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Tracing sewage and natural freshwater input in a Northwest Mediterranean bay: Evidence obtained from isotopic ratios in marine organisms

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ABSTRACT

Elemental carbon and nitrogen levels and isotope ratios were assessed in different biological compartments of a Northwest (NW) Mediterranean bay to trace the various sources of nutrient input from natural (river runoffs) and anthropogenic (harbor outflows, fish farms and urban sewage outfall) sources. Samples from transplanted mussels and natural sea grass communities (*Posidonia oceanica* leaves and epiphytes) were harvested from different locations throughout the bay during the touristic summer and rainy seasons. The results from the nitrogen analysis revealed that sewage and harbor outflow promote higher nitrogen levels, enrichment of ^{15}N in the tissues, and a higher seasonal variability in sea grass and epiphytes. In mussel tissues, the $\delta^{15}N$ was also influenced by sewage and harbor outflow, whereas $\delta^{13}C$ was influenced by terrestrial inputs. These results suggest that natural and anthropogenic nutrient inputs have a temporary and localized influence and affect the sensitivity of natural isotopic ratios to changes in hydrologic conditions, especially to rain and tourism.

Keywords : Sea grass ; Mussel caging ; Stable isotopes ; Sewage ; Pollution tracing ; Mediterranean Sea

1. Introduction

Coastal anthropic activities have global deleterious effects on marine ecosystems (Ralph et al., 2006). As often occurs, the sources of said effects can be multiple, and impacts result from the complex interactions between these sources (Peirano and Bianchi, 1995). As these disturbances are not always manifested as strong acute impacts but rather as moderate chronic impacts, integrators are required to trace diluted signals.

The ratio between stable isotopes, particularly those of carbon ($^{13}C/^{12}C$ ratio defined as $\delta^{13}C$) and nitrogen ($^{15}N/^{14}N$ ratio defined as $\delta^{15}N$), has been used by integrators to measure natural fluctuations in isotype levels (Fry and Sherr, 1984). In addition, when biological compartments (terrestrial plants, marine detritus, phytoplankton, etc.) are clearly identified by their isotopic signatures, the fate of the nutrients in marine ecosystems can be inferred, for instance, by using mixing models (Owens, 1987).

Natural variations in the isotopic composition of marine organisms are used to detect and quantify the impact of anthropic activity (Mac Clelland et al., 1997). For example, many studies have examined the extent of sea grass disturbance in the meadows that underlie fish farming facilities by assessing the isotope patterns of the sea grass (Dolenec et al., 2006; Vizzini et al., 2005). Water sewage outfall can also be monitored by examining its isotopic signature. Such an assessment can provide information about the dilution patterns of the plume (Costanzo et al., 2001; Tucker et al., 1999) and the recovery status of the sewage after outfall has ceased or improvements in sewage treatment have been made (Costanzo et al., 2005; Rogers, 2003). Furthermore, isotopes can also be used to discriminate nutrient input from storm water in an anthropized body of water (Dillon and Chanton, 2008).

The majority of studies involving the use of isotopes to examine anthropic activity use living organisms as bioindicators of ambient conditions. Filter feeder species such as bivalves (*Mytilus* sp. for example) are excellent candidates for use as bioindicators (Roméo et al., 2003). Owing to their large filtering capacity, they accumulate matter suspended in the water column and provide a long-term integrated signal of their food sources (Dubois et al., 2007; Gillikin et al., 2006; Machas and Santos, 1999); therefore, the use of bivalves is common in environmental studies. However, fractionation processes, such as the preferential use of one isotope over another

during biochemical processes, and the turnover rate of tissue cells must be taken into account when using isotopic analysis to assess the dietary signature of these organisms (Deudero et al., 2009; Dubois et al., 2007).

Macrophytes have also been used as bioindicators to trace dissolved carbon or nitrogen fluxes (Rogers, 2003; Costanzo et al., 2001). In the Mediterranean Sea, the endemic sea grass *Posidonia oceanica* (L.) Delile, a perennial species that is widely spread around the basin, is commonly used in isotopic studies (Vizzini and Mazzola, 2004). However, similar to other species living in oligotrophic environments, *P. oceanica* has a complex nitrogen budget, which involves the uptake of nitrogen through its leaves and roots and a well-developed processes of nitrogen recycling from older to younger leaves (Lepoint et al., 2002; Hemminga et al., 1999). Thus, the nitrogen isotope signature of *P. oceanica* leaves is influenced by these processes and will not exactly reflect the signature of dissolved inorganic nitrogen (Vizzini et al., 2003).

Epiphytic communities have also been used to assess the impacts caused by fish farms (Vizzini and Mazzola, 2004; Delgado et al., 1999) or urban disturbances (Piazzi et al., 2004). Epiphytes can reveal the complexity of the nitrogen budget in sea grasses because they are exclusively associated with the water column. However, it is important to take into account the heterogeneity of the epiphytic compartment, since it is composed of various taxa, including algae, which have different N acquisition strategies (Lepoint et al., 2007), hydrozoans, bryozoans and small invertebrates. This assembly of organisms has been shown to be very sensitive to disturbance (Piazzi et al., 2004).

In this study we used caged *Mytilus galloprovincialis* Lamarck 1819, *P. oceanica* leaves and epiphytes to examine the effects of various nutrient inputs on a Northwest (NW) Mediterranean bay. We assessed the isotopic ratios of carbon and nitrogen in these organisms, which were harvested from different parts of the bay and are diversely affected by nutrient input sources. This approach was intended to discriminate between the influence of sewage outfall *vs.* natural rainwater input in order to determine their impact on a highly anthropized and touristic area.

Although these organisms may not be the most appropriate for such a purpose, they were chosen because of their integrative properties, which allowed us to decrease short-term variations. In addition, these organisms were used within the framework of routine monitoring, thus reducing the costs of the study. Moreover, we sought to simplify the procedures so they can be implemented in the future by non-specialized operators. This latter point prompted us to test whether, despite its known heterogeneity, the global epiphytic community can be used without separating its different components.

2. Materials and methods

2.1. Study site

The six stations used in this study were located within the Golfe-Juan bay, in the NW Mediterranean Sea, France (Fig. 1). Three of these stations followed a depth gradient along the Golfe-Juan sewage pipe. Station S (8-m deep) was located at the entrance of the Golfe-Juan yachting harbor (19 ha, more than 1000 moorings) by the upper margin of the meadow; station M (16-m deep); and station D was located by the lower margin of the meadow (22-m deep), approximately 300 m away from the sewage outflow.

