

RESEARCH ARTICLE

First application of biomarkers approach in the zooplanktonic copepod *Acartia latisetosa* for the early management and conservation of transitional waters ecosystems

R. Minutoli¹, M.C. Fossi², G. Zagami¹, A. Granata¹ and L. Guglielmo¹

¹Department of Animal Biology and Marine Ecology, University of Messina, Italy

²Department of Environmental Science, University of Siena, Italy

Corresponding author: Dr Roberta Minutoli, Department Animal Biology and Marine Ecology, University of Messina Salita Sperone, 31 S. Agata 98166 – Messina, Italy;

Tel: +39-90-6765536, Fax: +39-90-393409, e-mail: rminutoli@unime.it

Abstract

- 1 - A very new and useful strategy for ecotoxicological studies is the application of biomarker techniques in zooplanktonic bioindicators. In fact an evidence of alteration at this level of the food chain, first or second, can be used as an early warning sign of risk to the health of an entire TW ecosystem, enabling local authorities to intervene promptly to avoid bioaccumulation and biomagnification phenomena. Ecological characteristics of this kind of environments are low biodiversity and peculiar spatial and seasonal distribution with high abundance peaks. These are favourable conditions in accordance with the biomarkers test protocols. Specific biomarkers of exposure and/or effects can be used giving informations about the kind of contaminant involved.
- 2 - The zooplanktonic organisms with largest distribution in all the TW environments are the copepods, so, in the field, it is possible to use a suite of biomarkers in a copepod species collected in a study area and in a reference site, not polluted, in order to evaluate its environmental “health status”.
- 3 - The aim of this study was to propose a suite of biomarkers (BPMO activity, EROD activity, NADPH-cytochrome C reductase, NADH-cytochrome C reductase, NADH-ferricyanide reductase, total proteins, esterases, porphyrins) and residue concentration (heavy metals) in the zooplanktonic copepod *Acartia latisetosa*, as a multi-disciplinary diagnostic tool for assessment of the health status of a TW environment in which the species was sampled. This was Lago Faro (Messina, Italy), a lake suspected of being polluted, respect to Lago Verde, a small brackish lake of a natural marine reserve (Marinello, Messina, Italy).
- 4 - The results show inductions of MFO activity (EROD, NADH-Cytochrome C reductase, NADH-ferricyanide reductase) and inhibition of acetylcholinesterase activity in *A. latisetosa* sampled in Lago Faro, suggesting contamination with xenobiotic lipophilic compounds and neurotoxic substances. But, in contrast with what was expected, samples collected in the reference lake were high in porphyrins and heavy metals.
- 5 - The results show and confirm that biomarkers in zooplankton species can be used to study early the “health status” of different TW environments.

Keywords: biomarkers, environmental monitoring, copepods, *Acartia latisetosa*, transitional water environments, Faro Lake, South Italy.

Introductions

One of the main topic of ecotoxicological researches is the evaluation of exposure of biological communities to contaminants and the effect of these compounds on them. To tackle this problem, the modern environmental toxicology has gradually combined biomonitoring studies, based on the evaluation of residue concentration in the environment and in the bioindicator organisms, with a new approach based on evaluation of the responses that an organism, population or natural community gives to chemical stressors in their environment. These responses, defined as biomarkers, constitute an integrated signal of the level of contamination in an environment, and are therefore also indicators of toxicological risk to which a natural population is exposed (Bayne *et al.* 1985, McCarthy & Shugart, 1990, Van der Oost *et al.*, 2003). A biomarker is defined as "a change induced by a contaminant in the biochemical or cellular components of a process, structure or function, that can be measured in a biological system" (NRC 1989). This change provides qualitative and semiquantitative information on the nature of chemical insult, and information on the relation between biological effects and levels of environmental contamination. Biomarkers evaluation in bioindicator organisms, sampled in a TW environment suspected of contamination and compared with organisms of the same species collected in a reference area, enables the potential danger to a community to be assessed (Depledge, 1989, McCarthy & Shugart, 1990, Fossi, 1991; Fossi *et al.*, 1998). The advantages of this approach with respect to conventional biomonitoring methods are as follows:

- biomarkers provide an integrated response to the overall exposure of the bioindicator species, including the sum of all the different intake routes and of exposure in time, within a given geographical area;

- biomarkers provide an integrated response to all the toxicological and pharmacological interactions of the mixture of compounds to which a sentinel organism is exposed.
- biomarkers provide an immediate response to exposure to a toxic substance.

