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

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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

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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: $\delta^{13}C = -18.18‰$, $\delta^{15}N = 6.12‰$)

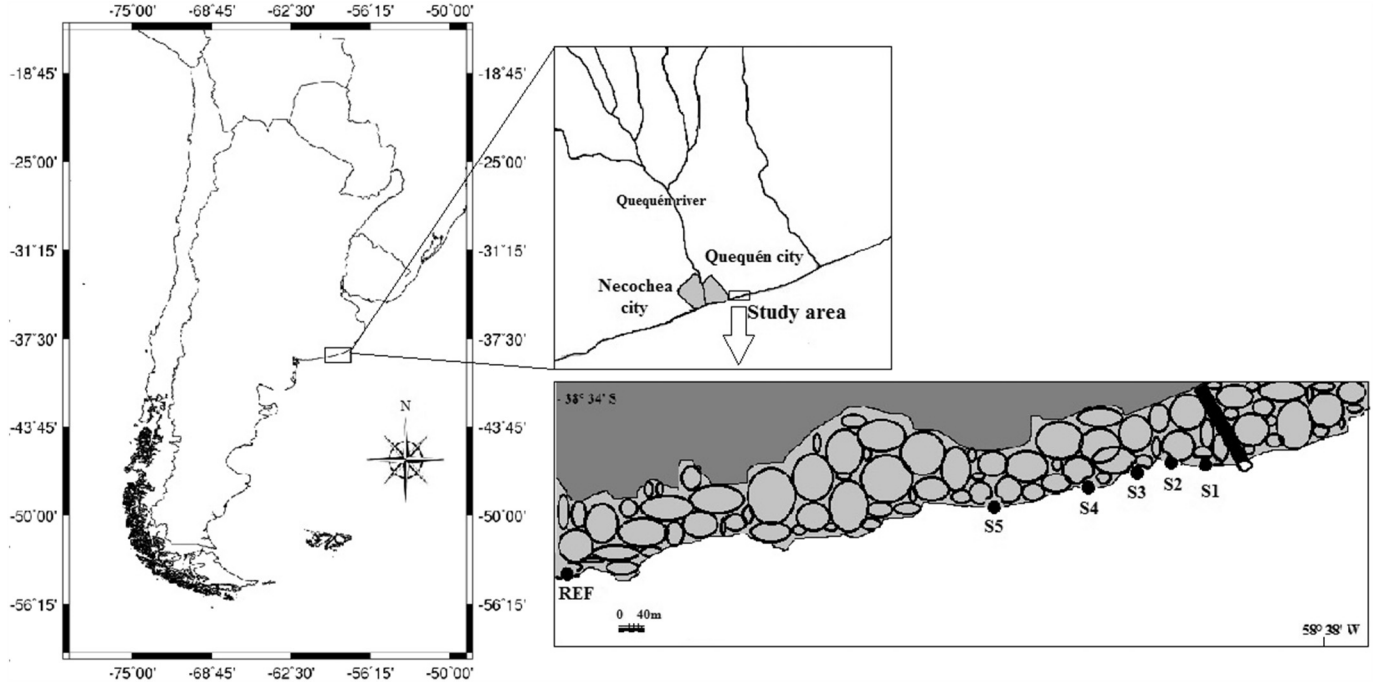

Fig. 1. Map showing the study area and sampling stations (S1–S5) at different distances (40, 80, 120, 180 and 280 m, respectively) from the Necochea-Quequén sewage outfall. REF is the reference station and it is 900 m from the outfall.