Three additive stations were located in other sectors of the bay. Station R was located in the west part of the bay, 500 m away from a small aquaculture farm, which processes approximately 200 tons of fish per year, and near the mouths of coastal streams that are dry during the summer and full of water during strong rains. Station V was located at Antibes Cape, in a cove reputed by fishermen as being 'very productive', and station T was located at the tip of Sainte Marguerite Island, facing the open sea. These three stations were 16-m deep, and stations V and T were considered as reference sites at the entrance of the bay.

From a global standpoint, the coastline of the Côte d'Azur is strongly urbanized, and the bay of Golfe-Juan has been considered to be 'subject to urban contamination' by previous studies (Bodin et al., 2004).

Fig. 1. *Location of the sampling stations in the Golfe-Juan bay (NW Mediterranean Sea, France). Stations S (8-m deep, located at the entrance of the Golfe-Juan yachting harbor), M (16-m deep) and D (22-m deep, located 300 m away from the sewage outflow) follow a depth gradient along the Golfe-Juan sewage pipe. Station R was located in front of the rain outflow, 500 m away from an aquaculture farm. Station V was located in Antibes Cape, in a cove reputed by fishermen as being 'very productive'. Station T, which was considered as the reference site, was located at the tip of Sainte Marguerite Island, facing the open sea.*

2.2. Water input evaluation

Outflow from the Golfe-Juan sewage treatment plant (20,000 and 38,000 population equivalents (PE) during the winter and summer, respectively, with a peak of ~59,000 PE) was quantified daily throughout the duration of the experiment based on the data provided by the plant manager. In this part of the urban coast, domestic wastewater and rainwater are collected and driven to the treatment plant. To identify the increase in sewage outflow due to significant rain events, data for daily rainfall levels was obtained from the Meteo France database. We also quantified the accumulation of rainfall during each time period of the survey.

The concentrations of suspended particulate matter (SPM), ammonium (NH_4^+) and total Kjeldahl nitrogen (TKN) were determined weekly in the treated water before it was released into the bay. The same water samples were filtered through GF/F Whatman filters that were previously combusted at 450 °C for 4 h; these were then oven-dried at 60 °C for >3 days and stored in a dry environment for later isotopic analysis of SPM.

2.3. Sea grass sampling

P. oceanica leaves were collected in April, June and November of 2007 from each site. Young juvenile leaves were used to determine the isotopic signature of the plant; older leaves were scrapped with a razor blade to collect epiphytes. Additional *P. oceanica* leaves were collected in April, June and September of 2008. Young leaves and epiphytes were oven-dried at 60 °C for > 3 days and ground to a fine powder using a ball mill (MM301, Retsch, Germany). The samples were then stored in a dry environment for later analysis.

2.4. Mussel caging

Caging operations were carried out in April, August and November of 2007 at each site. The mussels used in these experiments were purchased from an aquaculture farm located in Languedoc-Roussillon, which harvests mussels from the open sea (Andral et al., 2004). Sixty individuals were placed into 30 × 30-cm homemade cages made from 2-cm hole-size polypropylene mesh, and 20 individuals were kept unexposed and used as controls. All stations were equipped with mooring screws, and the cages were maintained 2 m above the ocean floor by floats.

The cages were exposed for 21, 42 and 38 days in April, August and November of 2007, respectively. The exposure period was shorter in April because of technical incidents with the local fishermen, which obliged us to remove the cages prematurely. After exposure, the cages were collected and transported back to the laboratory for treatment. Ten individuals were used to determine the condition index (CI). For this purpose, the soft tissues and shells were separated and oven-dried at 60 °C for >3 days, and the CI was determined for exposed and non-exposed (control) individuals according to the following formula by Andral et al. (2004):

CI = 100 (dry flesh weight/dry shell weight)

Ten other individuals were used for isotope analysis. Rather than using the entire organism for this analysis, we used the digestive gland, as recommended by Deudero et al. (2009), since this tissue has a higher turnover rate. For this purpose, individual digestive glands were isolated, oven-dried at 60 °C for >3 days and ground to a fine powder using a mortar and pestle. Control individuals were processed in the same manner. The samples were then stored in a dry environment for later analysis.

2.5. Isotope analysis

Isotopic ratios and elemental C and N compositions were assessed using an isotopic ratio mass spectrometer (VG Optima, Isoprime, UK) equipped with an elemental analyzer (Carlo Erba, Italy). Isotopic carbon and nitrogen ratios are expressed using the δ notation as follows:

$$\delta X = \frac{(R_{\text{standard}} - R_{\text{sample}})}{R_{\text{standard}}} \times 1000 \qquad (1)$$

In this formula, X is ^{13}C or ^{15}N, R is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio and the standards are vPDB (Vienna Pee Dee Belemnite) and atmospheric N_2 for carbon and nitrogen, respectively. The experimental precision, which is based on the standard deviation of replicate measurements of a standard, was 0.7‰ for carbon and 0.2‰ nitrogen. The elemental composition data are expressed relative to the dry weight of the measured element (‰ D.W.).

For mussel tissues, the observed C:N ratios (w:w) were always greater than 3.5, the value above which lipid normalization is recommended (Post et al., 2007). To account for the influence of lipid content on the $\delta^{13}C$, mathematical delipidation was performed after analysis using the following equation (Post et al., 2007):

$$\delta^{13}C_{\text{norm}} = \delta^{13}C_{\text{raw}} - 3.32 + 0.99 \times C:N$$

2.6. Statistical analysis

Statistical analysis was performed using R environment software (R Development Core Team, Vienna, Austria). In most cases, when the number of replicates was low, significant differences were determined using the non-parametric Kruskal-Wallis test, and the post hoc test was performed according to the Nemenyi procedure (Zar, 1999). When the number of replicates was sufficient, an ANOVA was performed after controlling for homoscedasticity using the Bartlett test. Post hoc tests were performed using the Tukey test.

