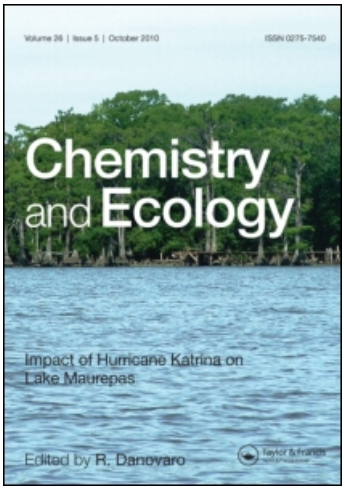

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R. Minutoli[a]; M. C. Fossi[b]; A. Granata[a]; S. Casini[b]; L. Guglielmo[a]
[a] Department of Animal Biology and Marine Ecology, University of Messina, Messina, Italy [b] Department of Environmental Sciences, University of Siena, Siena, Italy

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Use of biomarkers in zooplankton for assessment of the 'health status' of marine and brackish environments: a short overview

R. MINUTOLI*†, M. C. FOSSI‡, A. GRANATA†, S. CASINI‡ and L. GUGLIELMO†

†Department of Animal Biology and Marine Ecology, University of Messina, Salita Sperone 31, 98166 S. Agata, Messina, Italy
‡Department of Environmental Sciences, University of Siena, Via Mattioli 4, 53100 Siena, Italy

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The biomarker approach has been used for 25 years to study the environmental quality of marine, brackish and freshwater ecosystems. Biomarkers may indicate *health status* and can be applied to organisms of all zoological phyla by destructive or non destructive methods. For 5 years we have been using this approach in zooplankton to detect ecotoxicological alterations at low levels of the food chain due to contaminants. Here we review our approach to validate and apply biomarker techniques in zooplankton. We discuss advantages, limitations, some results and future research. We indicate that biomarkers in zooplankton can be used as new indices of trophic status and ecological integrity of Italian marine coastal and lagoon environments, to be included among the tools specified by Italian law D.Lgs. 152/2006.

Keywords: Environmental health status; Biomarkers; Zooplankton; Marine coastal environment; Lagoon

1. Introduction

One applied ecotoxicological approach to determining the health status of marine, brackish or freshwater environments is evaluation *in situ* of the toxic effects of a contaminant or a mixture of contaminants on an ecological community. At the beginning of the 1980s, the international scientific community started to reflect on the possibility of using responses of bioindicator organisms to contamination as diagnostic and prognostic tools for assessing environmental quality [1–3]. This practical modern approach considers the real effect that a toxic substance has on a biotic compartment. Environmental chemical analysis and conventional toxicity tests are often unsuitable for biomonitoring programs. Past environmental studies were based on environmental chemistry (evaluation of contaminant levels in abiotic compartments, like water or sediment), classical toxicology (laboratory toxicity tests on specific species to demonstrate the effect of specific contaminants) or bioindication (presence/absence of a species in an environment as an indicator of environmental quality). However, these methods

*Corresponding author. Email: rminutoli@unime.it

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do not consider interactions between different contaminants and between contaminants and chemical or physical parameters that may modify the toxicity of a substance, nor do they consider the effects of a toxic substance on species at different developmental stages.

Thus the biomarker approach, often accompanied by conventional methods, has been used by the scientific community to study the effects of contaminants or mixtures of contaminants on organisms *in situ* for the last 25 years. The novelty of the biomarker approach is that it is based on the concept that the effects of a contaminant are felt at different levels of structural complexity [20]. The aim of environmental monitoring using biomarkers is to identify (diagnosis) and predict (prognosis) early signs of exposure or effect through the study of immediate responses [4]. The study of *environmental health status* starts with analysis of *early adverse effects* as biological markers. The modern biomarker approach has been used to demonstrate toxic effects on natural marine, brackish and freshwater communities [5–8]. The role of biomarkers in ecotoxicology is not to provide quantitative information on levels of exposure of an organism to a contaminant, but to provide indications of alterations.

Many studies have been published on the application of destructive and non-destructive biomarkers to invertebrates, fishes, reptiles, birds and marine mammals, especially top predator species, but not to zooplankton [1, 5, 6, 8–10, 21]. Indeed, it seems unlikely that herbivorous or primary carnivorous organisms would show ecotoxicological effects of exposure to xenobiotic compounds. Zooplankton have much shorter biological cycles than, for example, the fishes that feed on them, and do not show biomagnification phenomena, being at the start point of the food chain. Papers on the application of biomarkers to crustaceans have only considered crabs, like *Carcinus aestuarii* [6], and never zooplankton crustaceans.

