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Short Note

Determination of $\delta^{15}N$ in *Anemonia sulcata* as a pollution bioindicator

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A R T I C L E I N F O

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A B S T R A C T

$\delta^{15}N$ concentration can reveal the anthropogenic pollution of an ecosystem. To study this pollution level, this isotope can be analyzed from water or animal tissue. Sewage pipes pour anthropic waters, rich in $\delta^{15}N$, to the sea. In the sewage pipes surroundings live several organisms, like the cnidarian *Anemonia sulcata* (Pennant, 1777). As anemones have zooxanthellae, they incorporate nitrogen from the environment. To test for the possible effects of wastewater effluents on the coastal ecosystem through the content of the isotope $\delta^{15}N$, *A. sulcata* specimens were collected near a sewage pipe in North Tenerife (Spain), in contiguous zones to look for a $\delta^{15}N$ gradient. The obtained results show that there is a gradient of $\delta^{15}N$ concentration, and the highest levels were found nearest the sewage pipe (mean = 5 $\delta^{15}N$ ‰) and the lowest in the control zone (mean = 3.26 $\delta^{15}N$ ‰). Being so, *A. sulcata* has proven to be a sensitive organism whose $\delta^{15}N$ concentration can be used as bioindicator for pollution and human involvement in the ecosystem in which it lives.

1. Introduction

Nowadays, marine ecosystems are being affected by multiple human-induced stressors, such as fishing, climate change and pollution (Halpern et al., 2008; Fujiwara, 2012). These stressors produce effects at the local, regional and global scale, like habitat loss or degradation, trophic network disturbances and reductions in diversity and some species abundance (Blight et al., 2015; Fitzgerald et al., 2007).

Thus, effluents are discharged into aquatic systems, either freshwater or into the coastal environment. This may constitute a large input of organic and inorganic substances to these ecosystems (Gearing et al., 1991; Waldron et al., 2001). The denitrification that occurs during treatment usually results in an effluent with organic matter enriched with $\delta^{15}N$ (Ramírez-Álvarez et al., 2007). The $\delta^{15}N$ proportion of the organic matter may vary due to the isotopic fractionation associated with waste treatment.

Purification plants in big cities can deliver a high content of $\delta^{15}N$ to the environment as a result of the extraction of organic matter from municipal and industrial sewage systems (Rozic et al., 2015). Marine organisms may absorb this $\delta^{15}N$, and there are several studies that relate the concentration of $\delta^{15}N$ in these organisms with anthropic pollution, presenting very high values of this isotope due primarily to inadequate infrastructure of septic systems (Dolenec et al., 2005; Dolenec et al., 2007).

Stable isotopic proportion is usually studied to reveal animal trophic position (Post, 2002), either on land (De Visser et al., 2008) or in coastal communities (Leakey et al., 2008). For freshwater ecosystems, it has been suggested that $\delta^{15}N$ signatures in macroinvertebrates would be detected near wastewater effluents (Morrissey et al., 2013). It also appears that changes in isotopic rates in the food web are more detectable in invertebrates than in fish (Loomer et al., 2015) because of physiologic differences between these animals.

Anemonia sulcata is a cnidarian species that lives in the intertidal areas of the Atlantic Ocean and Mediterranean Sea. These anemones have a symbiosis with zooxanthellae located in the tentacles that provide the necessary nutrients for its development (Fitt et al., 2000, Tytler, 1982). This species is considered a pollution bioindicator in the intertidal of the coasts and is being used for coastal communities monitoring in Tenerife, Spain (Lozano et al., 2016). Therefore, we propose to study the possible effects of wastewater effluents on the