3. Results

3.1. Freshwater input

3.1.1. Rainfall and sewage flow

The annual weather cycle in the Côte d'Azur is characterized by a sunny and almost dry period extending from spring to late summer with most of the annual rainfall occurring in autumn (October-November) and early spring (March). In the spring of 2007, the rain was scarce and caused no noticeable rise in freshwater input into the sewage pipe (Table 1). In contrast, autumnal rain raised the output approximately 24%. However, regardless of the rain levels, the sewage outflow peak occurred in August, during the height of the tourist season (over 5900 m^3/d).

Table 1 *Daily water outflow, influence of rain and accumulated rainfall levels during the survey period. Outflow was measured at the exit of the treatment plant. The influence of the rain was estimated as a % increase in outflow volume between dry and rainy days. Accumulated rainfall levels were obtained from Meteo France.*

	Mean daily water output (m^3/day)	% Increase in rainy days	Cumulated rainfall (mm)
April 07	5108	4	24.6
June 07	5675	0	17.4
August 07	5909	1	2.1
November 07	5103	24	83.7

3.1.2. Particulate matter and nutrient input

During our study, the sewage plant discharged an average of 300 kg SPM/d into the bay. The TKN output was of approximately 270 kg/d, of which 85% consisted of NH_4^+ (230 kg of N/d). This level of N represents a total of approximately 100 tons per year.

The elemental composition of SPM varied throughout the year, with lower nitrogen concentrations occurring in November (1.4 ± 0.1% D.W.) than in August (2.2 ± 0.3% D.W.). The delta values of SPM also changed throughout the seasons. The greatest variation was observed for the $\delta^{15}N$, which shifted from +3.0 ± 0.6‰ in April to +2.8 ± 0.7‰ in August and then to -0.4 ± 0.5‰ in November (the scarcity of data prevent us from performing statistical analysis).

3.2. P. oceanica community

3.2.1. Epiphytes

Nitrogen concentrations in the epiphytic community reached a minimum in June in all the stations, except at station M in which the minimum values were unexpectedly observed during the month of November (Fig. 2a). These seasonal changes in nitrogen concentration were significant at all the stations, except at station D, which was the deepest site, where nitrogen levels remained relatively stable throughout the seasons ($H = 2.14$, $p = 0.343$). The decomposition of variance test performed using season as the single factor revealed lower seasonal variability at station D when compared to other sites (Table 2).

The mean $\delta^{15}N$ varied significantly throughout the seasons at sites S ($H = 8.18$, $p = 0.016$), D ($H = 9.84$, $p = 0.007$) and V ($H = 7.97$, $p = 0.019$) (Fig. 2b). When analyzed based on site instead of season, the $\delta^{15}N$ values showed significant differences ($H = 27.05$, $p < 0.001$), with the highest $\delta^{15}N$ values occurring at sites S (5.2 ± 0.4‰), R (4.1 ± 0.1‰) and D (4.1 ± 0.2‰) and the lowest values occurring at site T (3.2 ± 0.2‰).

3.2.2. P. oceanica leaves

P. oceanica leaves exhibited variations in nitrogen concentrations (% D.W.) throughout the seasons, varying from 1.3% to 3.6% D.W. with an overall annual average of 2.0% D.W. (Fig. 2c). These seasonal fluctuations were greater at sites D (deeper site close to a sewage pipe extremity), R (near a fish farm) and S (shallow site near the harbor entrance) than at sites V (Antibes Cape) and T (close to the open sea), as shown by the

decomposition of variance test performed using season as the single factor (Table 2). The residual variance was significantly higher at site D in comparison to the other sites (Bartlett's $K^2 = 55.49$, $p < 0.001$).

The $\delta^{15}N$ values varied throughout the seasons and between sites (Fig. 2d). An ANOVA performed using site and season as orthogonal factors revealed a significant interaction term ($F_{25,168} = 7.14$, $p < 0.001$). The leaves collected from station T exhibited the minimum $\delta^{15}N$ values during each season, and those collected from station S exhibited the maximum values, except in November of 2007 when their nitrogen isotopic signature was as low as that observed in leaves collected from station T. Nevertheless, a significant site effect was detected ($F_{5,168} = 36.71$, $p < 0.001$), with mean $\delta^{15}N$ values decreasing between station S (5.5 ± 0.2‰) and station T (3.7 ± 0.1‰).

Non-metric multidimensional scaling (nMDS) was performed on the four variables related to nitrogen (percent content and $\delta^{15}N$ in epiphytes and leaves) for data collected in April, June and November (Fig. 3). For stations T and V, the most distant from any source of impact, the results are close together and distinct from those of stations D and S. Station S, which was located near the entrance of the harbor, had the most scattered distribution, indicating that it had a higher seasonal variability than the other less impacted locations. Station M, though located halfway between two impacted sources, was only moderately affected, which is likely due to the water circulation in the bay.

Table 2 *Decomposition of variances for the nitrogen variables ‰N and $\delta^{15}N$ in Posidonia oceanica leaves and the epiphytic community. The ANOVA was performed using season as a single fixed factor.*

Posidonia oceanica leaves				Epiphytes			
%N		$\delta^{15}N$		%N		$\delta^{15}N$	
MS season	MS resid	MS season	MS resid	MS season	MS resid	MS season	MS resid
1.47	0.09	2.53	0.37	0.20	0.01	0.37	0.20
1.39	0.10	5.77	0.55	0.34	0.01	6.43	0.12
0.60	0.08	3.04	0.45	0.08	0.01	0.04	0.21
3.52	0.41	3.02	0.37	0.03	0.02	2.70	0.35
0.33	0.06	4.39	0.31	0.21	0.03	0.18	0.22
0.39	0.11	2.02	0.20	0.09	0.01	1.85	0.24

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Fig. 2. *Seasonal patterns during April, June and November for (a) nitrogen concentration (% D.W.) and (b) $\delta^{15}N$ values (‰) in an epiphytic community of old Posidonia oceanica leaves harvested from different sites of the Golfe-Juan bay. Seasonal patterns during April, June and November 2007 and April, June and September 2008 for (c) nitrogen concentration (% D.W.) and (d) $\delta^{15}N$ values (‰) in young Posidonia oceanica leaves harvested at different sites of the Golfe-Juan bay. Asterisks indicate significant differences. Mean + 1SE. n = 6.*