The aim of our studies has been to apply biomarkers to zooplankton, since evaluation of ecotoxicological risk at this level of the food chain can be used as an early warning sign of risk to the health of the marine, brackish or freshwater ecosystem to which they belong. Alterations at the first or second levels of the food chain could provide early notice of contamination, enabling local authorities to intervene promptly to avoid biomagnification and consequences at higher levels.

This paper reviews the validation of this approach, reports some results and suggests biomarkers in zooplankton as new indices of trophic status and ecological integrity of Italian marine coastal and lagoon environments, for inclusion in the list of tools specified in Italian law D.Lgs. 152/2006 before 2008.

2. Methods, results and discussion

We first focused on the advantages and limitations of using zooplankton as bioindicators of ecotoxicological health [11]. The advantages that prompted our research were: (i) zooplankton is a major component and plays a fundamental role in all marine, brackish and freshwater ecosystems; (ii) zooplankton is a basic link in the food chain, evidence of alteration of which could enable consequences at higher levels to be avoided; (iii) bioaccumulation in zooplankton seems unlikely because they are at the bottom of the food chain; we were curious to know whether they are affected by exposure to xenobiotic compounds; (iv) zooplankton forms very large populations, so sampling does not modify population dynamics; (v) nearly all zooplankton species have narrow distributions, so they can provide information about the health status of a limited area.

On the other hand, there are practical difficulties related to the small size of zooplankton organisms. We therefore developed methodological protocols for organisms only a few millimeters in size.

To test a biomarker, more than 100 mg of material of a single species is needed. Different species, even of the same genus, have different biochemical characteristics. Monospecificity of at least 90% in samples is therefore essential. The small size of the organisms does not permit particular organs or biological material to be obtained selectively, but this was obviated by standardizing large samples of zooplankton and using monospecific pools of whole live organisms. Because it is necessary to sort live samples, the latter cannot be collected in environments with high biodiversity. The zooplankton approach is therefore best applied to confined environments, such as coastal marine areas, lagoons and brackish lakes, where one species abounds at specific sample stations in certain seasons. Once we had obviated these sampling difficulties, we adapted existing analytical protocols for biomarker measurements to these new organisms.

So far we have tested, modified and validated the following biomarkers in zooplankton (table 1) [2, 11–14]: acetylcholinesterase activity, mixed function oxidase activities ((7-ethoxyresorufin-O-deethylase (EROD), benzo(a)pyrene monooxygenase (BPMO), NADPH-cytochrome C reductase, NADH-cytochrome C reductase, NADH-ferricyanide reductase), porphyrin concentrations (coproporphyrin, uroporphyrin, protoporphyrin, total porphyrins), vitellogenin and zona radiata proteins (Vtg and Zrp); and residue concentrations of organochlorines, polycyclic aromatic hydrocarbons (PAHs) and heavy metals.

Spectrophotometric assays were performed with a Shimatzu UV mini 1240 photometer. Spectrofluorimetric assays were done with a Perkin Elmer LS 50B luminescence spectrometer. Acetylcholinesterase (AChE) activity was determined by the method of Westlake *et al.* [8] modified by Fossi *et al.* [11]. Spectrofluorimetric assay was carried at 30 °C. From 2.5 to 20 µl of sample was used for enzyme readings to check for linearity of enzyme activity in relation to sample concentration. BPMO activity was measured by the method of Kurelec *et al.* [15], using 100 µl of sample as enzyme source and incubating the reaction mixture for 1 hour. EROD activity was measured by the method of Lubet *et al.* [16]. NADPH cytochrome C reductase, NADH cytochrome C reductase and NADH ferricyanide reductase were assayed by the method of Livingstone and Farrar [1]. All tests were carried out at 30 °C.

For assay of porphyrin concentration, 0.4 ml of homogenate in water was spiked with 1.6 ml of 50:50 methanol/perchloric acid mixture. The porphyrin extract in the upper layer was used for spectrofluorimetric separation. Quantitative determination of porphyrins was performed according to Grandchamp *et al.* [17].

Table 1. Biomarkers and residue levels evaluated in zooplanktonic samples.