Many studies have been published until now on this topic (Fossi *et al.*, 1997; Fossi *et al.*, 1998; Stronkhorst *et al.*, 2003; Pérez *et al.*, 2004; Behrens *et al.*, 2005), but none has been concerned with zooplankton.

It must be underlined that this work follows other studies in which the possible application, conceptual and methodological, of biomarkers in zooplankton was validated (Fossi *et al.*, 2001; Minutoli *et al.*, 2002a, 2002b; Minutoli *et al.*, 2004; Minutoli *et al.*, 2007; Fossi *et al.*, 2002). Zooplankton is an essential component of all the TW environments and an essential link in all the food chains. So the ecotoxicological risk to zooplankton, evaluated by the biomarker approach, can be used as an early signal of threats to ecosystem health. It is in fact useful to show effects at a so low level of the food chain, in order to prevent bioaccumulation and biomagnification phenomena through higher trophic levels.

Furthermore the ecological peculiarity of TW environments are a low biodiversity and a specific spatial and seasonal distribution with high abundance peaks. These are favourable conditions for the biomarkers test protocols, because there is the necessity of monospecific samples, live organisms and of about 200-300 mg for any biomarker tested. It will be, so, more easy inside zooplanktonic samples from TW areas, to sort under the stereomicroscope the live organisms of a selected species that is present with a monospecificity of almost the 90%.

After the validation of the methodological protocols (Fossi *et al.*, 2001; Minutoli *et al.*, 2002a, 2002b; Minutoli *et al.*, 2004; Minutoli *et al.*, 2007; Fossi *et al.*, 2002),

this paper represents the first application of biomarkers in zooplankton, to evaluate the environmental “health status” of a selected ecosystem respect a reference area in which the same species was collected.

Material and Methods

Study areas and species

A suite of biomarkers was investigated in a zooplanktonic crustacean, the copepod *Acartia latisetosa*. This herbivorous species reaches a maximum size of about 0.92 mm and is typical of confined environments (Mauchline, 1998). It was sampled in two lakes, the study area and the reference one. The investigated environment was Lago Faro (Messina, South Italy), selected because suspected of being polluted, being in the centre of a village with many inhabitants, specially during the summer, in the northeastern Sicily near Capo Peloro (Fig. 1). It has a peculiar

Ganzirri by a channel, to the Tyrrhenian Sea by another channel which remains closed, and to the Strait of Messina by a further channel which does not always ensure good water exchanges. Poor exchanges lead to modifications in environmental parameters and ecological factors in the biotope, reflected by low species diversity and various euryvalent species.

Samplings were carried out during the 13th, 19th and 26th of July 2001 (respectively samples named F1, F2, F3) during the density peaks of the species, using a zooplankton net with 180 µm mesh size and 30 cm mouth.

In Lago Faro, *A. latisetosa* shows spatial and temporal segregation with seasonal density peaks in few sample stations (Zagami & Guglielmo, 1995), where we have collected the zooplanktonic material. For any sampling, ten trawls lasting 10-15 minutes each were performed. All the