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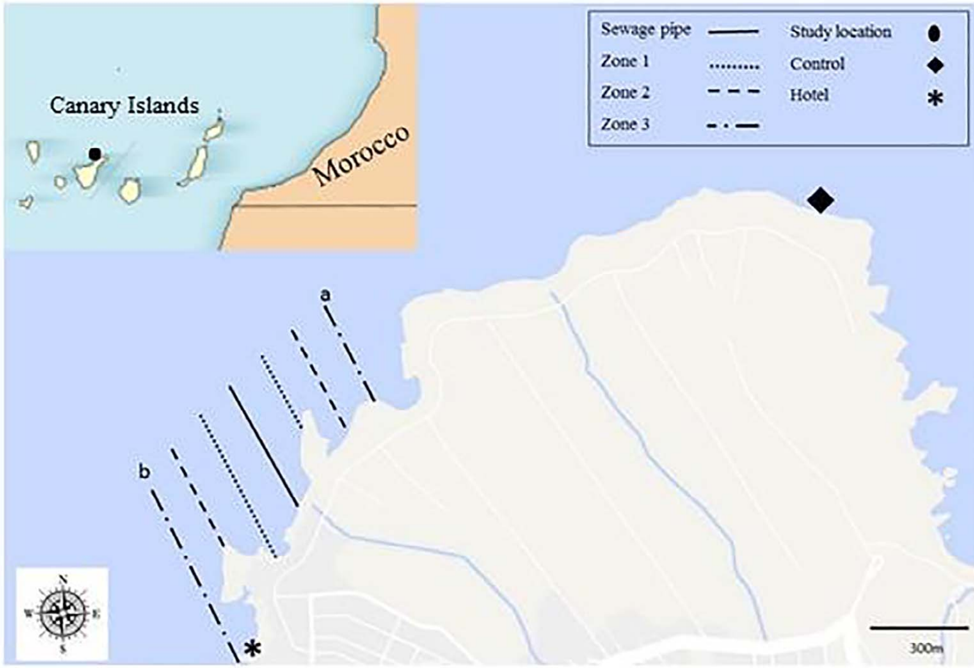

Fig. 1. Map of the sampling area in Punta del Hidalgo, Tenerife.

coastal ecosystem through the content of the isotope δ^{15}N in the macroinvertebrate *A. sulcata* (Pennant, 1777).

2. Material and methods

Anemonia sulcata samples were collected in the intertidal zone of the locality Punta del Hidalgo in Tenerife (Canary Island, Spain), (Fig. 1), in February 2017. The study area was divided into 3 zones according to the distance to the sewage pipe (28°34′5.17″N 16°19′35.45″W), on both sides of it (Fig. 1). Each zone was separated by a distance of 130 meters from the adjoining one, starting from the pipe (Zone 1). A control zone was also included (28°34′41.46″N 16°19′23.74″W). In each zone, a longitudinal transect through all the sampling area was performed, randomly collecting 10 anemones from different pools.

All the samples were frozen immediately after sampling and kept at −20 °C till further processing. Each anemone specimen was lyophilized to remove water from the tissues, pulverized using an agate mortar and pestle. Dry samples were preserved in a desiccator at room temperature until the analyses were carried out. Organisms sampled at each location were analyzed individually. Nitrogen isotopic analysis were performed at Servizos de Apoio á Investigación (SAI) of the University of A Coruña. Samples were weighed in tin capsules and measured by continuous flow isotope ratio mass spectrometry using a FlashEA1112 elemental analyzer (ThermoFinnigan, Italy) coupled to a Deltaplus mass spectrometer (FinniganMat, Bremen, Germany) through a Conflo II interface. Nitrogen stable isotope abundance was expressed as δ^{15} N parts per thousand (‰) relative to VPDB (Vienna Pee Dee Belemnite) and Atmospheric Air, according to the following equation:

$$\delta(‰) = [(R_{Sample}/R_{Reference}) - 1] \times 1000$$

As part of an analytical batch run, a set of international reference materials for δ^{15}N (IAEA-N-1, IAEA-N-2, IAEA-NO-3) and δ^{13}C (NBS 22, IAEA-CH-6, USGS 24) were analyzed. The precision (standard deviation) for the analysis of δ^{13}C and δ^{15}N of the laboratory standard (acetanilide) was ± 0.15‰ (1-sigma, n = 10).