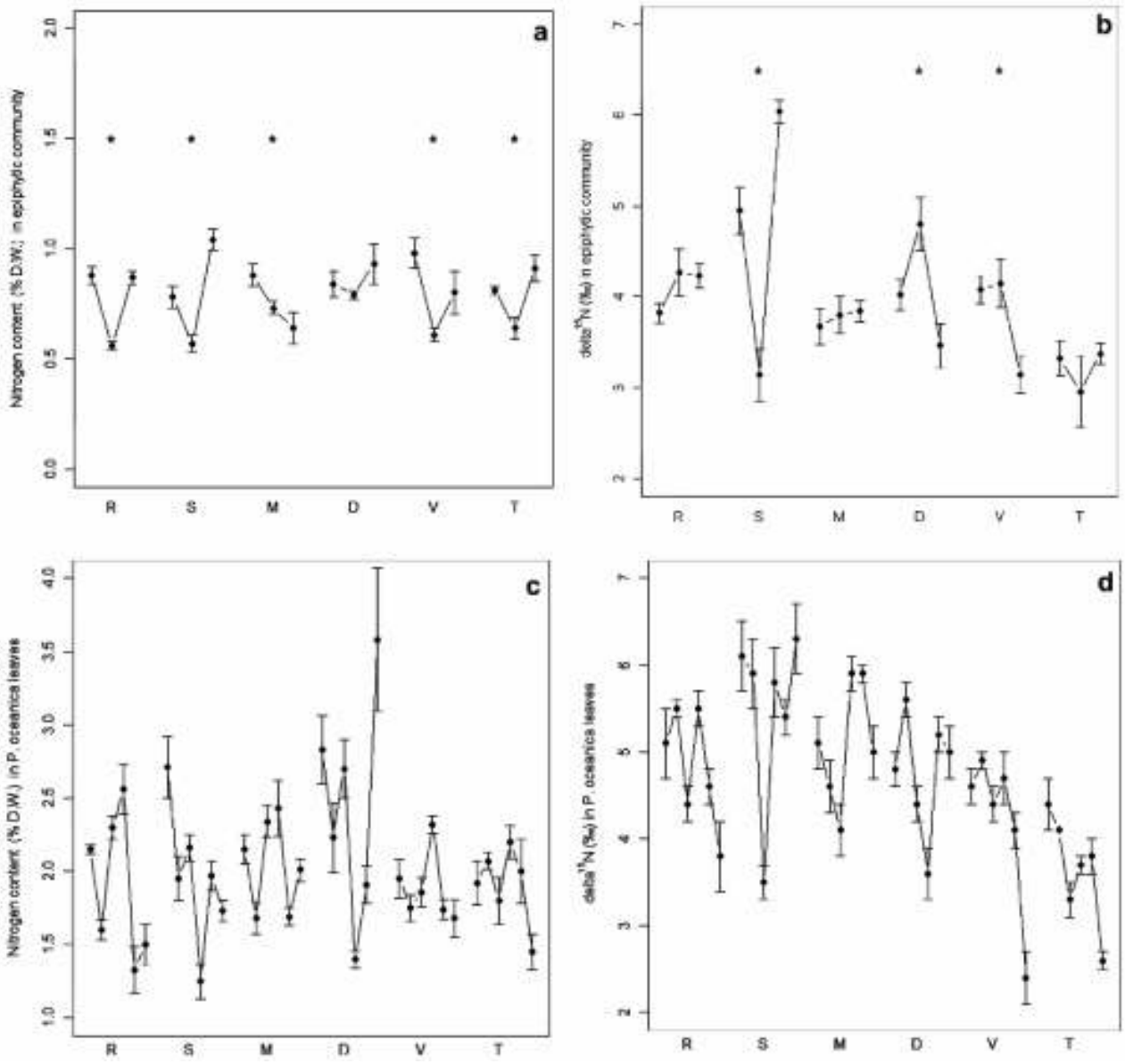


Fig. 3. *Nitrogen (%N and $\delta^{15}N$) nMDS analysis in young Posidonia oceanica leaves and the epiphytic community for samples collected in April, June and November of 2007 at different sites of the Golfe-Juan bay. Sites are pooled according to putative impact and were assessed based on their distance from the pollution source. White dots indicate low impact sites T and V; grey dots indicate intermediate impact sites R and M; and black dots indicate high impact sites D and S (see Fig. 1 for the locations of the stations).*

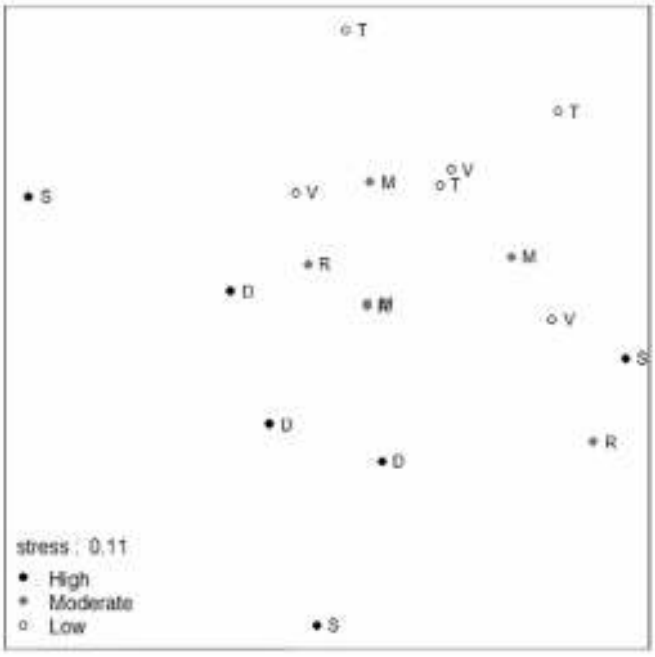


Table 3 *Results of the Mytilus galloprovincialis caging experiments performed in April, August and November of 2007. Mortality rates (%), dry meat weights (g) and condition indices (CI) for mussels collected from each site and for unexposed mussels (control). The C:N ratios (w:w), lipid-normalized $\delta^{13}C_{norm}$ (‰) values and the $\delta^{15}N$ (‰) obtained from the digestive glands of the mussels. Mean (SE). n = 6.*