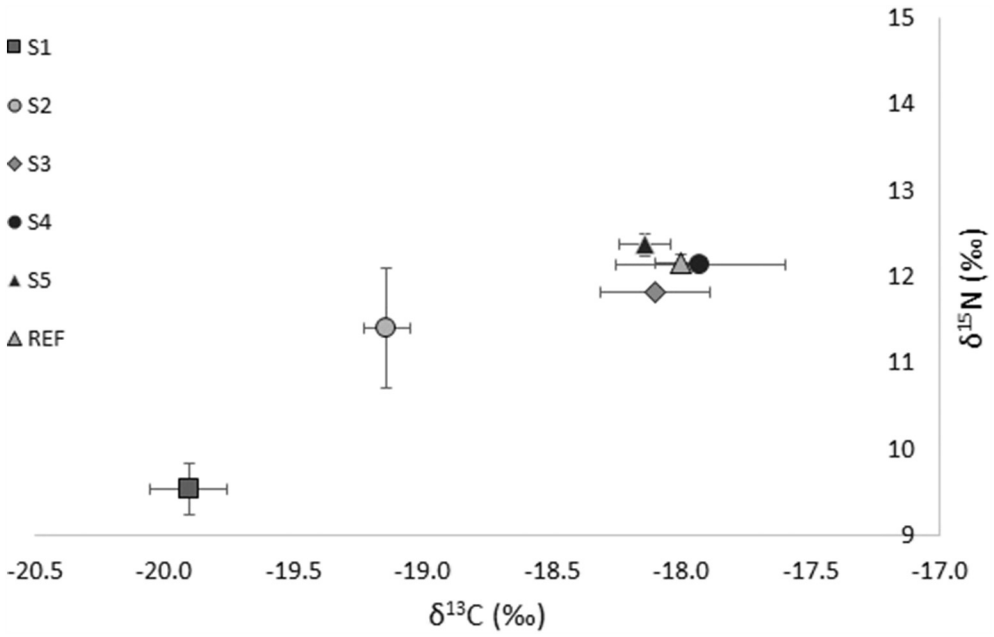

Fig. 2. Carbon and nitrogen stable isotopes ratios of barnacles *Balanus glandula* at different distances from Necochea-Quequén sewage outfall (S1: closest–S5: farthest) and a reference site (REF).

were calibrated against VPDB and AIR reference standards for carbon (L-SVEC, NBS-19 and NBS-22) and nitrogen (IAEA N1 and IAEA N2) (Coplen et al., 1992; Gonfiantini, 1978). Replicates of internal standards showed analytical uncertainties to be on the order of ± 0.2‰ for both $\delta^{13}C$ and $\delta^{15}N$ values.

$$\delta^{13}C = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{VPDB}}{(^{13}C/^{12}C)_{VPDB}} \times 1000‰; \quad \delta^{15}N = \frac{(^{15}N/^{14}N)_{Sample} - (^{15}N/^{14}N)_{AIR}}{(^{13}C/^{12}C)_{AIR}} \times 1000‰.$$

2.3. Biochemical analysis

Soluble protein was measured by the method described by (Bradford, 1976), using serum bovine albumin (Sigma A9647) as the standard. Peroxidases activity was determined according to (Lamela et al., 2005) using Pyrogallol 5% (*w/v*) (Sigma P0381) as substrate and hydrogen peroxide (1.6 vol.) to activate the assay. After 20 s, absorbance was registered at 420 nm. Glutathione-S-transferase activity was measured following (Habig et al., 1974), modified to microplate lecture and measuring the conjugation between the substrate, 1-chloro-2,4-dinitrobenzene (CDNB; Sigma 138,630), and reduced glutathione (Sigma G4251). Absorbance at 340 nm was recorded every minute during 10 min. Total antioxidant competence against peroxyl radicals (ACAP) was measured in a 96-well microplate format according to (Amado et al., 2009). Briefly, the method based on the exposure of tissue homogenate with and without ABAP at 37 °C in which peroxyl radicals are produced by thermal decomposition of ABAP. The thermal decomposition of ABAP and ROS formation was monitored for 30 min, with fluorescent readings every 5 min (488 ex. 525 em). The relative difference area with and without ABAP was considered a measure of antioxidant capacity, with greater differences reflecting lower antioxidant capacity.

Phenoloxidase activity measurement was adapted from (Palmer et al., 2011) using L-DOPA (3 mg ml^{-1}) (Aldrich 333,786) as substrate and recording absorbance at 490 nm. The activity of two types of proteinases was assessed, those which act at pH 7.5 and pH 6, using 1% azocasein as the substrate in 50 mM Tris–HCl, pH 7.5 (García-Carreño, 1992) and 6 (Celis-Guerrero et al., 2004), respectively. Lipases activity was determined according to (Versaw et al., 1989). β-naphthyl caprylate (Goldbio N-100) dissolved in dimethyl sulfoxide (DMSO) was used as substrate and absorbance was recorded at 550 nm.