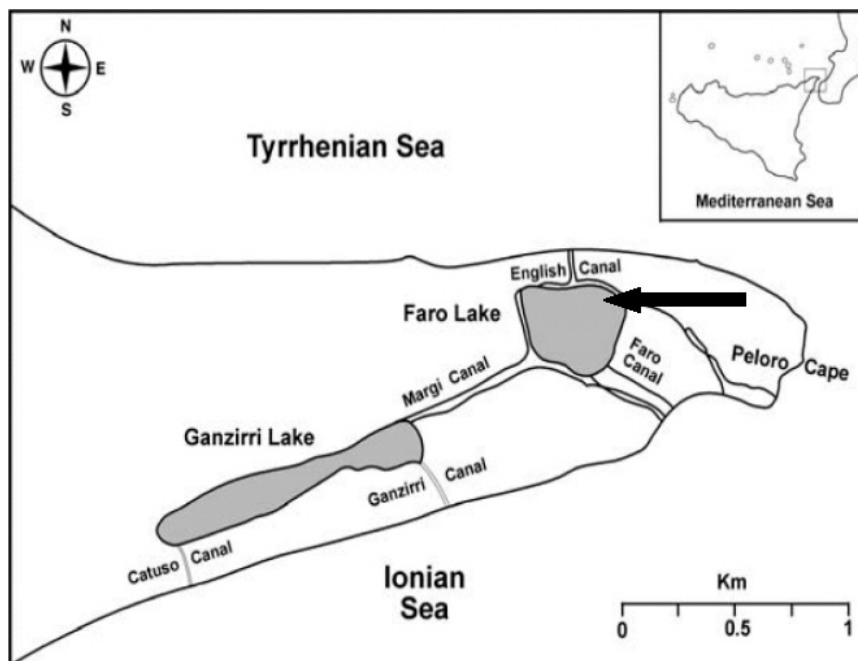


Figure 1. Study area, Lago Faro, in the northeastern Sicily, Italy.

funnel shape with a mean depth of 2 m and a maximum of 28 meters only in the centre. It has an area of 263,600 m², a major NW-SE axis of 661 m and hosts mollusc farms. Lago Faro is connected to the next Lago

samples, were brought alive, refrigerated and oxygenated, to the laboratory. An aliquot of each was always fixed in 4% neutralized formalin for qualitative analysis of the taxa present, to ensure that the sample showed at

least 90% of monospecificity, and so useful for the biomarker analyses. All the alive sample was anyway every time observed at the stereomicroscope in order to discard the organisms of other species present, using a pipette. All the 3 samples collected were always frozen at -80°C . For the analyses, 3 subsamples of about 500 mg of wet weight each one, from any samples, were used.

The reference environment selected was Lago Verde, a small brackish lake in a nature reserve (Marinello, Messina, South Italy). It has a mean depth of 1.60 m and maximum of 3 m (Crisafi *et al.*, 1981). Species segregation occurs in this environment and the only zooplanktonic species is *A. latisetosa*. Samplings were carried out on the 15th, 20th and 30th of July 2001 (respectively samples named V1, V2, V3), using a zooplankton net with 180 μm mesh size and 30 cm mouth. Three trawls were performed for any sampling, each lasting 30 min, more time than in Lago Faro because of the lower zooplanktonic biomass. Also these 3 samples were brought alive, refrigerated and oxygenated, to the laboratory, where they were treated as those from Lago Faro. Then for the analyses, also in this case, 3 subsamples of about 500 mg of wet weight each one, from any samples were used.

Biomarkers and trace element analyses

Benzo(a)pyrene monooxygenase (BPMO) activity, 7-ethoxyresorufin-O-deethylase (EROD) activity, NADPH-cytochrome C reductase, NADH-cytochrome C reductase, NADH-ferricyanide reductase, total proteins, Acetylcholinesterase activity (AChE), level of Coproporphyrin, Uroporphyrin and Protoporphyrin, total Porphyrins, heavy metals (As, Hg, Cd, Pb) were evaluated in the 3 monospecific samples of *A. latisetosa* from Lago Faro and 3 monospecific samples of *A. latisetosa* from Lago Verde.

All the biomarkers activities and residue concentrations were evaluated in homogenates

from monospecific pools of whole organisms. The spectrophotometric assays were performed with a Shimadzu UV mini 1240 photometer. The spectrofluorimetric assays were carried using a Perkin Elmer LS 50B luminescence spectrometer.

BPMO activity was measured by the method of Kurelec *et al.* (1977), using 100 μl of sample as source of enzymes and incubating the reaction mixture for 1 hour. EROD activity was measured by the method of Lubet *et al.* (1985).

NADPH cytochrome C reductase, NADH cytochrome C reductase and NADH ferricyanide reductase were assayed by the method of Livingstone and Farrar (1984). All tests were carried out at 30°C .

Acetylcholinesterase (AChE) activity was determined by the method of Westlake *et al.* (1981) modified by Fossi *et al.* (2001). Spectrofluorimetric assay was carried at 30°C . From 2.5 to 20 μl of sample was used for enzyme readings in order to check for linearity between the enzyme activity and sample concentration.

Porphyrim concentrations were determined in pools of whole organisms; 0.4 ml of homogenate in water was spiked with 1.6 ml of 50:50 methanol/perchloric acid mixture. After 20 potter-mixing and 4 vortex-mixing, the samples were stored in the dark for 10 minutes and then centrifuged for 5 minutes at a low speed. The porphyrin extract in the upper layer was used for spectrofluorimetric separation. Quantitative determination of porphyrins was performed by the method of Grandchamp *et al.* (1980).