Site	Season	Mortality rate (%)	Tissues dry weight (g)	Condition Index (without unit)	C:N ratio (without unit)	δ^{13}Cnorm (‰)	δ^{15}N (‰)
Z	April	-	na	na	4.8 (0.2)	-18.6 (0.8)	6.5 (1.1)
	August	-	0.64 (0.17)	97 (20)	5.8 (0.6)	-19.8 (0.4)	8.0 (0.2)
	November	-	0.67 (0.32)	87 (28)	5.0 (0.4)	-20.1 (0.3)	7.4 (0.4)
R	April	35	0.39 (0.17)	70 (34)	4.4 (0.2)	-19.7 (0.4)	5.2 (0.7)
	August	0	0.71 (0.13)	102 (18)	5.4 (0.3)	-20.8 (0.6)	5.8 (0.4)
	November	3	0.47 (0.17)	60 (14)	4.8 (0.1)	-19.8 (0.3)	6.2 (0.5)
S	April	17	0.38 (0.08)	61 (17)	4.3 (0.3)	-19.5 (0.3)	5.8 (1.1)
	August	0	0.70 (0.14)	100 (18)	4.9 (0.1)	-20.0 (0.4)	6.2 (0.3)
	November	0	0.63 (0.14)	74(7)	4.8 (0.1)	-19.5 (0.2)	6.5 (0.4)
M	April	30	0.42 (0.15)	63 (18)	4.4 (0.1)	-19.4 (0.6)	4.5 (1.0)
	August	1	0.71 (0.17)	98 (25)	5.4 (0.3)	-20.9 (0.6)	5.7 (0.4)
	November	9	0.58 (0.18)	73 (16)	4.7 (0.3)	-19.4 (0.2)	6.2 (0.5)
D	April	20	0.59 (0.17)	78 (19)	4.5 (0.1)	-19.2 (0.3)	4.5 (1.1)
	August	0	0.63 (0.11)	93 (17)	5.2 (0.3)	-20.8 (0.9)	5.4 (0.5)
	November	8	0.73 (0.30)	95 (38)	4.9 (0.3)	-19.7 (0.5)	6.3 (0.4)
V	April	12	0.62 (0.28)	110 (18)	4.4 (0.1)	-18.8 (0.3)	5.1 (0.3)
	August	0	0.59 (0.16)	89 (21)	5.4 (0.3)	-20.0 (0.5)	6.2 (0.3)
	November	4	0.64 (0.24)	87 (30)	5.0 (0.4)	-19.9 (0.4)	6.2 (0.4)
T	April	17	0.55 (0.26)	76 (44)	4.5 (0.2)	-18.9 (0.7)	5.2 (0.9)
	August	5	0.65 (0.22)	103 (32)	5.2 (0.3)	-20.4 (0.6)	5.7 (0.5)
	November	6	0.56 (0.09)	80 (16)	4.5 (0.2)	-19.6 (0.4)	6.2 (0.3)

na, data not available.

3.3. Mussel caging

3.3.1. Physiological indices

The mussels exhibited low mortality rates (below 10%) during experiments performed in August and November, but they exhibited higher mortality rates in April (up to 30%).

The dry weight of the mussel tissues showed a seasonal pattern, with weights increasing between spring and summer and decreasing in autumn (Table 3). Significant differences in weight for mussels located at different sites were found only in April ($H = 16.69$, $p = 0.005$), during which mussels from station V had a higher dry weight than those from stations R and S.

The physiological CI_{dry} exhibited a similar seasonal pattern, with lower values in April, a maximum reached in summer and decreasing in autumn (Table 3). With respect to dry weight, significant differences between sites were found only in April ($H = 26.31$, $p < 0.001$), during which we observed a higher CI_{dry} at site V.

3.3.2. Lipid content and mathematical delipidation

The C:N ratio of the digestive gland, which allowed us to estimate the lipid content, also varied throughout the seasons, with significantly higher values occurring in August ($H = 78.53$, $p < 0.001$). When plotting the $\delta^{13}C$ against the C:N ratio for the digestive gland, we observed a clear correlation ($r = -0.79$, $p < 0.001$, Fig. 4). The correlation between the C:N ratio and the $\delta^{13}C$ has been described previously by Post et al. (2007) as an effect of lipid content, prompting us to apply a mathematical correction on the raw $\delta^{13}C$ values to estimate lipid-free isotopic ratios. This led to noticeable corrections (up to +2.5‰ in August when the lipid content was higher).

Fig. 4. *Individual $\delta^{13}C$ values (‰) of mussel digestive glands plotted against the ON ratio (w:w) obtained from caging experiments performed in the Golfe-Juan Bay in April (O), August (x) and November 2007 (♦).*