All assays were made in triplicate with two control trials. Pe and lipases activities were measured with a Shimadzu UV-2102 PC, UV–visible Scanning Spectrophotometer, while GST, PO and proteinases activities were measured with a Biotek EPOCH microplate spectrophotometer. Pe, PO, proteinases and lipases activities are expressed as the change of absorbance per minute per mg protein (Abs min^{-1} mg $protein^{-1}$). GST activity is expressed as units per mg protein (U mg $protein^{-1}$). One unit of enzyme is the quantity of enzyme that catalyzes the formation of 1 μmol of product per minute.

2.4. Data analysis

Results are presented as means ± standard error. The isotopic signature and the biochemical biomarkers were compared among sites trough generalized linear models (GLM). Models with isotopic signature or biochemical biomarkers as dependent variable and site as fixed factor were developed, specifying Gaussian or Gamma family, as appropriate, according to the distribution of each data set. In order to determine the significance of the factor “site” on the isotopic signature or the biochemical biomarkers (which means, if there are differences in these variables according to the distance to the sewage outfall), models were contrasted with a null model (without any independent variable) through the Akaike's Information Criteria. When the Akaike's number of a model with the factor “site” is lower (with a difference of at least 7) and the Akaike's weight is considerably higher than those of the null model (Burnham et al., 2011), it is an indication of significant differences in the analyzed variables due to such factor. When significant differences among sites were found, Tukey's tests were applied to the constructed model in order to make post-hoc multiple comparisons and detect which sites differ in the stable isotopes values and each biochemical parameter. The relation between the isotopic signature and each biochemical biomarker was tested through Pearson or Spearman correlation tests, according to the normality and homoscedasticity of each one. All statistical analyses were conducted in R 2.13.0.

