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Linking stable isotopes and biochemical responses in *Balanus glandula* under sewage influence

M.V. Laitano[a,*], M. Díaz-Jaramillo[b], Y.E. Rodriguez[a], E. Ducós[c], H.O. Panarello[c], A.V. Fernández-Gimenez[a]

[a] Instituto de Investigaciones Marinas y Costeras (IIMyC), Universidad Nacional de Mar del Plata (UNMdP), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Laboratorio de Fisiología de Organismos Acuáticos y Biotecnología aplicada, Funes 3350, 7600 Mar del Plata, Argentina
[b] IIMyC, UNMdP, CONICET, Laboratorio de Ecotoxicología y Contaminación Ambiental, Funes 3350, Mar del Plata 7600, Argentina
[c] INGEIS, Instituto de Geocronología y Geología Isotópica, CONICET-UBA, Universidad Nacional de Buenos Aires, Pabellón INGEIS - Ciudad Universitaria, 1428 Buenos Aires, Argentina

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ABSTRACT

In the present study, we analyzed the influence of untreated sewage exposure on carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopic composition and several biochemical responses in the barnacle *Balanus glandula*. The main objective was to evaluate whether changes in stable isotopes signature do reflect biochemical sub-lethal effects in a sewage influence gradient. Stable isotopes analysis showed differences in isotope signatures between close sewage influence and distant sites, being $\delta^{13}C$ signatures stronger than that of $\delta^{15}N$. Regarding biochemical effects, although organisms close to the effluent would be clearly exposed to contaminants (increased GST activity) the oxidative stress would not be too evident (peroxidases and ACAP not affected). The most affected physiological aspect was the digestive one, reflected in increased alkaline proteases and lipases activities. A clear relation between $\delta^{15}N$ and GST activity was found, showing to $\delta^{15}N$ as an indicator of potential exposure to chemical contaminants.

1. Introduction

One of the main sources of coastal marine pollution is the sewage effluents through which domestic and industrial residuals reach the sea. In developed countries, the implementation of different degrees of treatments has improved the quality of these liquid wastes (Fytili and Zabaniotou, 2008), whereas this issue is still a challenge for the governments of developing countries. However, fortunately, they are currently attempting to face it (Kivaisi, 2001). This is the case of Latin America countries like Argentina, where most sewage effluents receive none or minimal treatment (Sato et al., 2013). Only in the recent few years, some secondary treatment systems can be found in Argentina, but they constitute exceptions and usually they still do not reach the desired quality of effluents (Iribarnegaray et al., 2017).

The assessment of anthropic impact in natural environments through the study of pollution sublethal effects over organisms has become a widespread scientific method. In particular, biochemical biomarkers are considered early warming tools since it is assumed that they are more sensitive than individual or population level biomarkers, which would be evident subsequently (Jemec et al., 2010). For instance, the enzyme Glutathione-S-transferase (GST) is an indicator of exposure to contaminants since it takes place in phase II of the biotransformation process; also, this enzyme acts as an antioxidant. The biochemical biomarkers related to oxidative stress processes (e.g., peroxidases enzymes (Pe), antioxidant capacity against peroxyl radicals (ACAP)) are in particular widely used in environmental health assessment since it is a very well-known effect of pollution (Benedetti et al., 2015). All these biomarkers present sensitivity to sewage pollution in different marine invertebrate species (Galloway and Depledge, 2001; Machado et al., 2014; Zanette et al., 2015).

Further, there are some enzymes that although presenting potential effectiveness as sewage biomarkers, they are poorly studied in this topic. For example, the enzyme phenoloxidase (PO) takes part in the melanin formation process, which constitutes a common immune response to pathogens in invertebrates (Söderhäll and Cerenius, 1998). Since the bacterial load discharged to the environment by a sewage effluent is in general extremely high (Edwards, 1998), the PO activity of invertebrates living under such conditions may indicate an influence of this pollution source. On the other hand, the behavior of digestive enzymes in invertebrates shows a great plasticity according to the

* Corresponding author.
E-mail address: vlaitano@mdp.edu.ar (M.V. Laitano).

