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NOTES

Potential use of ecotoxicological biomarkers in *Serratella ignita* (Ephemeroptera) larvae for Alcantara river (Sicily, Italy) water quality assessment

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ABSTRACT

Benthic macroinvertebrates are important components of aquatic river ecosystems. These organisms are often used for biological monitoring since they are good indicators of the aquatic freshwater environment health status *which can be negatively affected by human, agricultural and industrial activities. Many studies focused on the use of observed changes in macroinvertebrate communities or populations, but studies using biochemical biomarkers in these species are almost absent. The aim of this paper was to test the employment of ecotoxicological biomarkers in Ephemeroptera larvae to assess the water quality of the Alcantara river (Sicily) from its headwater to its mouth. This river represents the main source of potable water for all the counties situated in the Alcantara valley and for the city of Messina, and is the primary irrigation source for the large surrounding agricultural lands. Acetylcholinesterase activity (AChE) and Benzo(a)pyrene monooxygenase (BPMO) activity were evaluated in* Serratella ignita *(Ephemerellidae) larvae from different sampling sites along the river to show the potential presence of xenobiotic substances. Environmental parameters, such as temperature, dissolved oxygen, pH, conductivity and flow velocity, were also measured at different sampling sites. The biomarker approach in invertebrates represents an early warning signal of ecotoxicological alterations, providing information on which contaminants exert toxic effects on different biota. The results showed that the highest inhibition of AChE activity in* S. ignita *larvae was in Randazzo and Castiglione di Sicilia villages (*i.e. *high and medium level of the river). Here, agricultural activities are intense, with high production of fruit, olives, citrus and the heaviest usage of fertilizers, pesticides, and neurotoxic substances like organophosphate insecticides and carbammates in all the Alcantara valley. Regarding BPMO activity, the highest value was recovered in* S. ignita *larvae from the river mouth, which was characterised by the highest entry of water run off of streets and loaded with xenobiotic lipophilic compounds. Both AChE and BPMO were not linked to water temperature values. The data reported in the present work represent the first attempt to monitor levels of two widely recognised enzymatic biomarkers in benthic macroinvertebrates. Results indicate the possible use of this approach in macroinvertebrate larvae to study the* health status *of Alcantara river. The river appears to be impacted by contamination mainly from waste discharges and, upstream, from large pasture areas. Considering the world-wide distribution of* S. ignita*, the biomarker approach used to assess water quality may be applicable not only to Alcantara river, but also to other lotic ecosystems.*

Key words: responses to pollutants, river health status*, Ephemerellidae larvae, AChE, BPMO, Alcantara river*.

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INTRODUCTION

Benthic macroinvertebrates are important representatives of the aquatic organisms living in river ecosystems (Girgin, 2010), and they are often employed for biological monitoring (Alam *et al*., 2007; Imandoust and Gadam, 2007; Nouri *et al.,* 2008; Igbinosa and Okoh, 2009; Pejman *et al*., 2009).

Ephemeroptera are the most represented order of riverine insect species. They are heterometabolous and hemimetabolous with a gradual metamorphosis, sub-aerial adults and aquatic larvae, few mm to 2.4 cm max long as mature nymphs. The larvae are equipped with a primitive mouth apparatus and abdominal tracheobranchia that can be agitated to increase oxygenation; they show detritivorous habits, feeding on decomposing particles of animal or plant origin or periphyton adhering to the stream substratum.

Many papers have been published on the use of observed changes in macroinvertebrate communities or populations (Engel and Voshell, 2002; Ogbeibu and Oribhabor, 2002; Neumann *et al.,* 2003; Sandin, 2003) to assess the effects of pollutants. By contrast, studies using biochemical biomarkers in these species are rare (Berra *et al.,* 2004), or they have been carried out under laboratory-controlled conditions (Ibrahim *et al.,* 1998; Kheir *et al*., 2001; De Coen *et al.,* 2001; Hyne and Maher, 2003) in order to establish cause-effect relationships of specific contaminants and to predict the effect of such contaminants on natural populations (Clements, 2000). Benthic macroinvertebrate larvae are commonly exploited for measuring biomarkers for several reasons: i) they are easily available, widely distributed, relatively abundant, and easy to collect; ii) many taxa are fairly sedentary and thus representative of local conditions; iii) they are closely associated with sediments; and iv) they are close to the base of food chains (Hare, 1992; Berra *et al.,* 2004).

The aim of this paper was to test the possible employment of ecotoxicological biomarkers in larval stages of an aquatic macroinvertebrate species, *Serratella ignita* (formerly *Ephemerella ignita*, Poda 1761, Insecta: Ephemeroptera), to assess the water quality of the Alcantara river (Sicily, Italy) from its headwater to its mouth. *Serratella ignita* is a typical European species, present in most countries, and also present in streams and rivers of Palaearctic regions, such as Asian Turkey, Caucasian Russian republics, Georgia, Armenia, Lebanon, Syria, Israel, Jordan, Egypt, Iran, Iraq (Fauna Europaea, 2012).