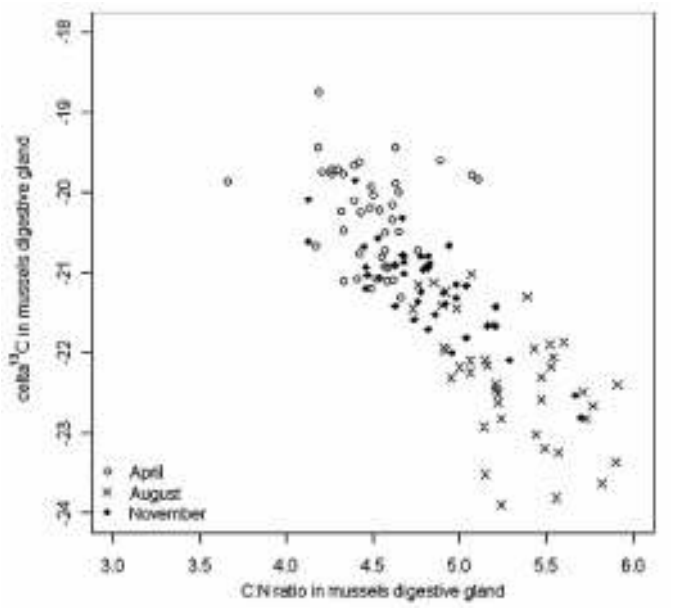
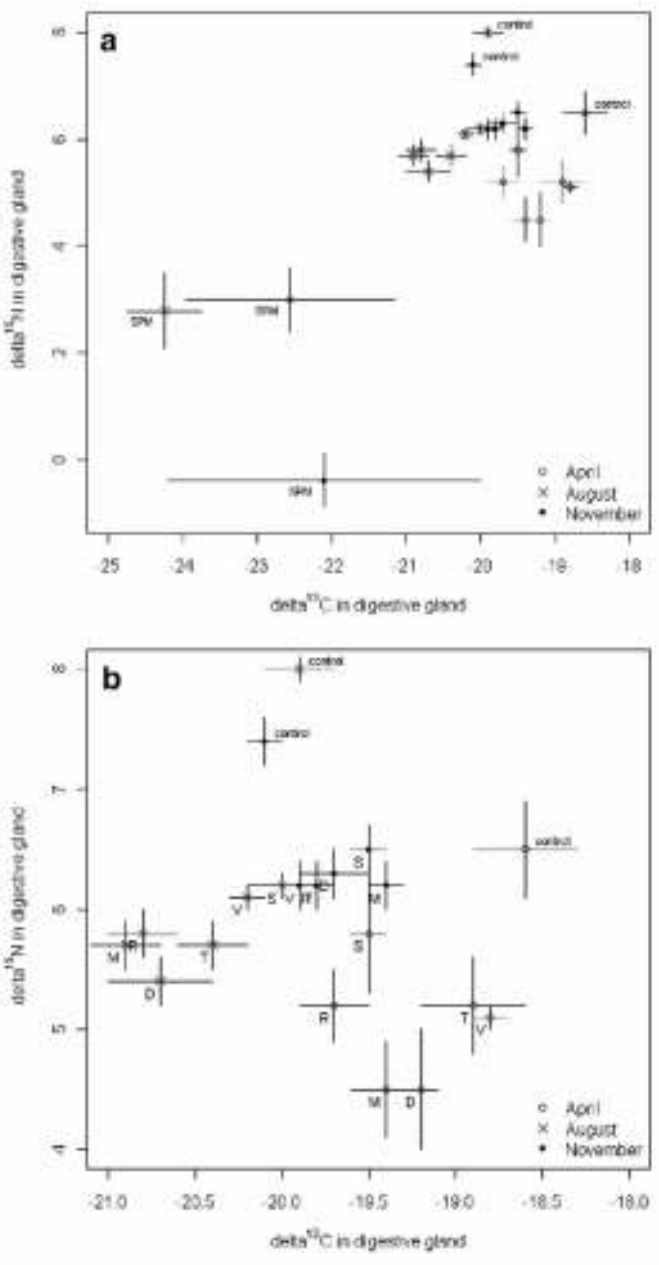


Fig. 5. *Carbon and nitrogen delta values for caging experiments performed in April (O), August (×) and November 2007 (♦). (a) General overview including sewage suspended particulate matter (SPM). (b) Focus on mussels. Bars represent the standard error, n = 6.*



3.3.3. $\delta^{u}C$ in mussel tissues

The lipid-normalized carbon isotopic ratios ($\delta^{13}C_{norm}$) obtained from mussel digestive glands exhibited seasonal variations. We observed lower ^{13}C-depleted values in April (-19.2 ± 0.1‰, mean ± 1SE), higher values in August (-20.5 ± 0.1‰) and intermediate values in November (-19.7 ± 0.1‰). Interestingly, the spread of the values was lower during November (Bartlett's $K^2 = 13.24$, $p = 0.001$), resulting in no significant differences between the sites during this season (Fig. 5).

We observed significant differences with respect to sites during the spring and summer. In April, the $\delta^{13}C_{norm}$ was significantly less negative in digestive glands from mussels collected at stations V and T than in those collected from station R ($H = 13.84$, $p = 0.017$). In August, the digestive glands of mussels collected from station S had a significantly ^{13}C-enriched signature compared to mussels from stations M and R ($H = 11.93$, $p = 0.035$).

When considering the difference between exposed and non-exposed mussels (referred to as control in Fig. 5), two major features were observed. In April and August, the digestive glands from exposed mussels exhibited lower ^{13}C levels ($\Delta\delta^{13}C$ = -0.6 ± 0.4‰ in April and $\Delta\delta^{13}C$ = -0.6 ± 0.3‰ in August), whereas an enrichment in ^{13}C levels was observed during the month of November ($\Delta\delta^{13}C$ = +0.4 ± 0.2‰). When an ANOVA was performed including the control mussels and using site and season as fixed orthogonal factors, the opposed behavior observed in November appeared as a significant site × season effect ($F_{12,105} = 3.54$, $p < 0.001$; Table 4). When the same ANOVA was performed excluding the data for November, no site × season effects were detected. As previously described, site ($F_{6,70} = 5.5$, $p < 0.001$) and season ($F_{1,70} = 118.63$, $p < 0.001$) had significant effects on the $\delta^{13}C$; this values decreased significantly during exposure at stations R, M and D. Finally, a significant difference in annual $\delta^{13}C$ values was observed between stations V (highest mean $\delta^{13}C$) and R (lowest mean $\delta^{13}C$).

3.3.4. $\delta^{15}N$ in mussel tissues

An ANOVA was performed on the raw data using site and season as fixed orthogonal factors (Table 4). For the $\delta^{15}N$ values, the interaction term site × season was not significant between the three seasons. The exposure of mussels resulted in the depletion of ^{15}N in the digestive glands as follows: $\Delta\delta^{15}N$ = -1.4 ± 0.6‰, -2.1 ± 0.2‰ and -1.2 ± 0.2‰ for April, August and November, respectively. The mean $\delta^{15}N$ was significantly different from one season to another ($F_{2,105} = 38.24$, $p < 0.001$), with the highest and lowest values occurring in November and April, respectively (Fig. 5). Overall, the $\delta^{15}N$ value was significantly higher ($F_{6,105} = 18.72$, $p < 0.001$) at station S (6.2 ± 0.2‰) than at stations D (5.4 ± 0.2‰) and M (5.5 ± 0.2‰).

Table 4 *ANOVA performed on the isotopic ratios obtained from the digestive glands of the mussels using site (7 levels: 6 sites and 1 control pool) and season (3 levels: April, August and November) as fixed and orthogonal factors.*

	df	MS	*F*	p_level
$\delta^{13}C_{norm}$				
Site	6	0.70	3.15	**
Season	2	16.76	75.21	***
Site x season	12	0.79	3.54	***
Residuals	105	0.22		
$\delta^{13}C_{norm}$ *(without November)*				
Site	6	1.55	5.50	***
Season	1	33.47	118.63	***
Site x season	6	0.39	1.39	
Residuals	70	0.28		
$\delta^{15}N$				
Site	6	7.57	18.72	***
Season	2	15.47	38.24	***
Site x season	12	0.52	1.28	
Residuals	105	0.40		

Significance levels: *$p < 0.5$; **$p < 0.1$; ***$p < 0.01$.