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environmental conditions, making them potential biomarkers to assess pollution effects (Charron et al., 2015). Due to the known changes in the environmental organic matter generated by wastewater discharges, it is very likely to find some variation in the activity of these enzymes, in particular in filter-feeders invertebrates.

Several authors have used carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotopes of aquatic organisms as tracers of sewage contamination (e.g., Waldron et al., 2001). The relative abundances of the lighter and heavier isotopes of some elements are stable and characteristic of each environment; e.g., the fraction of heavy ^{13}C relative to the abundance of the light ^{12}C is different between freshwater and marine matrices (Peterson and Fry, 1987). Further, due to processes occurring along the food webs, stable isotopes ratios may reveal the origin of nutrients used by primary producers as well as the diet composition of organisms (Post, 2002). Since the marine environment under the influence of sewage undergoes changes in the sources of nutrients and organic matter, which have a different isotopic signature (human, terrestrial, freshwater), the background levels of stable isotopes of the receiving system are also altered. Hence, the communities living there reflect these changes through carbon and mainly nitrogen stable isotopes composition (Mancinelli and Vizzini, 2015).

Although the exposure evidence provided by stable isotopes offers valuable information, the presence of a sewage plume does not necessarily indicate adverse effects on organisms. In this sense, it is interesting to test the relation between stable isotopes signatures and alterations in sentinel species in order to determine if stable isotopes do reflect the real impact of this type of pollution. In this context, in the present work, we propose to test whether carbon and nitrogen isotopic signature are related with biochemical biomarkers (Pe, GST, ACAP, PO, lipases and proteases) alteration in a barnacle species under the influence of an untreated sewage outfall.

2. Material and methods

2.1. Study site and sampling

The studied area consisted on an intertidal loess platform located at the Argentinean Coast (Southwest Atlantic) which is under the influence of a sewage outfall coming from two cities, Necochea and Quequén (115,457 habitants; 2010 census INDEC Argentina) (Fig. 1). Although Necochea-Quequén effluent is relatively small, the discharged residual waters do not receive any treatment, and the effects of sewage pollution over the receiving marine community have already been reported (López Gappa et al., 1990; Tablado et al., 1994; Tablado and Gappa, 2001). In addition, in the first few meters from the outfall, macroinvertebrate species are absent, whereas a little further away, only the limpets *Siphonaria lessoni* and the barnacles *Balanus glandula* begin to appear (*pers. obs.*). Thus, we chose *B. glandula* as the sentinel species for this study because in a previous work it has shown high sensitivity to pollution at the biochemical level (Laitano and Fernández-Gimenez, 2016). In addition, this barnacle has a broader distribution since it is an invasive species in many parts of the world (Spivak and Schwindt, 2014).

The specimens were collected by hand at six locations from the outfall towards west, following the direction and approximately the distances of a previously reported pollution gradient (López Gappa et al., 1990). The areas were sited at about 40 m (S1), 80 m (S2), 120 m (S3), 180 m (S4), 280 m (S5) from the outfall. A sixth site at about 900 m was chosen as reference site (López Gappa et al., 1990) (Fig. 1). At each location, three sites were randomly selected. Samples (3 per station) were carried to the laboratory in cold conditions, immediately dissected and pooled. Subsamples of soft tissues were dried in an oven at 60 °C and processed for the stable isotopes analysis. For the biochemical analysis, soft tissues were homogenized (1/2 w/v) in phosphate buffer (50 mM, pH 6) on ice, then samples were centrifuged 30 min at 10,000 rpm and 4 °C and the supernatant (protein extract) was carefully removed and stored at − 80 °C until analysis.