Biomarkers
Benzo(a)pyrene monooxygenase (BPMO)
7-ethoxyresorufin-O-deethylase (EROD)
NADPH-cytochrome C reductase
NADH-cytochrome C reductase
NADH-ferricyanide reductase
Acetylcholinesterase activity (AChE)
Coproporphyrin
Uroporphyrin
Protoporphyrins
Total Porphyrins
Vitellogenin (Vte)
Zona radiata proteins (Zrp)
Residue levels
Organochlorines (OCs)
Polycyclic aromatic hydrocarbons (PAHs)
Heavy metals As, Hg, Cd, Pb, Zn, Fe, Cu, Mn

Immunochemical analysis of Vtg and Zrp was performed in homogenate of whole organisms by indirect ELISA according to Goksoyr [18]. A 96-well microplate was used and each sample was tested in triplicate, adding 10 μg of protein to each well. Dilution of primary antibodies was 1:1000 for anti-Vtg (PO-2) and 1:3000 for anti-Zrp (O-173). The results were expressed as adsorbance at 492 nm.

Freeze-dried samples were extracted in a Soxhlet apparatus for analysis of chlorinated hydrocarbons (OCs) and PAHs. Extraction of OCs was carried out by high resolution capillary gas chromatography with a Perkin-Elmer Series 8700 GC and a 63Ni ECD, according to Marsili [7], revealing op'- and pp'-isomers of DDT and its derivatives DDD and DDE, and about 30 PCB congeners. PAHs were analysed with an HPLC/fluorescence system. Extraction was carried out according to Holoubek *et al.* [19] with some modifications [7]. Lyophilized organisms were digested with HNO_3 in a teflon bomb according to Stoeppler and Backhaus [19]. Levels of metals were determined by atomic emission ICP/AES (Zn, Fe, Cu, Mn), atomic absorption spectrometry with transverse heated graphite furnace and Zeeman background correction (Pb, Cd), flow injection Mercury system (Hg) techniques and a combination of flow injection Fias and atomic absorption spectrometry with transverse heated graphite furnace (As).

These new studies of biomarkers in zooplankton have so far been carried out in the following zooplankton crustacean species [2, 11–14] (Table 2): copepods *Acartia margalefi* and *Acartia latisetosa* collected in brackish Lake Ganzirri (Messina, Italy); mysid *Siriella clausi* collected in brackish Lake Faro (Messina, Italy); mysids *Diamysis bahirensis*, *Siriella armata* and *Mysidopsis gibbosa*, collected in the lagoon of Stagnone di Marsala (Palermo, Italy); krill *Euphausia crystallorophias* and *Euphausia superba* collected during the 2000–2001 Antarctic expedition; amphipod *Streetsia challengeri* and euphausiid *Meganicthyphanes norvegica* – stranded specimens from the Ionian coast of Messina.

The first analyses were done selecting a specific biomarker of effect of neurotoxic substances, such as OPs and carbamates, acetylcholinesterase activity. The data obtained was compared internally and with data obtained with two species of benthic decapods, *Eriphia verrucosa* and *Pachygrapsus marmoratus* [2, 11, 13], used for comparison because they are from two orders used in coastal marine biomarker studies to determine whether zooplankton organisms had assayable activities and whether they could be used for future research. All these tests showed high activity of the biomarker with respect to the reference species. For example mean AChE activities in the various pools of the species were: 10.05 μmol/min/g for *A. margalefi*; 3.30 for *A. latisetosa*; 79.70 for *S. clausi*; 49.97 for *D. bahirensis*; 7.48 for *S. armata*; 14.20 for *M. gibbosa*; 4.49 for *E. crystallorophias*; 1.66 for *E. superba*; 2.74 for *S. challengeri*; 13.26 for *M. norvegica*. The two crab species showed the following activities: 5.78 for *E. verrucosa* and 6.38 for *P. marmoratus*, often lower than the much smaller zooplankton. All the species used for the AChE experiment also showed a linear increase in enzyme activity with increasing concentration of the samples from 2.5 to 5, 10 and 20 μl.

A preliminary study was carried out, proposing a suite of biomarkers (BPMO activity, NADPH-cytocrome C reductase, NADH-ferricyanide reductase, esterases, porphyrins, vitellogenin and zona radiata proteins) and residue levels (organochlorines, PAHs and heavy metals) in the euphausiid *M. norvegica*, for ecotoxicological study of the Mediterranean whale sanctuary [12]. Very little difference in BPMO was detected between sites, with values ranging from 0.75 to 2.68 U.A.F./mg prot/h. Larger differences between sites were found for reductase activities. Esterases (AChE), porphyrins (copro-, uro-, proto-porphyrins), vitellogenin and zona radiata proteins were detectable in this zooplankton species. Total PAHs ranged from 860.7 to 5037.9 ng/g d.w., carcinogenic PAHs from 40.3 to 141.7 ng/g d.w., HCB from 3.5 to 11.6 ng/g d.w., DDTs from 45.3 to 163.2 ng/g d.w. and PCBs from 84.6 to 210.2 ng/g d.w.