3. Results and discussion

3.1. Stable isotopes

Carbon and nitrogen stable isotopes of *B. glandula* at different

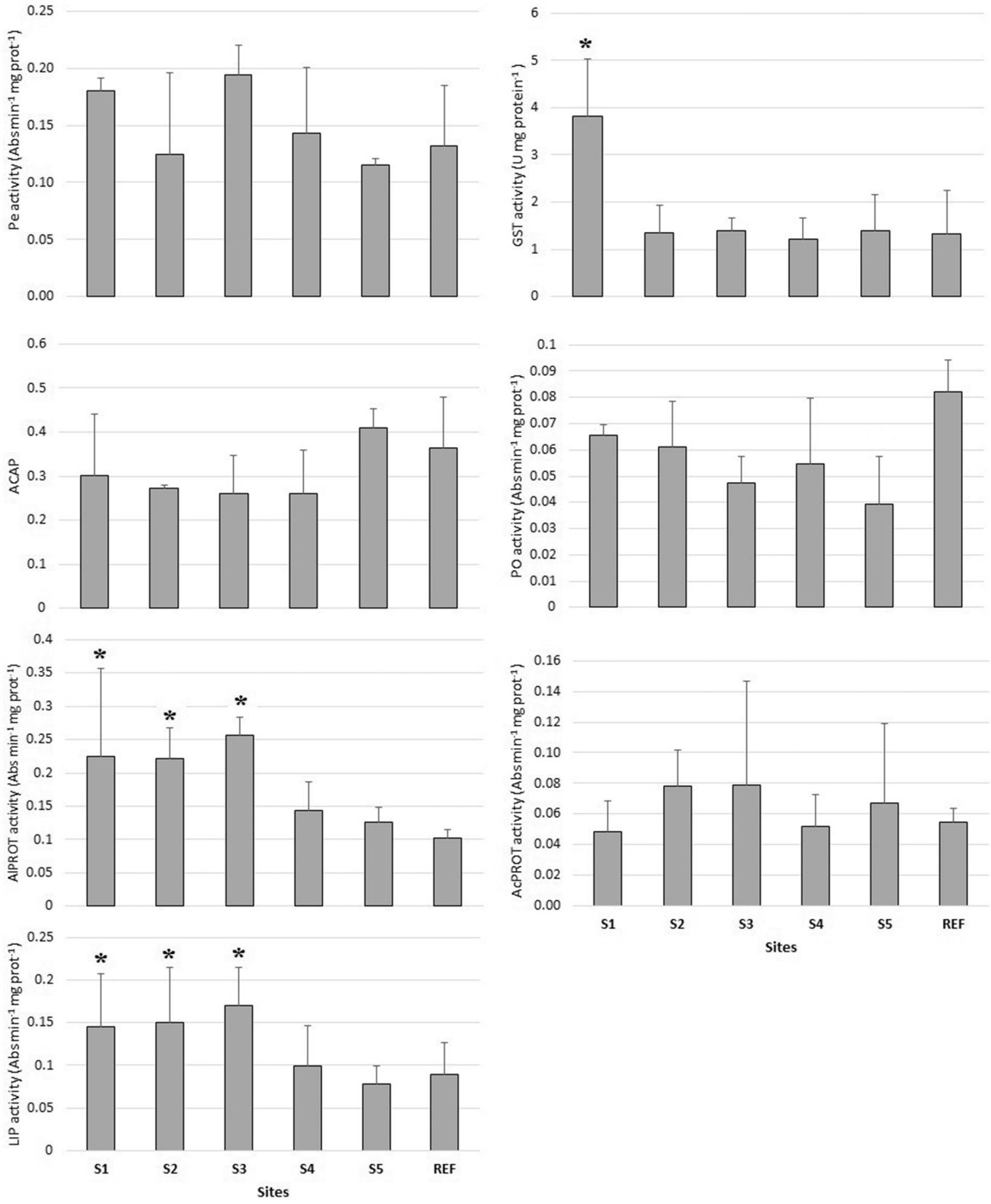

Fig. 3. Biochemical responses (mean and standard deviation) measured in barnacles *Balanus glandula* at different distances from Necochea-Quequén sewage outfall (S1–S5) and a reference site (REF). Pe: peroxidases; GST: glutathione-S-transferase; ACAP: antioxidant capacity against peroxyl radicals; PO: phenoloxidase; AlPROT: alkaline proteases; AcPROT: acid proteases; LIP: lipases. Asterisks indicate significant differences with the reference site.

Table 1
GLMs. Akaike's number (AICc) and Akaike's weight (wAICc) for both, the null model (without independent variable; m_0) and that with site as independent factor (m_1), for the different measured parameters. Lower AICc (a difference of at least 7) and higher wAICc indicates that is the best-fitted model when comparing m_0 with m_1. In bold: lower AICc and higher wAICc of m_1, which means significant differences among sampling stations. Pe: peroxidases; GST: glutathione-S-transferase; ACAP: antioxidant capacity against peroxyl radicals; PO: phenoloxidase; Al PROT: alkaline proteases; Ac PROT: acid proteases; LIP: lipases.

Variable	AICc	wAICc
$\delta^{13}C$	**m_0: 45.7**	**m_0: < 0.001**
	m_1: 6.5	**m_1: 1**
$\delta^{15}N$	**m_0: 57.4**	**m_0: < 0.001**
	m_1: 29.5	**m_1: 1**
Pe	m_0: − 47.3	m_0: 0.63
	m_1: − 41.4	m_1: 0.37
GST	**m_0: 112.5**	**m_0: < 0.001**
	m_1: 94.1	**m_1: 1**
ACAP	m_0: − 27.8	m_0: 1
	m_1: − 6.7	m_1: < 0.001
PO	m_0: − 156.9	m_0: 0.37
	m_1: − 158	m_1: 0.63
Al PROT	**m_0: − 95.8**	**m_0: < 0.001**
	m_1: − 111	**m_1: 1**
Ac PROT	m_0: − 55.9	m_0: 1
	m_1: − 43.8	m_1: < 0.001
LIP	**m_0: − 53.8**	**m_0: 0.01**
	m_1: − 62.9	**m_1: 0.99**

Table 2
Parameters of correlation analyses (p-value and rho) between biomarkers significantly different among study sites and carbon and nitrogen stable isotopes signatures ($\delta^{13}C$ and $\delta^{15}N$, respectively). GST: glutathione-S-transferase; PO: phenoloxidase; Al PROT: alkaline proteases; LIP: lipases; In bold: significant correlation.