The biomarker approach in invertebrates has already been demonstrated to be valid as it represents an early warning signal of physiological alterations and provides information on which level of the biotic compartment suffers the toxic effects of the contaminants (Fossi *et al.,* 2001, 2002; Minutoli *et al.,* 2002a, 2002b, 2007, 2008). Biochemical changes include i) the induction of detoxification enzymes, such as those of the hepatopancreatic mixed function oxidase system which are capable of degrading xenobiotics, or ii) the reduction in the activity of enzymes sensitive to inhibition, such as esterases (Callaghan *et al.,* 2002).

The Alcantara river is of great importance in Sicily, being the main source of potable water (about 35 water sources along its length, with a total annual volume for civil use of 9,500,000 m^3) for all the counties situated in the Alcantara valley and for the city of Messina, and being the primary irrigation source for the large surrounding agricultural lands (about 5000 ha of irrigated area).

METHODS

Study area

Located in Eastern Sicily, between the cities of Messina and Catania, the catchment basin of the Alcantara river occupies a valley, wide in some places and narrow in others, flowing finally into the Ionian sea (Fig. 1). The waters have slowly eroded the hard lava rock of the mount Etna, giving rise to considerable landscape vitality with mufflers, rapids and waterfalls, but especially with narrow and deep *gorges*. Second only to the Simeto among the most important rivers in Sicily, the basin has a surface area of about 557 km^2, a length of about 52 km and the basin maximum altitude coincides with the peak of mount Etna (about 3274 m), while the average altitude is about 900 m. The Alcantara river rises south of Floresta in the Nebrodi mountains, at an altitude of 1250

Fig. 1. Study area and sampling sites along Alcantara river in April 2010.

m asl; the mouth, situated between the villages of Giardini Naxos and Calatabiano, is a linear delta formed from fine alluvial gravel.

The Alcantara basin comprises 26 municipalities. The main sources of pollution affecting the river are urban wastes, produced in the valley and flowing in the river, and in most cases the purification plants are not working or are still to be built. Purified wastewaters directly or indirectly entering into the river correspond to a total of 15,700 of the 34,107 inhabitants in the area. Along the river there are point sources of industrial pollution, like the unloading of a processing citrus industry located between Calatabiano and Giardini Naxos (above site 1). Between Francavilla and Castiglione di Sicilia (at sites 2 and 3), there are two paper mills located on the land immediately adjacent to the river bed which directly discharged waste water, without any treatment, in the recent past. Thanks to the Regional Forest Corp's repression activity, this illegal procedure is now prohibited.

Diffuse non-point pollution comes from the use of the land surrounding the river. In this regard, it should be mentioned that the Alcantara river is lined with farmlands in its sections: proceeding from the mouth upstream there are citrus groves, often accompanied by horticultural crops and, on higher hills, even by olive groves; far upstream, between Castiglione di Sicilia and Mojo Alcantara, the citrus groves give way to orchards and, between Mojo and Randazzo, to vineyards (above site 3). Further upstream agricultural activities disappear and give way to pasturable lands and woods along a short stretch in the Nebrodi Park (above site 4). Furthermore, urban areas are generally a source of diffuse pollution for surface waters; in relation to their distance from the riverbed and to related slopes, waters of urban washing can entirely be poured into the river at speeds even higher than from the areas devoted to agriculture or zootechny. In reference to this, not all urban agglomerations in the valley are equally relevant: influence is determined by the dimension of the urban settlement, the position (distance from the riverbed, slopes, etc.), and the nature of soils.

Sampling sites and field activity

Sampling was conducted in April 2010 at four sites along the Alcantara river (Fig. 1):

Site 1: Giardini Naxos (37°48'29.01"N, 5°15'15.25"E);
Min/max depth: 5 cm/1.5 m
Altitude: 10 m
Wet riverbed width: 25 m

Site 2: Francavilla (37°53'31.22"N, 15°8'16.18"E)
Min/max depth: 2 cm/6 m
Altitude: 320 m
Wet riverbed width: 15 m

Site 3: Castiglione di Sicilia (37°53'21.22"N, 15°6'20.73"E)
Min/max depth: 2 cm/0.5 m
Altitude: 430 m
Wet riverbed width: 20 m

Site 4: Randazzo (37°56'59,3" N, 14°55'16,3" E)
Min/max depth: 2 cm/1 m
Altitude: 1062 m
Wet riverbed width: 6 m

At each site, about one hundred macroinvertebrates were collected manually under rocks along the banks were the water depth was 10 cm maximum and the flow velocity near zero. *Serratella ignita* specimens were identified using a stereomicroscope (Leica Wild M10; Leica Geosystems, Heerbrugg, Switzerland) and sorted live from the debris directly in the field. Specimens of the same size from each site were pooled and immediately frozen in liquid nitrogen.

