene. The chromatographic separation was performed on a Supelcosil™ LC-PAH HPLC chromatographic column (250 × 4.6 mm i.d. particle size 5 μm, Supelco) with an acetonitrile:water gradient of 60:40 imposing a ramp to 100:0 within 30 min and hold 10 min, with a flow rate of 1.5 mL min⁻¹. PAHs were quantified using TLC Polynuclear Aromatic Hydrocarbon Mix by Supelco Inc. (USA) as the external standard; the standard mix contained 16 compounds. Detection limits were evaluated as mean blank + 3 SD (IUPAC) and ranged from 0.01 to 0.5 ng g⁻¹ dry weight for PAHs. A certified reference material (HS-6 harbour sediments, purchased from NRC, Canada), procedural blanks and replicate samples were used for the quality control procedures, and their reproducibility and recovery were high (70 %–80 %).

When the concentrations of PAHs were below the detection limit, both in the sand and in sandhopper samples, the data were not considered for the statistical analysis. When only one of the data was below the detection limit, we arbitrarily assigned the lower detection limit value (0.01 ng g⁻¹ dry weight) and included it in the analysis.

Total lipids were determined for each sampling site by a gravimetric procedure reported by Kannan et al. (1998). Samples of 10 sandhoppers (wet weight range 0.460–0.690 g) were homogenized and extracted in soxhlet for 16 h in dichloromethane:hexane 3:1 and concentrated to a volume of 10 mL; 2 mL were used to determine the total lipid concentration (Table 1). The relationship between PAH concentrations in amphipods and sand was evaluated by the Spearman rank-order correlation coefficient test (Siegel and Castellan 1989).

Since sandhoppers are supralittoral sandy beach-dwelling grazers and scavengers (Wildish 1988), we investigated PAHs uptake via food. Two groups of 50 adult individuals of *T. saltator* were collected on the beach of Morto Vecchio River mouth in November 2010. The sandhoppers were kept in the laboratory in Plexiglas containers with artificial sand (Bio Dry®) wetted with artificial sea water (Tropic Marin® Sea Salt, Wartenberg, Germany) for 5 days before the start of the experiment. During this period the sandhoppers were fed with dry fish food (SERA bioflakes, Sera, Germany). Then, 10 individuals per container

Table 1 Total lipid content (g g^{-1}) in sandhoppers collected at the different sampling sites

Localities	Total lipids (g g^{-1})
FM	0.142
A	0.138
C	0.117
RS	0.112
P	0.139
O	0.111
AM	0.095
AR	0.070

(control group) were placed in 10 Petri dishes (diameter 10 cm) containing plastic sponge wetted with artificial sea water (salinity = 39 ‰). The artificial sea water consisted of artificial sea salt diluted in double-distilled water. The saline concentration was checked with a hand refractometer (Salt refractometer, Sper Scientific106 ATC, Scottsdale, Arizona). Each control group was left in the presence of 0.2 g of dry fish food (SERA bioflakes, Sera, Germany) for 24 h at 24°C. The experimental groups (40 individuals) were maintained at the same temperature, but the food was contaminated with Chry (concentration in the food = 20 ng g^{-1}) dissolved in methanol. The solution was concentrated by Rotavapor and dried under a gentle stream of ultrapure nitrogen. At the end of the treatment, the sandhoppers were killed by freezing (T = −20°C). For both controls and experimentals, the total lipid content was determined as described above (controls, 0.040 gg^{-1}; experimentals, 0.051 gg^{-1}), and the Chry concentration for each sample (compared to the total lipids content) was determined. Samples were pre-treated with anhydrous sodium sulphate and then soxhlet-extracted for 16 h with a mixture of methylene chloride and hexane (3:1 v/v). An aliquot of extract was used for fat determination as described above; the remaining extract was concentrated and cleaned up on an acid silica/silica gel column (Kannan et al. 1998). A gas chromatograph (6890 Inert, Agilent Technologies) coupled with a mass spectrometric detector (5976 SD, Agilent Technologies) was used to determine Chry. A DB-35MS (30 m × 0.25 mm × 0.25 μm) was used for separation and identification of Chry. The column oven temperature was programmed from 50 to 130°C at a rate of 25°C min^{-1}, from 130 to 260°C at a rate of 8°C min^{-1} and from 260 to 300°C at a rate of 5°C min^{-1}. Injector and detector temperatures were 300 and 150°C respectively. Helium was used as carrier gas and MSD was operated at an electron impact energy (EI) of 70 eV. Chry was determined by selective ion monitoring (SIM) at m/z 228. The recovery percentage was evaluated by adding an aliquot of Chrysene d12 (Supelco) at the beginning of the

analytical procedure; recovery percentages were greater than 80 % for each sample and blank procedures did not show the presence of Chrysene.