2.2. Stable isotopes analysis

Measurements of $\delta^{13}C$ and $\delta^{15}N$ for each sample were made on a Carlo Erba Elemental Analyzer (CHONS) coupled to a Finnigan MAT Delta V continuous-flow isotope ratio mass spectrometer (CF-IRMS) through a Thermo ConFlo IV interface using internal standards. These standards (caffeine: $\delta^{13}C = -39.33‰$, $\delta^{15}N = 7.02‰$; sugar: $\delta^{13}C = -11.41‰$; and collagen: $\delta^{13}C = -18.18‰$, $\delta^{15}N = 6.12‰$)

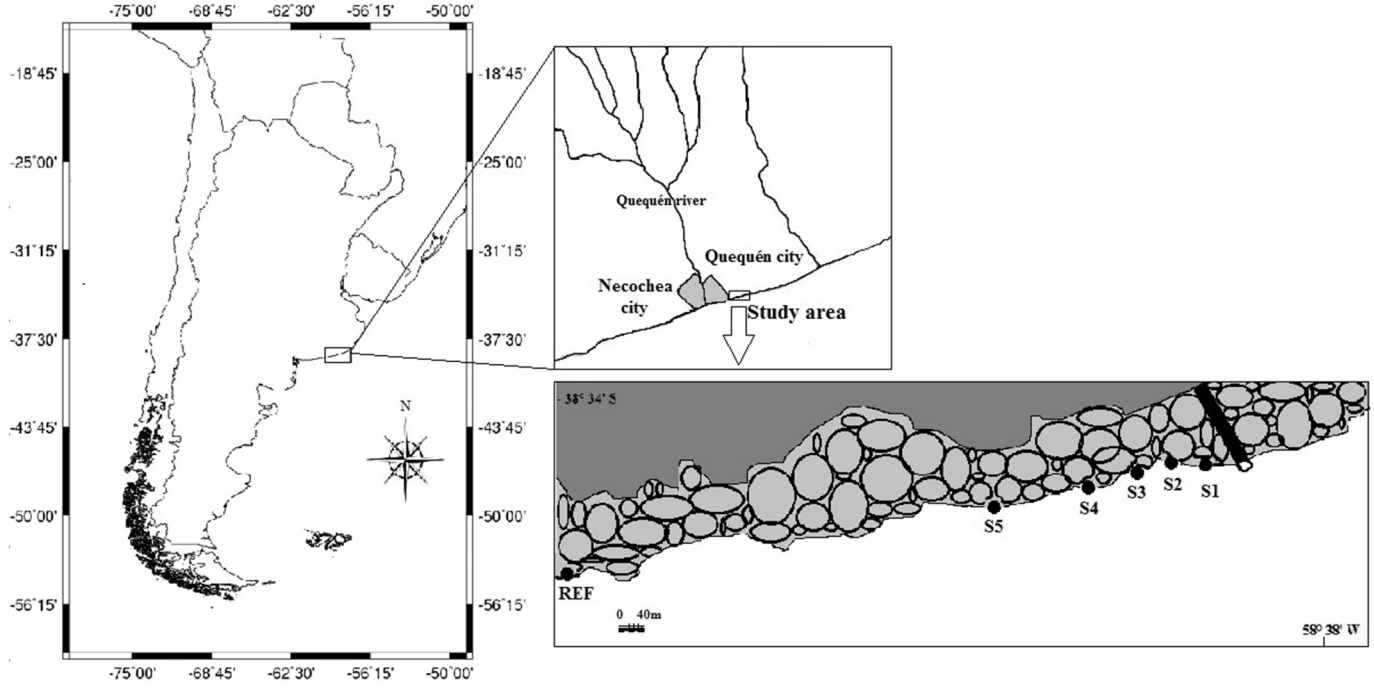

Fig. 1. Map showing the study area and sampling stations (S1–S5) at different distances (40, 80, 120, 180 and 280 m, respectively) from the Necochea-Quequén sewage outfall. REF is the reference station and it is 900 m from the outfall.