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Epilithon $\delta^{15}N$ signatures indicate the origins of nitrogen loading and its seasonal dynamics in a volcanic Lake

Fiorentino Federico[a], Cicala Davide[a], Careddu Giulio[a], Calizza Edoardo[a], Jona-Lasinio Giovanna[c], Rossi Loreto[a,b,*], Costantini Maria Letizia[a,b]

[a] Department of Environmental Biology, Sapienza University of Rome, Via dei Sardi 70, 00185 Rome, Italy
[b] CoNISMa,Piazzale Flaminio 9, 00100 Rome, Italy
[c] Department of Statistical Sciences, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy

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A B S T R A C T

The intensification of agricultural land use and urbanisation has increased nutrient loads in aquatic ecosystems. Nitrogen loads can alter ecosystem structure and functioning, resulting in increased algal productivity, algal blooms and eutrophication. The principal aim of the present paper is to extend the use of epilithic $\delta^{15}N$ signatures to a lake ecosystem in order to evaluate the potential impact of anthropogenic nitrogen discharges (organic and inorganic) that can also reach coastal waters.

Epilithic associations were collected from volcanic rocks in different seasons in shallow water along the entire perimeter of Lake Bracciano and analysed for their nitrogen stable isotope signatures. Furthermore, some stones were moved from an unpolluted site to a polluted one in order to verify the effect on the nitrogen signature of the epilithic association. The epilithon's $\delta^{15}N$ signatures provided strong evidence of the space-time variability of N inputs. The differing quality of nitrogen loads was reflected in high isotopic variation within the lake, especially at the beginning of summer (1.7‰ $\leq \delta^{15}N \leq$ 13.3‰), while in winter, when anthropogenic pressure was lowest, the $\delta^{15}N$ signature variation was less accentuated (3.1‰ $\leq \delta^{15}N \leq$ 7.6‰). At all sampling times, spatial variability was found to be related to the various human activities along the lake shore (especially tourism and agriculture), while seasonal variation at all sampling sites was related to the intensity of anthropogenic pressures (higher in summer and lower in winter).

Our results showed that epilithic algal associations and the physicochemical properties of the water did not influence the $\delta^{15}N$ signature, which in contrast was strongly related to the site-specific effect of human activities around the lake. Thus, the distribution of $\delta^{15}N$ across space and time can be used to direct nutrient reduction strategies in the region and can assist in monitoring the effectiveness of environmental protection measures.

1. Introduction

Inland waters account for only 2% of the Earth's surface (Wetzel, 2001) but provide several ecosystem services of importance to human communities (Page et al., 2012; Pimentel et al., 2004; Smith, 2003). On the other hand, the intensification of agricultural land use, industrial activity and urbanisation has caused an increase in nitrogen loads in aquatic ecosystems (Derse et al., 2007; di Lascio et al., 2013; Galloway et al., 2003; Matson et al., 1997; Vilmi et al., 2015; Vitousek et al., 1997). Associated with phosphorus, nitrogen inputs can alter ecosystem structure and functioning, resulting in increased algal productivity and algal blooms (Dodds et al., 1989; Maberly et al., 2002; Page et al., 2012). Physicochemical environmental monitoring methods only provide snapshots of ecosystem trophic conditions, and therefore several sampling times are required, although even then, pulse inputs are likely to be missed (Danilov and Ekelund, 2001; DeNicola et al., 2004; Gartner et al., 2002; Vilmi et al., 2015). There is a need therefore for improved and integrated management of N pollution in fresh waters.

Isotope analysis is widely employed in trophic ecology, microbial ecology and nutrient cycling studies (Calizza et al., 2013a,b; Careddu et al., 2015; Costantini et al., 2014; di Lascio et al., 2013; Mancinelli et al., 2013; Rossi et al., 2007). In addition, the nitrogen isotopic signature, $\delta^{15}N$, has been successfully used to monitor coastal marine habitats by determining the type of nitrogen input (Orlandi et al., 2014). Inorganic fertilisers have a $\delta^{15}N$ range of −4‰ to +4‰, while for organic fertilisers and organic waste (including compost and animal excretion) $\delta^{15}N$ can range from +6‰ to + 38‰ in exceptional cases (Cole et al., 2004; Dailer et al., 2010, Derse et al., 2007; Jona-Lasinio

* Corresponding author at: Department of Environmental Biology, Sapienza University of Rome, Via dei Sardi 70, Rome 00185, Italy.
E-mail address: loreto.rossi@uniroma1.it (R. Loreto).