Results and Discussion

Nap, AceP, and A were not recorded in the sand or in sandhoppers (Table 2) at all the localities.

The correlation between the concentrations in *T. saltator* and in sand did not reach statistical significance for any of the other 13 PAHs analysed (Spearman rank-order correlation coefficient test, r_s max = 0.586, n = 8, *p* = NS). However, the comparison between the mean sand and sandhopper concentrations for each PAH, independently of the localities, showed a significantly higher concentration in sandhoppers than in sand for Phe, Chry and BbF (see Table 3). The comparisons did not reach full statistical significance for BkF and BghiP (see Table 3).

In all the other cases, the difference was not significant. Comparison of the mean total concentration of the 13 PAHs obtained for sand and sandhoppers (Table 2) showed a significantly higher total PAH concentrations in sandhoppers than in sand (SE $_{sand}$ = 4.746, ES $_{T.\ saltator}$ = 6.948, t = 3.880, df = 7, *p* < 0.01).

In the experiment on the uptake of PAHs (in this case Chry) via food, the control sandhoppers had a significantly lower concentration (mean = 6.646 ng g^{-1}, SE = 0.249) than the experimental ones (mean = 56.698 ng g^{-1}, SE = 1.331). The comparison of the means was highly significant (t = 36.956, df = 8, *p* < 0.01, Student's test). Therefore, sandhoppers can accumulate Chry via food: the Chry concentration in the experimental sandhoppers was far higher (700 %) than in the control ones.

With the development of environmental geochemistry, the fingerprints of PAHs from pyrolytic or petrogenic origin can be assessed using molecular indices based on ratios of selected PAH concentrations. These criteria are based on peculiarities in PAHs composition and distribution as a function of the emission source. A value of the ratio between low molecular weight (LMW, two or three rings) and high molecular weight (HMW, four to six rings) higher than one indicates a petrogenic source. Some authors (Sicre et al. 1987; Colombo et al. 1989; Budzinski et al. 1997; Baumard et al. 1998; Soclo et al. 2000; Magi et al. 2002) have also suggested that the Phe/Ant ratio is very high (>10) in petrogenic PAHs pollution, whereas Phe/Ant <10 is typical of pyrolitic sources. In addition, a predominance of fluoranthene over pyrene (Flu/Py > 1) is characteristic of pyrolytic products, while pyrene is more abundant than fluoranthene (Flu/Py < 1) in petroleum-derived PAHs (Budzinski et al. 1997). The PAH concentrations were below the method detection limit for most samples.

Table 2 Concentration of PAHs in *T. saltator* (ng g^{-1} of total lipids, in bold) and sand (ng g^{-1}) samples collected at various sampling sites along the coast of Tuscany

Localities	Fl	Phe	Ant	Flu	Py	BaA	Chry	BbF	BkF	BaP	DBA	BghiP	IP	Total
FM	–	–	–	–	**15.28**	–	**9.718**	–	–	–	**2.983**	**16.831**	–	**44.812**
	–	–	–	–	0.01	–	0.01	–	–	–	0.01	0.01	–	0.04
A	**0.434**	**6.086**	**1.367**	**0.237**	**15.367**	**0.359**	**7.719**	**7.41**	**4.076**	**0.82**	**0.01**	**17.338**	**0.884**	**62.098**
	0.01	1.02	0.463	0.116	10.586	19.86	2.086	0.683	0.073	0.076	1.07	1.923	0.01	37.998
C	**1.803**	**7.376**	**3.589**	**9**	**18.29**	**0.01**	**9.23**	**0.01**	**0.01**	–	–	–	**9.914**	**59.205**
	0.156	0.136	0.106	0.113	0.185	0.145	0.149	0.171	0.308	–	–	–	0.01	1.744
RS	**0.01**	**7.973**	**3.687**	**9.285**	**0.01**	**0.01**	**15.294**	**0.01**	**0.01**	**0.01**	–	**0.01**	–	**36.246**
	0.536	0.3	0.263	0.563	0.243		0.14	0.106	0.386	0.066		0.406-	–	3.31
P	**2.733**	**2.518**	**1.194**	**2.633**	**1.554**	**1.438**	**2.611**	**1.841**	**2.827**	**2.374**	–	**2.23**	**1.482**	**25.435**
		2.228		1.913	1.756	0.253	2.833	1.256	1.286	3.52		0.166	0.01	19.694
O	**0.01**	–	–	**0.270**	**0.01**	–	**0.01**	–	–	–	–	**0.45**	–	**0.75**
	2.496	–	–	2.616	–	–	2.116	–	–	–	–	0.01	–	7.563
AR	**0.01**	**6.914**	**8.214**	–	**0.01**	–	**2.571**	**8.357**	**8.286**	**2.214**	**7.214**	–	–	**43.772**
	0.085	0.01	0.01	–	0.085	–	0.075	0.01	–	0.01	0.01	–	–	0.491