At all sites, environmental parameters [temperature, dissolved oxygen (DO), pH, conductivity, and flow] were measured simultaneously for all invertebrate samplings by a multiparameter probe (YSI 6600 V2-type multiparameter water quality sonde; YSI Inc., Yellow Springs, OH, USA).

Biomarker analysis

Acetylcholinesterase (AChE) activity and benzo(a)pyrene monooxygenase (BPMO) activity, together with total protein content, were evaluated in S. *ignita* larvae from the four sampling sites to show the presence of xenobiotic substances. The activities were evaluated in homogenates obtained from whole organisms. Three independent replicates were carried out for each biomarker, starting each one from a pool of fifteen specimens. The spectrophotometric assay was performed with a Shimadzu UV mini 1240 photometer (Shimadzu, Columbia, MD, USA). The spectrofluorimetric assay was carried out at 30°C using a Perkin Elmer LS 50B luminescence spectrometer (PerkinElmer, Waltham, MA, USA). Each activity was read three times or more if the obtained values were dissimilar, and then averaged.

Acetylcholinesterase activity was determined using the method by Westlake *et al.* (1981), modified by Fossi *et al.* (2001), *i.e.* using 10 μL of sample for enzyme readings. The absorbance change for each sample and its double, at 410 nm for 3 min, was registered and directly converted into enzymatic activity using a factor of 44.1, and expressed as μmoles/min/g larvae.

Benzo(a)pyrene monooxygenase activity was measured by following the method by Kurelec *et al.* (1977): 100 μL of sample was used as source of enzymes and the reaction mixture was incubated for 1 h. The excitation and emission wavelengths were 396 and 522 nm, respectively. The reading order was: H_2SO_4 (autozero), quinine sulfate (100% of emission), blank of the sample, sample, double sample. The final enzymatic activity was expressed as arbitrary fluorescence units/mg proteins/h (F.U./mg prot/h). Protein content was quantified by the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) into 200 µL of sample. The homogenate of larvae was previously diluted 1:10 with TRITON 0.02%. The readings were done at 595 nm.

The results obtained were statistically analysed with a one-way ANOVA, in order to determine if the values along the river were significantly different ($P<0.05$). Tukey's *post hoc* comparison test (at a=0.05) was also carried out to determine in which station(s) the investigated biomarkers were significantly different.

RESULTS

The lowest temperature was detected at site 4, whereas the highest value was recorded at site 3 (Tab. 1). Dissolved oxygen concentration varied from a maximum of 11.60 mg L^{-1} at site 3 to a minimum of 10.59 mg L^{-1} at site 2. pH was always basic. Conductivity showed an increasing trend from site 4 to site 1.

Mean biomarker values for AChE and BPMO measured in specimens from the four sampling sites, and their standard deviations, are shown in Fig. 2.

Differences among the sampling sites were found. With regard to esterases, the highest inhibition of AChE activity was shown in the samples from site 4 Randazzo, followed in descending order by site 3 Castiglione di Sicilia, site 1 Giardini Naxos and site 2 Francavilla. The analysis of variance and the Tukey's *post hoc* comparison test revealed a significant difference among all the sites (ANOVA; $F_{3,8}=41.21$, $P<0.05$; Tukey test $P<0.05$, site 4>site 3>site 2 >site 1).

Benzo(a)pyrene monooxygenase activity was higher in samples from site 1 Giardini Naxos, followed in descending order by site 4 Randazzo, site 2 Francavilla and site 3 Castiglione di Sicilia. Also in this case, the analysis of variance and Tukey's *post hoc* comparison test revealed a significant difference among all the sampled sites, the smallest only between site 2 and 3 (ANOVA; $F_{3,8}=449.20$, $P<0.05$; Tukey test $P<0.05$, site 4>site 3>site 2 >site 1).

Both AChE and BPMO were not linked to temperature values, which can influence enzymatic activity. In fact, the samples from site 4 – with the lowest temperature –, and from site 3 – with the highest temperature – did not show the lowest and highest activities, respectively.