Once all the samples were analyzed, one-way analysis of variance (ANOVA) by permutations with Euclidean distances (Anderson, 2001) were performed in order to determine if anemones living near the outfall have higher levels of δ^{15}N compared to the ones further away from the pipe. A fixed factor "Zone" was used, with 4 levels: Zone 1–3 and control Zone (Fig. 1). Pair-wise test were performed between all the established zones, using a significant level of p = .05.

3. Results

Table 1 shows the data obtained using the described methodology. There are significant differences (p-value < .05) in the δ^{15}N concentration in *Anemonia sulcata* regarding the surveyed zones (Table 2). Pair-wise comparisons of these zones show differences (p-value < .05) between all locations except when regarding Zone 3 (Table 3). Fig. 2 shows there is a wide variation in Zone 3, which was divided into 2 zones: Zone 3a for the 5 samples near the control zone (samples 1–5), and Zone 3b for the 5 samples near a hotel (samples 6–10) (Fig. 4).

The analysis carried out using this new zone division shows again differences (p-value < .05) for the δ^{15}N concentration in *A. sulcata* for the factor Zone (Table 4). Pair-wise comparisons of the new zones show differences (p-value < .05) between all locations except between Zones 3a and control (Table 5). It is important to note that the sample size is the half for these Zones 3a and 3b (Fig. 4).

Table 1
δ^{15}N proportion for each *Anemonia sulcata* sample in the surveyed zones.

	δ^{15}N (‰)			
Sample	Control Zone	Zone 1	Zone 2	Zone 3
1	3.3	6.2	4	2.9
2	3.8	5.2	4.3	3.2
3	3.3	4.6	4.2	3.5
4	2.9	5.5	4.5	2.8
5	3.2	5.4	4.2	3.6
6	3.8	4.4	3.8	5.7
7	3.2	5.1	4.1	5.7
8	3.1	4.6	4.1	6.2
9	3.2	4.6	4.3	5.8
10	2.8	4.4	3.8	6.2
Mean	3.26	5.00	4.13	4.56

Table 2
PERMANOVA results calculated from the Euclidean distance dissimilarity matrix for the $\delta^{15}N$ concentration in *Anemonia sulcata* at the different suveyed zones (fixed factor, 4 levels).

Source of variation	df	SS	MS	Pseudo-F	p (perm)
Zone	3	0.979	0.326	8.749	0.000*
Residual	36	1.343	0.037		
Total	39	2.322			

* P < .05.

Table 3
Pair-wise comparison of the studied zones (4 levels), (p- values). 0 is control Zone.

Groups	t	p (perm)
1, 2	4.541	0.000*
1, 3	1.047	0.305
1, 0	8.638	0.000*
2, 3	0.699	0.486
2, 0	6.867	0.000*
3, 0	2.689	0.019*

* P < .05.

4. Discussion

There are numerous studies about the influence of submarine sewage pipes on marine biota as well as physical-chemical oceanography of the ocean, which can alter the chemical composition of organisms, which is why many researchers have looked for possible bioindicators of anthropogenic pollution, like that caused by submarine sewage pipes (Dolenec et al., 2011, Lozano et al., 2016). One of the best indicators is $\delta^{15}N$, because when the concentration is higher than natural average is associated to an anthropic origin (Jennings et al., 1997; Costanzo et al., 2001 Lipschultz & Cook, 2002; Dolenec et al., 2005; Orlandi et al., 2014).

Anemonia sulcata has zooxanthellae in its tentacles, which is why they have the same mechanism of nitrogen absorption as algae. In these anemones, zooxanthellae concentration varies depending on the amount of light that reaches the hosting anemone (Tytler, 1982). Anemones living in the intertidal will have similar concentrations of zooxanthellae between them, while zooxanthellae concentration will differ compared to the anemones found in the subtidal. We can therefore infer that both the anemones with zooxanthellae and the algae can absorb and assimilate the nitrogen coming from the pipe, explaining the $\delta^{15}N$ concentration variation in their tissues. The closer the organism is to the pipe, the more concentration of $\delta^{15}N$ it will contain (Costanzo et al., 2001, Lipschultz & Cook, 2002).