For the trace element analysis, freeze-drying organisms were digested with HNO_3 in a teflon bomb (Stoeppler and Backhaus, 1978). Levels of metals were determined by atomic absorption spectrometry with a transverse heated graphite furnace equipped with Zeeman background correction (Pb, Cd), flow injection mercury system (Hg) and a combination between flow injection Fias

and atomic absorption spectrometry with a transverse heated graphite furnace (As).

All the results obtained were statistically analysed with a parametric test, the Student t- test, in order to analyse if the means (F and V) showed significant differences between them.

Results

Table 1 shows the mean biomarker values obtained for the 3 samplings of *A. latisetosa* carried out in Lago Faro (F) and in Lago Verde (V) during July 2001. Table 2 shows trace element concentration mean values for

the 3 samplings of *A. latisetosa* carried out in Lago Faro (F) and in Lago Verde (V) in the same month. The Standard Deviations was calculated for any mean.

Very little difference in BPMD activity was detected between organisms of the two lakes.

EROD activity was very little higher in Lago Faro samples (0.091 pmol res/min/mg prot versus 0.041 of the Lago Verde sample).

Greater differences between sites were found for reductase activities, except NADPH-cytochrome c reductase. NADH-cytochrome C reductase and NADH-ferricyanide reductase

Table 1: Biomarker mean values, evaluated in *Acartia latisetosa* samples from Lago Faro (F) e Lago Verde (V), Standard Deviation, Student t-test performed on biomarker means data sets (**:p <0.01; *: p <0.05).

BIOMARKERS EVALUATED	F	± S.D.	V	± S.D.	t- value
BPMD (U.A.F./mg prot/h)	2.4	0.34	2.41	0.19	0.029 n.s.
EROD (pmol res/min/mg prot)	0.091	0.0	0.041	0.02	1.952 n.s.
NADPH- cytochrome C reductase (nmol/mg prot/min)	4.7	0.82	4.63	0.11	0.153 n.s.
NADH- cytochrome C reductase (nmol/mg prot/min)	4.49	0.49	2.4	0.18	6.918**
NADH- ferricyanide reductase (nmol/mg prot/min)	185.3	14.35	136.4	7.64	5.209**
AChE (umol/min/g zoop)	14.22	3.41	20.68	1.73	2.921*
Uroporphyrin (pmol/g zoop)	27	12.69	22.5	1.12	0.611 n.s.
Coproporphyrin (pmol/g zoop)	23.4	7.79	22.5	0.56	0.199 n.s.
Protoporphyrin (pmol/g zoop)	17.1	12.73	60.3	10.13	4.276*
Total Porphyrins (pmol/g zoop)	61.8	6.63	105.3	7.87	7.321**

Table 2. Heavy metal residues mean levels evaluated in *Acartia latisetosa* samples from Lago Faro (F) e Lago Verde (V), Standard Deviation, Student t-test performed on residues means data sets (**:p <0.01; *: p <0.05), (fw: fresh weight; nd: not detectable).

Heavy metal evaluated	F	± S.D.	V	± S.D.	t- value
As ppm fw	0.8	0.1	1.70	0.36	4.166 **
Hg ppm fw	0.21	0.02	0.30	0.03	3.714*
Cd ppm fw	0.05	0.02	0.09	0.03	1.621 n.s.
Pb ppm fw	nd		nd		

were higher in F mean value than V.

With regard to esterases, high inhibition of AChE activity (31.24%) was found in sample from Lago Faro (activity 14.22 mol/min/g zoop versus V 20.68).

Total porphyrins were higher in reference sample than from Lago Faro, and in particular the protoporphyrin.

Residue analysis (Table 2) showed higher concentrations of As, Hg and Cd in reference TW area samples of *A. latisetosa*.

The statistical test (Tab.1, Tab.2) applied to biomarker and heavy metal residues results showed significant differences (** $p < 0.01$; * $p < 0.05$), by Student t-test, for NADH-cytochrome C reductase, NADH-ferricyanide reductase, AChE, protoporphyrin, total porphyrins tests, and for Arsenicum and Mercury levels, to be considered as differences in ecotoxicological status between the two environments.