DISCUSSION

The results obtained suggest that the stretches of the Alcantara river that are more contaminated by neurotoxic substances, such as organophosphate insecticides (OPs), carbammates (CBs) and heavy metals, are in correspondence to Randazzo and Castiglione di Sicilia (high and

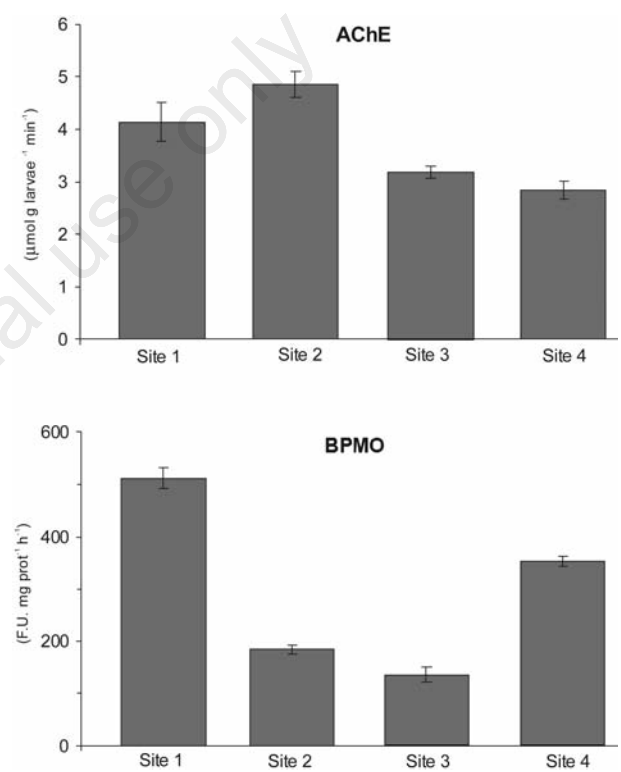

Fig. 2. Biomarker mean values of Acetylcholinesterase (AChE) activity and benzo(a)pyrene monooxygenase (BPMO), evaluated in *Serratella ignita* larvae collected in the four sampling sites along Alcantara river. Three replicates were done for each site. Standard deviation is also shown.

Tab. 1. Physico-chemical parameters at each sampling site along Alcantara river.

Site	Temperature (°C)	DO (mg L^{-1})	pH	Conductivity (µS cm^{-1})
1	15.39	10.59	8.86	850
2	14.22	10.61	8.17	810
3	15.86	11.60	8.73	620
4	10.01	11.14	8.90	370

DO, dissolved oxygen.

medium river), where in fact agricultural activities take place, with high production of fruit, olives, citrus and the highest employment of fertilisers and pesticides in all of the Alcantara valley. Acetylcholinesterase activity is the primary target of xenobiotic neurotoxic pesticides designed to control invertebrate pests (Hassall, 1990). Inhibition resulting from irreversible binding at the AChE active site leads to the accumulation of acetylcholine in the synapse, resulting in the disruption of normal function. The measurement of AChE activity to detect inhibition resulting from pesticide binding has been used successfully in several studies (Crane *et al.*, 1995; Ibrahim *et al.,* 1998; Berra *et al.,* 2004), as well as for heavy metals (Tsangaris *et al*., 2007; Richetti *et al*., 2011).

As for xenobiotic lipophilic compounds, such as polycyclic aromatic hydrocarbons (PAHs) and polyhalogenated aromatic hydrocarbons (PHAHs), the results obtained suggest that the stretch of the Alcantara river that most contaminated is represented by the river mouth. This area is certainly the most urbanised along the river, with the highest entry of water runoff of streets, and the presence of the unloading of the citrus industry located between Calatabiano and Giardini Naxos. A lower but still high induction, compared to sites 2 and 3, of the BPMO activity was measured in site 4 Randazzo, thus unexpectedly suggesting such a contamination in an area close to the wellspring. A reason for this is that Randazzo is the largest village of the Alcantara valley and is located along the right bank on a high ridge. Because of its location and the permeability of lava rock, it produces the highest input of water runoff of the streets, sewers and small urban leachate.

Biomarkers employing enzyme activity measurements to detect sublethal levels of pollution are increasingly used in ecological risk assessments of aquatic ecosystems (Berra *et al.,* 2004).

CONCLUSIONS

Results admit the possibility of using biomarkers in macroinvertebrate larvae to study the Alcantara river *health status.* Its water quality appears to be affected by contamination mainly from waste discharges and large pasture areas. Data reported in the present work represent the first attempt to measure the levels of two widely recognised enzymatic activities, AChE and BPMO, in *Serratella* larvae from this river. A series of experiments is being running to test a large suite of biomarkers in these macroinvertebrates. The ultimate purpose is to seasonally monitor the river water quality throughout the year, considering that population in this freshwater ecosystem increases during summer.

Considering the wide European and world-wide distribution of *Serratella ignita*, the biomarker approach used in this study to assess water quality is applicable not only to the Alcantara river, but to any lotic ecosystem.

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