Table 4
PERMANOVA results calculated from the Euclidean distance dissimilarity matrix for the $\delta^{15}N$ concentration in *Anemonia sulcata* at the different suveyed zones (fixed factor, 5 levels). p values calculated through the Monte Carlo permutation test.

Source of variation	df	SS	MS	Pseudo-F	p (perm)
Zone	4	2.022	0.506	59.083	0.001*
Residual	35	0.299	0.009		
Total	39	2.322			

* P < .05.

Table 5
Pair-wise comparison of the studied zones (5 levels). 0 is control Zone.

Groups	t	p (perm)
1, 2	4.541	0.000*
1, 3a	6.755	0.000*
1, 3b	3.279	0.01
1, 0	8.638	0.000*
2, 3a	6.274	0.000*
2, 3b	13.529	0.000*
2, 0	6.867	0.000*
3a, 3b	12.862	0.008*
3a, 0	0.333	0.756
3b, 0	14.316	0.000*

* P < .05.

The presented results show a higher concentration of $\delta^{15}N$ in the anemones close to the sewage pipe (Zone 1) compared to the $\delta^{15}N$ found in anemones living further away (Zones 2 and 3) (Fig. 3). For Zone 3, which is the furthest from the pipe, there are two subzones (Zone 3a and 3b). Furthest to the town (Fig. 1) the lowest $\delta^{15}N$ concentrations in anemones tissue were found, similar to the control samples. On the other hand, the area closest to the town has the highest $\delta^{15}N$ concentrations (Fig. 4), which may be explained because there is a hotel and a recreational area there, which seem to affect the $\delta^{15}N$ concentrations in anemones in a way like the sewage pipe does. Although general sea currents surrounding Tenerife island are dominated by the Canary Current and the trade winds, both with a NE-SW main direction, it has been demonstrated that during winter this main current

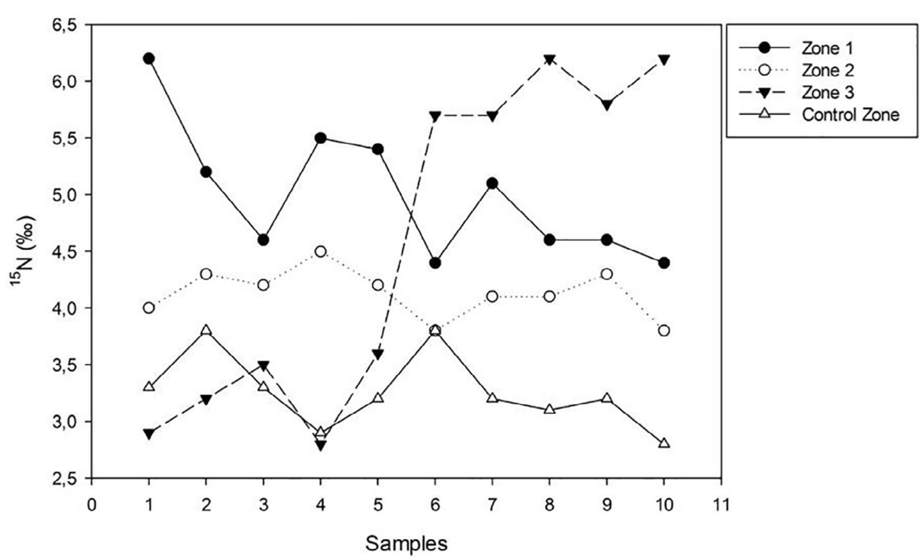

Fig. 2. Distribution of the nitrogen concentration for Zones 1–3.