Table 2. Zooplanktonic species in which the biomarkers were evaluated.

Copepods
Acartia margalefi
Acartia latisetosa
Misyds
Siriella clausi
Diamysis bahirensis
Siriella armata
Mysidopsis gibbosa
Euphausiids
Euphausia crystallorophias
Euphausia superba
Meganycthiphanes norvegica
Amphipods
Streetsia challengeri

The preliminary results demonstrated that this multi-disciplinary ecotoxicological approach can be used in *M. norvegica* as an early indicator of the health status of the Mediterranean whale sanctuary. This kind of investigation can provide information to local authorities, who can intervene and take administrative and legislative action to curb pollution.

After this step, the biomarkers were applied in their true sense, as a multi-disciplinary diagnostic tool for assessment of the health status of a selected study area with respect to a reference area. A suite of biomarkers (BPMO activity, EROD activity, NADPH-cytochrome C reductase, NADH-cytochrome C reductase, NADH-ferricyanide reductase, total proteins, esterases, porphyrins) and residues (heavy metals) in the zooplankton copepod *Acartia latisetosa* was used to assess the health status of two environments in which the same species was sampled [14]. One was Lake Faro (Messina, Italy), which is thought to be polluted. The other, Lake Verde, a small brackish lake in a marine reserve (Marinello, Messina, Italy), was selected as the reference area.

EROD activity was higher in the Lake Faro (0.091 pmol res/min/mg prot) than the Lake Verde sample (0.041 pmol res/min/mg prot). Greater differences between sites were found for reductase activities. With regard to esterases, high inhibition of AChE activity (31.24%) was found in the sample from Lake Faro. Both samples showed a linear increase in enzyme activity with increasing concentration of samples. Porphyrins (copro, uro, protoporphyrins) were higher in the reference sample than in that from Lake Faro. Residue analysis showed higher concentrations in the reference lake sample of the copepod. The results suggested that Lake Faro is contaminated with lipophilic xenobiotics, such as PAHs and PHAHs. In fact, basal MFO activity in hepatopancreas is enhanced when organisms are exposed to this class of contaminants. Inhibition of AChE activity in *A. latisetosa* from Lake Faro could be due to exposure to neurotoxic substances, such as OPs and carbamates. We were unable to compare our values with those of others, because there have been no papers on biomarkers in zooplankton. Ecotoxicological and ecological interpretation of the results are discussed in the manuscript [14].

Many laboratory experiments were also carried out. The main ones were: copepods *Acartia latisetosa* collected in Lake Ganzirri (Messina, Italy) were kept under controlled lab conditions and exposed to the organophosphoric insecticide Parathion (20 μg/l); mysids *Siriella clausi*, collected in Lake Faro, were exposed in the lab to different concentrations of benzopyrene. Biomarkers were evaluated before and after treatment in both cases to detect and standardize the relation between quantity of contaminant and response [11]. For example, moderate

inhibition (19%) of acetylcholinesterases activity was found in the sample treated respect the control [11].

All the results confirmed that biomarkers in zooplankton can be used to study the health status of different environments and that they provide an early sign enabling action to be taken before bioaccumulation and biomagnification affect higher levels of the food chain.

After this short review of our research on this topic, we propose biomarkers in zooplankton as new indices of trophic status and ecological integrity of Italian marine coastal and lagoon environments, to be included among those specified by D.Lgs. 152/2006 before 2008. This tool has the following advantages: (i) zoopankton is found in all marine coastal and lagoon environments; (ii) in these environments, zooplankton richness may be very high, but biodiversity is low, making it possible to sort live samples to obtain a selected zooplankton species for biomarkers analysis; (iii) biochemical alterations detected through biomarkers are based on many intermediate metabolites that provide information about the real effects of contaminants or mixtures of contaminants on organisms, populations or communities, up to ecosystem level; (iv) detection of alterations at the lowest or second lowest level of the food chain will enable legal action to be taken at an early stage.

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