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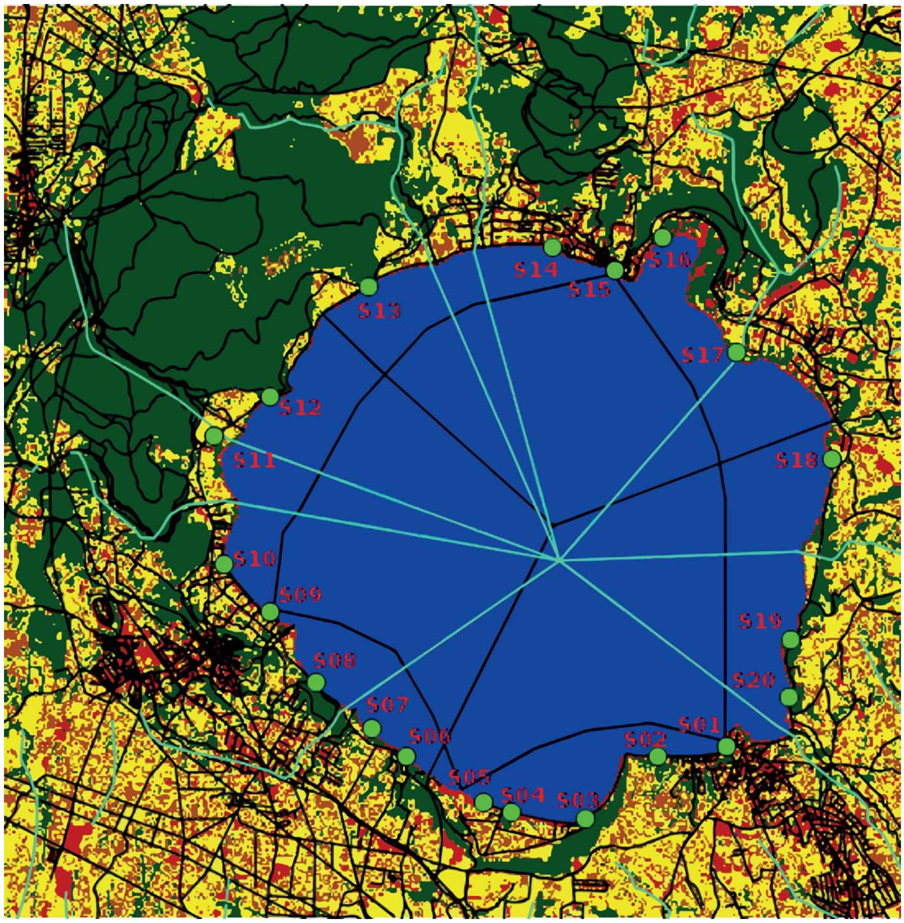

Fig 1. Bracciano (RM) land cover based on Corine Land Cover second level. Legend: green = vegetation, yellow = crops, blue = lake, red = built up, brown = soil, black = roads, light blue = streams. Green points indicate the sampling sites.

et al., 2015; Kendall et al., 2007).

Given their ability to assimilate and store N inputs in their tissues, macroalgae (in marine ecosystems) and epilithon (in river ecosystems) can provide information on anthropogenic N loadings based on changes in their isotopic signatures (Cejudo et al., 2014; Cole et al., 2004; Costanzo et al., 2005; Jones et al., 2004; Pastor et al., 2014; Schiller et al., 2009; Yokoyama and Ishihi, 2006), even on short time scales (Orlandi et al., 2014), reflecting space-time variability in N availability (Jona-Lasinio et al., 2015; Vizzini et al., 2005). Several studies have been performed on macroalgal $\delta^{15}N$ signatures in marine coastal ecosystems (Cole et al., 2004; Dailer et al., 2010; Gartner et al., 2002; Titlyanov et al., 2011) and coastal brackish lakes (Jona-Lasinio et al., 2015) and lagoons (Vizzini et al., 2005). Recent studies have applied stable isotope analysis to epilithic $\delta^{15}N$ signatures to evaluate the effect of anthropic land use on rivers (Bentivoglio et al., 2016; Cejudo et al., 2014; Pastor et al., 2013, 2014; Peipoch et al., 2012; Schiller et al., 2009). However, in spite of its widespread presence, epilithon is poorly understood as an ecological indicator of nitrogen pollution.