Enzyme	$\delta^{13}C$	$\delta^{15}N$
GST	p > 0,05	**p < 0,05**
	rho = − 0.3	**rho = − 0.5**
PO	p > 0,05	p > 0,05
	rho = − 0.09	rho = − 0.3
Al PROT	p > 0,05	**p < 0,05**
	rho = − 0.26	**rho = − 0.5**
LIP	p > 0,05	**p < 0,05**
	rho = − 0.25	**rho = − 0.5**

distances from the Necochea-Quequén sewage outfall are presented in Fig. 2. Barnacles from the reference station presented similar values than those reference values previously reported in other marine filter-feeder species from the region (Laitano et al., 2016 and references therein), which confirms the lack of sewage influence at that station. The $\delta^{13}C$ values ranged from − 20.1 to − 17.6‰ and showed significantly depleted values in the two closest stations to the effluent outfall; compared with the reference site, S1 and S2 were 1.9 and 1.1‰ depleted. This depletion is a well-reported pattern in organisms of all trophic levels, or even sediments, exposed to sewage pollution (Gearing et al., 1991; Waldron et al., 2001). This carbon signature indicates the presence of terrestrial organic matter depleted in ^{13}C relative to that produced by phytoplankton (Peterson and Fry, 1987).

$\delta^{15}N$ values varied between 9.3‰ and 12.5‰, and only S1 (the closest site to sewage) displayed significant differences from the other sites, showing a less positive nitrogen isotope signature (2.6‰ depleted compared with the reference site; $p < 0.05$; Fig. 2). In general, studies on sewage pollution show that organisms exposed to such contamination present enriched ^{15}N values (Oakes and Eyre, 2015). However, this pattern corresponds to the incorporation of residues previously subjected to treatment, which, through microbial nitrification and denitrification processes that selectively utilize ^{14}N, enrich the effluent (Toyoda et al., 2011). The Necochea-Quequén sewage does not receive any treatment and, thus, barnacles are reflecting the depleted $\delta^{15}N$ characteristic of land-originated nitrogen compared with the marine origin one (Peterson and Fry, 1987), as has been reported in other species under untreated sewage outfalls influence (Rogers, 2003).

Thus, the stable isotopes results indicate that the influence of the studied sewage outfall would be in the first meters. The $\delta^{13}C$ was more sensitive than $\delta^{15}N$ because not only differ in the first two stations, but also these stations differ each other in $\delta^{13}C$, indicating a small gradient of sewage influence. In general, a different result is found in other studies in which $\delta^{15}N$ is a more sensitive tracer of anthropic organic matter than $\delta^{13}C$; however, this higher sensitivity of $\delta^{15}N$ is reported in primary producers and consumers which feed exclusively on them (e.g., grazers) (Vizzini and Mazzola, 2006). Rogers (2003) suggested that the $\delta^{13}C$ signature would be as good indicator as the $\delta^{15}N$ to filter-feeders because they assimilate the particulate sewage-derived organic matter directly, which would be the case of *B. glandula*. Consistently, other studies on the sewage influence on filter feeders organisms found the isotopic composition of both, carbon and nitrogen, as equally suitable tracers of such pollution (Dolenec et al., 2006; Piola et al., 2006).

3.2. Biochemical biomarkers

The different biochemical responses measured in *B. glandula* under sewage influence are presented in Fig. 3. Peroxidases mean activity varied between 0.115 and 0.238 Abs min^{-1} mg $protein^{-1}$, GST mean activity oscillated between 1.22 and 3.83 U mg $protein^{-1}$ while ACAP mean values did it between 0.26 and 0.41. The mean activity of the enzyme PO ranged from 0.039 to 0.082 Abs min^{-1} mg $protein^{-1}$. Alkaline and acid proteinases and lipases showed mean activities from 0.1 to 0.29, from 0.05 to 0.08 and from 0.079 to 0.197 Abs min^{-1} mg $protein^{-1}$, respectively.

GST, alkaline proteinases and lipases presented significant differences in at least one station respect to the reference site, whereas the other biomarkers did not show statistical differences among stations (Table 1, Fig. 3). GST showed markedly higher activity at the station S1 compared with all the other stations. This enzyme participates in detoxification processes and as an antioxidant agent (Barata et al., 2005). Therefore, in environmental studies, variations in its activity has been assumed as a response to organic contaminants presence (Zanette et al., 2015) as well as to oxidative stress (Lima et al., 2007). Since Pe activity and ACAP were similar among stations, indicating that the antioxidant system is not over stimulated by sewage influence, it is probable that the increased GST activity corresponds to the activation of biotransformation processes (Zanette et al., 2015). This would indicate that there is exposure to contaminants, but it would not be high enough to trigger oxidative stress.