FM Morto Vecchio River mouth (Pisa), *A* arno river mouth (Pisa), *C* calambrone (Livorno). *RS* rosignano solvay (Livorno), *P* piombino (Livorno), *O* ombrone river mouth (Grosseto), *AR* albegna river mouth (Grosseto), *AM* albegna seashore (Grosseto). PAHs: *Fl* fluorene, *Phe* phenanthrene, *Ant* anthracene, *Flu* fluoranthene, *Py* pyrene, *BaA* benzo[a]anthracene, *Chry* chrysene, *BbF* benzo[b]fluoranthene, *BkF* benzo[k]fluoranthene, *BaP* benzo[a]pyrene, *DBA* dibenz[a,h]anthracene, *BghiP* benzo[g,h,i]perylene, *IP* indeno[1,2,3-cd]pyrene, Total, sum of the 16 PAHs analysed

Table 3 Results of the Student's t test used to compare the mean sand and sandhopper concentrations for PAHs

	SE$_{sand}$	SE $_{T. saltator}$	t	df	p
Phe	0.345	1.187	3.369	5	<0.02
Chry	0.421	1.872	2.457	7	<0.05
BbF	0.171	1.533	2.652	5	<0.05
BkF	0.167	1.842	3.331	6	$0.05 < p < 0.1$
BghiP	0.296	3.252	2.212	5	$0.05 < p < 0.1$

However, for the samples showing PAH concentrations, the Phe/An ratio was <10 for both *T. saltator* and sand at all the sampling sites, indicating dominance of a pyrolytic origin. The Flu/Py ratio was >1 for sandhoppers and sand at Piombino, for sandhoppers at Albegna seashore, and for sediment at Rosignano Solvay, denoting a pyrolytic origin, whilst the ratio was <1 at Arno River mouth and Calambrone for both sand and sandhoppers, indicating a petrogenic origin. The results of this study show that *T. saltator* is able to accumulate PAHs with respect to the sand, particularly Phe, Chry and BbF. However, we found great variability of accumulation of the different PAHs by sandhoppers, as well as great variability among localities. This makes generalization difficult. However, it is likely that sandhoppers feed on organic matter (plants and animals) of marine and terrestrial origin that is differently contaminated by PAHs.

It should be underlined that the PAH concentrations determined in sandhoppers were not correlated with the PAH concentrations recorded in the sand of the sampling sites (Table 2). The highest total amount of PAHs in sandhoppers was recorded at Arno River mouth (62.098 ng g^{-1}) and the maximum value in the sand (37.998 ng g^{-1}) was also observed at that site; however, the sandhoppers from Calambrone showed a total PAHs value of 59.205 ng g^{-1}, whilst the value in the sand was only 1.744 ng g^{-1}. Therefore, we observed that sandhoppers can accumulate only a part of the 16 PAHs tested. However, the comparison between the sediment and biotic media does not provide relevant information about the true degree of contamination of a coastal area.

It is also difficult to explain the differences in PAH concentrations in the sand at different localities because of the presence of a huge number of environmental parameters that could influence the concentrations in this medium. On the other hand, a rather high concentration of PAHs is to be expected near Piombino (industrial harbour) and Arno River mouth (the main river of Tuscany flows through important industrial areas), but it is difficult to explain the low concentrations found near Calambrone (near the Livorno harbour) and at Morto Vecchio River mouth (located between the mouths of the Arno and Serchio rivers) where we found a large amount of plastic debris on the sandy shore.

The origin of PAHs contamination was indicated by the molecular ratios. The petrogenic origin of PAHs found in samples collected at Calambrone and the pyrolytic origin for the PAHs at Piombino (industrial harbour) and the

beach of Rosignano Solvay (plant) were not surprising. However, it is difficult to explain the pyrolytic origin of the PAHs at Albegna sea shore or the petrogenic origin of contamination at Arno River mouth.

Further studies are required to better understand the PAHs bioaccumulation in sandhoppers and the correlation with the lipid contents and the diet. Nevertheless, *T. saltator* appears to be a good bioindicator of this class of organic compounds because it is widespread in the supralittoral area of Mediterranean and North Atlantic and Baltic European sandy shores, and it is able to mediate environmental parameters that significantly influence PAH concentrations in sand.

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