Epilithon is defined as an association of non-planktonic algae, fungi and bacteria immersed in a polysaccharide matrix able to colonise stones on the bottom of lakes (Burns and Ryder, 2001; Castenholz, 1960, Harrison, 1998; Kahlert et al., 2002). Its abundance and composition are probably related to light (Watkins et al., 2001), temperature and nutrient availability (Maberly et al., 2002). Epilithic algal growth reaches its maximum in spring and its minimum in summer, with a second slightly smaller growth peak in late summer in stratifying lakes (Castenholz, 1960; Harrison, 1998).

Epilithon is a potentially interesting biological resource for monitoring aquatic ecosystems thanks to its high ability to colonise diverse substrates in all freshwater ecosystems and the ease of collection in littoral areas (Round, 1991; Sabanci, 2011), which allows more rapid sampling procedures than those required for phytoplankton and surface sediments (DeNicola et al., 2004). The littoral zone is a key habitat for primary and secondary productivity (Mancinelli et al., 2007; Vilmi et al., 2015), while epilithic associations contribute significantly to the productivity of the system as a whole and represent a trophic resource for benthic invertebrates (Fink et al., 2006; di Lascio et al., 2011; Hawes and Smith, 1994; Rossi et al., 2010), providing a key link between allochthonous nutrient inputs and aquatic consumers (Bentivoglio et al., 2016).

Our study focused on the Bracciano volcanic lake in the Central Italian Volcanic Lake District (CIVOILD; Costantini et al., 2012; Mazza et al., 2015), because of its high diversity of anthropic activities. A regional park since 1999, it is the main drinking water reservoir for the city of Rome, with direct water extraction (ACEA Sustainability Report, 2015). Previous research assessed the lake as oligo-mesotrophic (Ferrara et al., 2002; Margaritora et al., 2003; Mastrantuono et al., 2008). Moreover, in order to prevent anthropic discharges into the basin, in the early 80s, a centralised system was created to collect urban waste waters for a population of up to 40,000 inhabitants. Nevertheless, the present-day population of the CO.B.I.S. (the area served by the system, comprising the municipalities of Anguillara-Sabazia, Bracciano, Manziana, Oriolo-Romano and Trevignano Romano) is around 49,000 (ISTAT, 2016), which rises further in summer due to tourism, exceeding the system's capacity.

The aim of this study was to extend the use of epilithic $\delta^{15}N$ signatures to space-time monitoring of a tourism-oriented lacustrine ecosystem. We considered anthropogenic nitrogen input type and its variation across seasons, related to the intensity of tourism and agricultural activities in the littoral zone, on both short (i.e. weekend) and long (i.e. season) time scales. Specifically, we tested the hypothesis that (i) variations in organic and inorganic N inputs were reflected in increased or decreased δ^{15}N in epilithon, and (ii) the tourism-driven summer increase in anthropogenic pressure was reflected in a seasonal increase in isotopic signatures in tourism-impacted sites that was not seen in sites unaffected by tourism.

2. Materials and methods

2.1. Study area

Lake Bracciano (42°07′16″N; 12°13′55″E) is a volcanic lake located in central Italy, 32 km northwest of Rome (Lazio, Italy). It has a surface area of 57 km^2 and a perimeter of about 31.5 km. Its elevation is 164 m a.s.l. and its maximum depth is 165 m. The lake is oligo-mesotrophic, and warm monomictic from May to October and homothermic from November to February (Ferrara et al., 2002). It has a theoretical renewal time of 137 years, and is used as a drinking water reservoir for the city of Rome and the Vatican State. The combined action of climate change and water extraction produces dangerous water level variations that drastically change the shape of the shoreline and inshore bathymetry, causing substantial loss of areas where denitrification can take place as a result of the dry conditions during the frequently prolonged periods of minimum depth. Land use in the surrounding area is highly heterogeneous (Fig. 1), with the following types recorded:

- Vegetation: broadleaf woods, shrub-herbaceous vegetation, semi-natural areas
- Crops: annual crops, mixed crops, permanent crops, orchards
- Built up: the towns of Trevignano Romano (42°09′N; 12°15′E), Anguillara Sabazia (42°05′18″N; 12°16′39″E), Vigna di Valle (42°04′34″N; 12°12′28″E) and Bracciano (42°06′N; 12°11′E), as well as greenhouses, campsites and beach resorts
- Soil
- Roads
- Streams

Since 1999, the lake and its surroundings have been part of the regional park of Bracciano Martignano.