4. Discussion

The range of isotopic ratios that we determined for the sea grass community was comparable to that described in the literature. The $\delta^{15}N$ for *P. oceanica* leaves in pristine sites were reported to be 2.2 ± 0.9‰ in Corsica (Lepoint et al., 2003), 2.5 ± 0.4‰ in Croatia (Dolenec et al., 2006), 3.4 ± 0.9‰ in Sicily (Vizzini et al., 2005) and 3.7 ± 1.1‰ in the Balearic Islands (Fourqurean et al., 2007). In the current study, the values that we found for the site close to the open sea (between 2.6 ± 0.1‰ and 4.4 ± 0.3‰) fall within the range of those previously reported and allow us to consider this site as a reference, at least with respect to anthropic nitrogen levels. Thus, putative impacts can be identified by their ability to either increase the $\delta^{15}N$ or increase variability. The effect of anthropic impacts on the $\delta^{15}N$ in sea grasses and macrophytes is well documented (Holmer et al., 2008; Pergent-Martini et al., 2006; Costanzo et al., 2005; Vizzini and Mazzola, 2004; Tucker et al., 1999). For example, fish farming activities have been shown to promote the enrichment of ^{15}N in *P. oceanica* leaves; the isotopic ratios in the shoots of this plant range between 5.3 ± 0.5‰ and 7.2 ± 0.3‰ (Dolenec et al., 2006) and 5.6 ± 0.6‰ (Vizzini

and Mazzola, 2004) when grown in cages. In our study, the station located 500 m away from a fish farming facility exhibited $\delta^{15}N$ values that fluctuated seasonally between 3.8 ± 0.4‰ and 5.5 ± 0.2‰, which is a slightly lower range of values than those reported in the literature. However, this range of values represents a more significant enrichment of ^{15}N than that found in natural sites. The distance of this station from the farm may explain the lower $\delta^{15}N$ values. Indeed, Vizzini et al. (2005) showed that the $\delta^{15}N$ is higher closer to a farm and lower in natural conditions. In addition, this group also showed that while the $\delta^{15}N$ signature at a distance of 500 m was lower than at closer proximity to the farm, it was significantly higher than at a distance of 1 km away (500 m: 4.2 ± 0.8‰ *vs.* 1 km: 3.4 ± 1.0‰).

In our study, the most ^{15}N-enriched shoots were found at stations located near the entrance of the harbor or close to the sewage outfall. It could be argued that these stations are not located at the same depth, but natural depth effects on the nitrogen isotope signature have not yet been demonstrated (Lepoint et al., 2003; Grice et al., 1996). Harbor activities and sewage outfall are known to have deleterious effects on the nitrogen isotopic signature of sea grasses (Ralph et al., 2006; Ruiz and Romero, 2003; Pergent-Martini and Pergent, 1996). In Moreton Bay, Australia, a notable increase in the $\delta^{15}N$ was observed in sea grasses located in the open sea (2.5‰) and in stations close to urban centers (8.7‰) (Costanzo et al., 2001). This increase is often attributed to the enrichment of ^{15}N in sewage effluents, which tend to have higher $\delta^{15}N$ values than natural inorganic nitrogen sources. Indeed, Dillon and Chanton (2008) reported a $\delta^{15}N$ value of approximately -5.4 ± 2.6‰ in ammonium from rainwater sources, whereas waste-water ammonium had values of +16‰ to +25‰.

In the current study, the impacted sites are characterized by a much higher seasonal variability when compared to natural sites. The seasonal cycle of nitrogen content and the isotopic signature of P. *oceanica* leaves are well documented. In Mallorca, Fourqurean et al. (2007) reported an annual fluctuation of 0.6‰, whereas Vizzini et al. (2003) reported a fluctuation of +2‰ between summer and winter in Marsala, Italy. We found that the $\delta^{15}N$ value at the natural site was 1.9‰, and the maximal variability was observed near the entrance of the harbor, with a 2.7‰ difference between the maximum and minimum values. Interestingly, the variation of the $\delta^{15}N$ near the sewage outfall was low (2‰), possibly indicating a ^{15}N-enriched nutrient supply.

This seasonal pattern was opposed in the epiphytic community. It is well known that the epiphytic composition fluctuates throughout the year (Prado et al., 2008; Lepoint et al., 1999). This fluctuation is not surprising, since the epiphytic community is composed of structurally different organisms, such as calcareous algae and hydrozoans. In this study, we observed a significant temporal variability in the concentration of nitrogen, which is apparently due to fluctuations in the composition of the community, stressing the necessity of separating the different components of the community.

This temporal fluctuation, however, stabilized near the sewage outfall, possibly indicating a more stable composition of microalgal nitrophilous species (Coleman and Burkholder, 1994) or of nitrogen-enriched species, such as calcareous red algae (Lepoint et al., 2007; Piazzi et al., 2004). Indeed, we observed a higher variability in the nitrogen isotopic signature of the epiphytic community, with significant seasonal effects near the entrance of the harbor and the sewage outfall. Using spatial designs, anthropic impacts have been previously shown to enhance the variability of epiphytic communities (Balata et al., 2008; Piazzi et al., 2004); however, studies using temporal designs to describe anthropic impacts are still lacking (Prado et al., 2008).

The results obtained from the station near the sewage outfall are particularly interesting. Although the nitrogen content of epiphytes remained stable throughout the year, a peak in the $\delta^{15}N$ was detected in June. The heavier isotopic composition observed in the benthic community during the spring has been suggested to be a consequence of the fractionation that occurs upon nitrogen uptake by phytoplankton during the spring bloom. The preferential use of the lightest isotope by phytoplankton results in an increased $\delta^{15}N$ in the remaining substrate. In the Scheldt estuary in the Netherlands, the $\delta^{15}NH_4^+$ remains at approximately 11.4 ± 0.2‰ during the winter but increases to 70‰ in June (Brabandere et al., 2007). Culture experiments using *Skeletonema costatum* have also shown variations in the $\delta^{15}N$ of up to +50‰ for NH_4^+ and +12‰ for NO_3^-(Pennock et al., 1996). The higher $\delta^{15}N$ observed during the month of June in the epiphytic community located near the sewage outfall (also observed in *P. oceanica* leaves in 2007 and 2008) could be explained by the higher isotopic signature of dissolved nitrogen, which, in turn, may be caused by the phytoplanktonic bloom associated with the anthropic nitrogen supply that occurs during the summer.