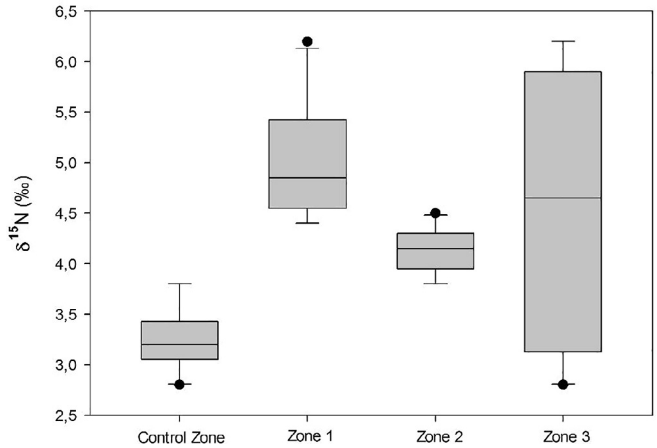

Fig. 3. Boxplots for the $\delta^{15}N$ proportion in *A. sulcata* in each of the four surveyed zones.

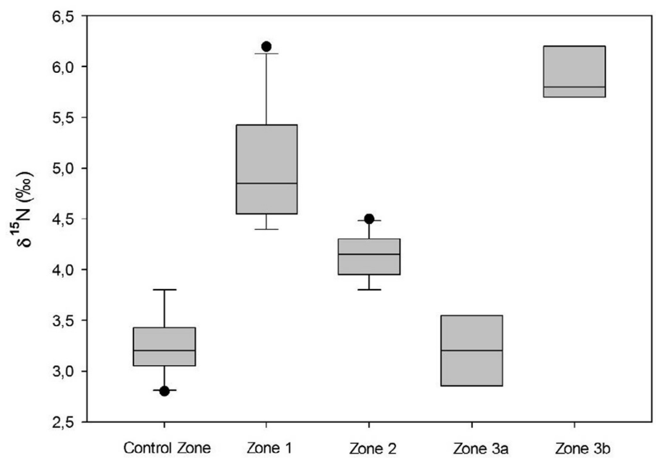

Fig. 4. Boxplots for the $\delta^{15}N$ proportion in *A. sulcata* for the five zones.

almost disappears (Hernández-Guerra et al., 2017). Being so, only small-scale sea currents, trade winds and tidal movements could influence dispersion of wastewater from the sewage. To our knowledge, small-scale currents have not been measured near the locality of study.

Our results show the highest mean concentration of $\delta^{15}N$ (‰) near the sewage pipe (Zone 1) and the urban nucleus ($\delta^{15}N$ (‰) = 5 and 5.92, respectively). These concentrations are lower than those presented by Dolenec et al. (2005) in their study ($\delta^{15}N$ (‰) = 7.3 and 11.1), which can be explained by the higher levels of pollution in the location and because their study was carried in the Mediterranean Sea, which is a closed sea, while the presented results are from an archipelago in the Atlantic Ocean. In recent years the oceans have undergone changes of all kinds, whether ecological or in the physicochemical parameters of water due to human activity. That is why the study of $\delta^{15}N$ can help us determine if an area is in a good condition. The variation of $\delta^{15}N$ can determine temporal trends and corroborate how the zones evolve, and determine if good recovery management is taking place, as it happens in our study (Halpern et al., 2008; Blight et al., 2015). The $\delta^{15}N$ set to $\delta^{13}C$ serves to establish the relationship in the food web, it also gives information about the diet, which is why this analysis can be complementary to recognize if an area is ecologically well preserved (Frederiksen et al., 2007; Vilchis et al., 2015; Blight et al., 2015).

Dolenec et al. (2005) recommended *A. sulcata* to be regarded as a bioindicator of anthropogenic pollution caused by sewage pipes, idea that is reinforced with the presented results. Because heavily anthropized marine areas lose their biological diversity (Dolenec et al., 2005, 2011), these bioindicator organisms are extremely relevant to assess and to monitor the conservation status of the coastal ecosystem.

5. Conclusions

Variation of $\delta^{15}N$ in anemones tissue serves as an indicator of their proximity to human outfalls to the sea. In the study area there is a clear gradient to one side of the coast in these concentrations, with higher levels on $\delta^{15}N$ in the anemones nearest the sewage pipe and the lowest concentrations in the further area. *Anemonia sulcata* has proven to be a sensitive organism whose $\delta^{15}N$ concentration can be used as bioindicator for pollution and human involvement in the ecosystem in which it lives.

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