Compared with the reference site, the activities of alkaline proteases and lipases were enhanced in the first three stations closer to the sewage outfall (S1, S2 and S3; Fig. 3). The influence of chemical contaminants potentially released by the studied sewage should be discarded since all the evidence available indicate an inhibition of digestive enzymes by pollutants (Chen et al., 2002; Dedourge-Geffard et al., 2013). On the other hand, the organic material released by the outfall could explain the increased alkaline proteases and lipases activities. The activity of these enzymes varies in response to changes in food type availability (Albentosa and Moyano, 2009), which would be the case of a filter-feeder exposed to sewage effluent. Raunkjær et al. (1994) studied the composition of four sewage outfall sludges and found them rich in lipids and proteins, which is consistent with the increased activities of proteases and lipases determined in the studied barnacles. From these results, proteases and lipases are proposed as biomarkers of physiological state alteration in organisms exposed to sewage pollution.

3.3. Stable isotopes and biomarkers link

$\delta^{13}C$ did not show significant correlation with any analyzed biomarker, whereas $\delta^{15}N$ was correlated with GST, alkaline proteases and

lipases activities (Table 2). This could be surprising since the carbon signature reflected statistically better the extent of influence of the sewage material. However, $\delta^{15}N$ showed a tendency to present lower values at the three first stations besides the significant difference of S1 (Fig. 2), which would be more similar to the biomarkers pattern mentioned above than $\delta^{13}C$ results.

Few previous studies established a relationship between stable isotopes signature and sewage pollution effects, but they did it in different conditions than this study. Morrissey et al. (2013) analyzed the relationship between isotopes composition and freshwater macroinvertebrates community indices exposed to secondary treatment plants. Another similar work which can be mentioned is that of Carballeira et al. (2011) that studied the relation of $\delta^{15}N$ with mollusks histopathological effects but exposed to land-based marine fish farms which, although present organic matter contamination, the chemical contaminants and routes of contamination would be different. Despite the differences, both studies reported a strong correlation between the analyzed pollution effects and $\delta^{15}N$. In the present study, we found such clear relationship when analyzing the impact of sewage on GST activity. Although a correlation between $\delta^{15}N$ and some digestive enzymes was also found, this does not mean that the nitrogen stable isotope analysis reflects such biochemical alterations since the $\delta^{15}N$ responded only in the first station whereas the biochemical responses were detected until S3.

The arising question now is why the digestive enzymes response is stronger than the isotopic response. One possible explanation would be that stable isotopes are time-integrated indicators of environmental condition whereas the digestive enzymes may depend on the organic matter recently released by the effluent. For instance, for limpets and mussels, it took them more than nine months to recover their background N signature after a sewage plant closure in New Zealand (Rogers, 2003). On the other hand, the differential activation of the digestive enzymes would be directly related to the different composition of the diet (Albentosa and Moyano, 2009). Thus, the variability in the digestive enzymes response would be higher than that of $\delta^{15}N$, which would explain the different responses. However, another possible explanation for the weaker $\delta^{15}N$ signal arises from the fractionation process. An organism does not directly assimilate the nitrogen signature of the consumed organic matter because it suffers the fractionation process, which will determine its final $\delta^{15}N$ (Peterson and Fry, 1987). Fractionation in animals is due mainly to differential higher excretion of the light ^{14}N isotope, leading to a more enriched body signature (Fry, 2006), which is precisely the contrary effect of the incorporation of sewage organic matter by barnacles in this study (depletion of $\delta^{15}N$). Thus, fractionation process could be counteracting the effect of sewage, leading to a weaker $\delta^{15}N$ signal.

Concluding, the stable isotopes analysis showed a sewage influence in the first few meters from the outfall, being the $\delta^{13}C$ more sensible than $\delta^{15}N$. The untreated wastewaters of the study area led to novel results compared to those obtained in most other studies of the same research topic. When analyzing the effects of sewage discharge on barnacles, a variety of responses have been found according to the different biochemical parameters. The most affected biochemical aspect was the digestive one, which in turn was measured for the first time in sewage-exposed animals and, thus, digestive enzymes are proposed as biomarkers of sewage pollution. A clear relationship between $\delta^{15}N$ and GST activity was found, showing that $\delta^{15}N$ is an indicator of potential exposure to chemical contaminants, besides the exposure to the organic ones. Further studies with different sewage conditions (size, treatment, environment, etc.) could assess this.

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