Discussions and Conclusions

The biomarker approach in the study of the environmental health status was applied until today in many species, vertebrates and invertebrates (Depledge, 1989, McCarthy & Shugart, 1990, Fossi, 1991; Fossi *et al.*, 1997; Fossi *et al.*, 1998; Stronkhorst *et al.*, 2003; Pérez *et al.*, 2004; Behrens *et al.*, 2005). With this work, a suite of biomarkers was evaluated for the first time in a zooplanktonic species, a crustacean of about 1 mm, and the applicative methodologies modified for this kind of organisms, were validated.

There were verified the applicative methodologies for the followed tests: Benzo(a)pyrene monooxygenase (BPMO) activity, 7-ethoxyresorufin-O-deethylase (EROD) activity, NADPH-cytochrome C reductase, NADH-cytochrome C reductase, NADH-ferricyanide reductase, total proteins, Acetylcholinesterase (AChE) activity, porphyrins and heavy metals.

The possible use of zooplanktonic species for the ecotoxicological assesment of a TW

area and for the comparison of different environments was evidenced.

In this study, the induction of Mixed Fuction Oxidases (MFO) activity (in particular of EROD activity, NADH-cytochrome C reductase, NADH-ferricyanide reductase) in *A. latisetosa* collected from Lago Faro, suggests that this brackish lake is contaminated by xenobiotic lipophilic compounds, as polycyclic aromatic hydrocarbons (PAHs), polyhalogenated aromatic hydrocarbons (PHAHs). In fact the basal activity of the MFO in the hepatopancreas is enhanced when the organism is exposed to this class of contaminants, in order to metabolize them. In this study, only the BPMO activity did not show at all differences between the two mean values from the two sites, maybe because this monooxygenase is highly degradable respect to the others.

The inhibition of AChE activity in *A. latisetosa* from Lago Faro can be explained with a contamination by neurotoxic substances, as organophosphate insecticides (OPs) and carbammates (CBs), that can modify the activity of the enzyme in the nervous system. These result maybe is due to the position of the lake. It is in fact in the centre of a village, Faro, in which there are many inhabitants with their anthropic activities, such as the farming of plants, flowers, vegetables, vineyards, with the employment of many pesticides.

With regard to porphyrins, elevated levels in this copepod from Lago Faro was expected, being suspected to be polluted, but higher values were found in the samples from the reference area, particularly the protoporphyrin. These intermediate products of the EME synthesis are accumulated in the liver of an organism for the exposition to polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzene hexachloro (HCB) or heavy metals as Lead (Pb), Arsenicum (As) and Mercury

(Hg) (Hugget *et al.*, 1992; Fossi & Leonzio, 1994). This result can be explained with the potential presence of metal objects on the bottom of Lago Verde in Marinello or more probably with a subterranean percolation of water from an adjacent, only few meters, small lake in which there is an ancient metal wreck. Furthermore, it is known that the protoporphyrin, that showed an higher value in the sample from the reference selected TW environment, is accumulated for exposition to heavy metals.

The trace element concentrations (As, Hg, Cd) confirm these results and maybe this hypothesis, with higher values in *A. latisetosa* from Lago Verde than Lago Faro.

At this step we can not compare our values with others, because the absence of papers about biomarkers applied to zooplankton in field.

The ecotoxicological results obtained, allow us to reflect about some ecological potential consequences. The ecotoxicological status of *A. latisetosa* in Lago Faro, evidenced

by the biomarker approach, can lead to ecological changes in its population, in all the food chain and in all this ecosystem. The biochemical alterations can involve many variations in the life cycle of *A. latisetosa*, even the disappearance of the species and the consequent alteration in the food web, and the ecotoxicological damages can be amplified for the biomagnification through the trophic levels.

In conclusion, the results demonstrate that this multi-disciplinary ecotoxicological approach, used from the 80s in many environmental situations and applied in vertebrates (fishes, birds, reptiles, etc.) and invertebrates as crabs, can be used also in zooplankton for TW environmental monitoring, not at all it can be an early indicator of alterations. In that way, carrying out a biomonitoring programme, early informations about the ecotoxicological status of a studied environment can be given to the local authorities, in order to intervene and to take administrative and legislative measures needed to save an entire TW environment.

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