During the caging experiments, the physiological indices of the transplanted mussels did not significantly differ from one site to the other; the only difference occurred in April at the site located close to Antibes Cape. Therefore, the data was not normalized according to the CI as is recommended when the physiological status of organisms differs widely (Andral et al., 2004). The physiological indices of the mussels showed temporal

patterns, increasing in weight during the summer and decreasing in the winter. This result is consistent with a previous caging study done in the Golfe-Juan bay near our station R (Bodin et al., 2004). This annual cycle could explain the homogeneous isotopic signature observed in mussel tissues in the month of November, during which reserve tissues can be remobilized to meet the nutritional balance in response to food shortage. As the other organs are generally ^{13}C-enriched compared to the digestive gland (Deudero et al., 2009; Piola et al., 2006), the incorporation of ^{13}C-enriched reserve tissues could lead to an increase in the isotopic signature of the digestive gland. Therefore, any results derived from caging experiments performed during periods of food shortage should be interpreted with caution, as nutritional stress can significantly impact isotopic composition, masking spatial differences during isotopic mapping.

Our decision to analyze the digestive gland instead of the total animal was based on a recent study that evaluated the turnover rates of different organs (Deudero et al., 2009). In addition, the digestive glands of *Pecten maximus* and *Crassostrea gigas* have been found to have the highest carbon incorporation index, suggesting that this organ is the most appropriate to detect short-term food source variations (Paulet et al., 2006). When using entire organisms of *Mytilus edulis*, Dubois et al. (2007) found a half-life of 8.9 days for the δ^{13}C and 14.1 days for the δ^{15}N. In our study, these values would represent an 81%, 96% and 94% replacement of tissue carbon and 64%, 87% and 82% replacement of tissue nitrogen during the months of April, August and November, respectively. As the turnover rate of the digestive gland is higher than for the whole animal, we can assume that isotopic equilibrium (i.e., the isotopic composition of mussel tissues is completely determined by the isotopic composition of the new diet) is approximately reached during our caging experiments. The exception to this may be for nitrogen during the month of April. This assumption is confirmed by the significant decrease observed in the δ^{15}N between exposed and unexposed individuals, even in April. This decrease reflects differences in the N dynamics between the original site of mussel cultivation (Aresquiers bank, in the Gulf of Lion) and the caging site (the Ligurian Sea).

If we consider only the data from April and August, the periods during which we observed a net weight gain, the isotopic signature of the digestive glands show spatial differences. On average, the highest δ^{15}N values were obtained at the location near the harbor entrance, which is influenced by harbor sedimentation and resuspended organic particles, and the lowest δ^{15}N values were obtained at the station located near the sewage outflow. It is important to note that, whereas inorganic and organic dissolved nitrogen from anthropic effluents is usually ^{15}N-enriched, the opposite is observed for sewage-derived particulate nitrogen (SPM-nitrogen). Tucker et al. (1999) demonstrated that the δ^{15}N ranged from +7 to +40‰ for dissolved nitrogen and from +1 to +3‰ for SPM-nitrogen, which is in agreement with our values (SPM δ^{15}N ranging from 0 to +3‰). Therefore, the low δ^{15}N values obtained from mussels grown near the sewage outfall suggest a higher contribution of sewage-derived SPM to the mussels' diet.

Spatial differences were also detected for the δ^{13}C, although these were less significant. Mussels grown at station R had ^{13}C-depleted tissues, possibly indicating an influence of terrestrial material (δ^{13}C = -26‰ for terrestrial C3 detritus and -23‰ to -30‰ for terrestrial C3 plants *vs.* -18‰ to -24‰ for phytoplankton and -3‰ to -15‰ for sea grasses) (Fry and Sherr, 1984). As this effect is significant even during the dry period, we hypothesize that the signature in this part of the bay is the result of ^{13}C-depleted material brought by rainy input and sediment. However, this hypothesis must be verified by performing an isotopic analysis of the sediment from different parts of the bay.

It is important to note that our study lacks information about the isotopic signature of other food sources, such as the sediment or the seawater POM. Tucker et al. (1999) published a review of the isotopic signature ranges observed in these food sources, and seawater POM is always found to have a higher δ^{15}N than effluent POM. These findings support the influence of sewage-derived SPM on the mussels' diet at the lower station, which is one of major points that remains to be addressed in our study.

5. Conclusions

One of the challenges of impact studies in anthropized areas is that, in most cases, the situation is not Manichean, with pristine sites clearly identified and point source pollution characterizing impacted sites. Reality is far less straightforward, often resulting in mixed effects from multiple and diffused pollution sources. In our study, we expected sewage outflow to have an indubitable effect; however, we found a more subtle situation. Overall, our study highlighted independent patterns based on the nature of the input, dissolved or particulate. The results of our analysis of the *P. oceanica* community, which reflects the nature of dissolved nitrogen, suggest that (i) a ^{15}N-enriched nutrient supply exists at the level of the sewage outflow and the harbor entrance; (ii) there is a higher seasonal variability at these sites compared to more pristine sites; and (iii) there is a slight ^{15}N-

enrichment at the site near the fish farm. Our analysis of transplanted mussels, with respect to SPM, indicates that (i) there is less disparity in the bay during autumn; (ii) there is a ^{15}N-enrichment at the harbor entrance; (iii) the ^{15}N-depleted sewage SPM influences mussel growth near the source of sewage outflow; and (iv) the rain input potentially influences the SPM at the site close to the rain outflow. Thus, a combined analysis of the isotopic signatures of diverse marine organisms gave us clues about the spatial differences that exist in nutrient supplies in a strongly anthropized bay. It is important to note, however, that the information provided by our analysis of stable isotopes was qualitative rather than quantitative. The spatial design that we used did not allow us to map a clear pollution gradient but rather highlighted the global and diffuse pollution that occurs in the Golfe-Juan bay.

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