So as to cover the whole perimeter of the lake (Fig. 1), twenty collection sites were selected in the littoral zone. The sites were located:

- In and near the towns (site 1–site 3, site 7, site 9, site 10, site 14, site 15, site 20)
- Close to tourism structures directly on the lake shore (campsites, beach resorts, garages etc.) (site 4, site 5, site 8, site 11, site 17)
- Cultivated fields (site 16 and site 18)
- Naturally vegetated areas (site 6, site 12, site 13, site 19)

A class was assigned to each sampling site in accordance with the dominant type of potential impact and its associated nitrogen input, on the basis of the human activities observed in the immediate vicinity (Table 1). We expected 'organic' inputs for those sites seasonally affected by tourism during summer; 'organic/inorganic' for those sites characterised by agricultural activity (as the type of fertiliser used was unknown) and 'non-impacted' for sites in naturally vegetated areas and in or near the towns. We expected sites in towns to be in the 'non-impacted' category given that the waste waters of urban areas in the catchment are directly channelled to the centralised collection system and discharged downstream. In addition to the catchment land-use classification, given that the lake is not directly served by a railway, we considered the cash earned by parking meters in parking areas on the lake shore used by non-residents (n = 6) to be a proxy for tourism pressure. Earnings were measured weekly from the beginning of May to the end of August. The parking meters do not operate from October to March. The number of non-resident cars per week in late March, April, and September was roughly the same as the May value, while from October to early March it was about half (COBIS, personal communication). The cost of parking is €1 per hour per car. To relate the number of non-resident cars to tourism pressure, one car was considered equivalent to 2.5 tourists (i.e. non-residents) as a mean value. The number of tourists was converted into g of N per day considering: 1 tourist = 6.16 g N/day (1 day = 24 h) (Pagnotta and Barbiero, 2003). Parking meters used by non-residents further away from the lake shore (n = 4) were also considered and compared with parking meters near the lake shore to confirm that the increase in car numbers was directly related to the increase in tourism-driven pressure on the lake.

Table 1
Expected and observed impact classes for each sampling site. The expected impacts were assigned before sampling in accordance with the human activities in the immediate vicinity of each sampling site. We considered the expected impact to be organic if it was related to tourism, "organic/inorganic" if it was related to agriculture. The observed impacts refer to the δ^{15}N measured at each site.

Site	IMPACT	
	Expected	Observed
1	Non-impacted	Non-impacted
2	Non-impacted	Non-impacted
3	Non-impacted	Moderate organic
4	Organic	Non-impacted
5	Organic	Moderate organic
6	Non-impacted	Non-impacted
7	Organic	Moderate organic
8	Organic	Moderate organic
9	Organic	Moderate organic
10	Organic	High organic
11	Organic	Moderate organic
12	Non-impacted	High organic
13	Non-impacted	Non-impacted
14	Non-impacted	Non-impacted
15	Non-impacted	Non-impacted
16	Organic/Inorganic	Inorganic
17	Organic	Non-impacted
18	Organic/Inorganic	Inorganic
19	Non-impacted	Non-impacted
20	Non-impacted	Moderate organic

2.2. Field procedures

We sampled at six times, from June 2015 to March 2016. Specifically, two samplings were conducted at the end of June (June 26: early summer 1, and June 30: early summer 2). Early summer 2 followed a public holiday for the entire municipality of Rome (June 29) after a weekend (June 27–28) during which tourism peaked in the lake area. Other samplings were conducted on July 28 (summer), September

23 (autumn), December 15 (winter) and March 31 (spring, 2016). At each site epilithon was sampled by scraping smooth volcanic rocks (size approximately 30 × 15 × 7 cm) in the littoral zone using single-edge sterilised razors. Two 5 × 5 cm patches were collected from each of three rocks sampled at each site, the rocks being 25 m apart along sampling transects parallel to the lake shore. The epilithon samples were stored in plastic Petri dishes and conserved on ice for transport to the laboratory. The temperature (°C), pH, total dissolved solids (ppm) and oxygen concentration (mg/l) and saturation (%) were recorded at each sampling site by multi-parameter probe (Hanna instruments HI 9829).

Following field samplings and data analysis, a transplant experiment was performed. We moved rocks from one site to another in order to test for site-specific effects on the epilithic $\delta^{15}N$ signature. Two rocks, duly labelled, from a site with non-impacted $\delta^{15}N$ signatures (site 3, as determined during previous samplings), were carefully moved to the site with the highest observed $\delta^{15}N$ (site 10, 9.9 km from site 3). Rocks of the same size and smoothness as those used for field sampling were chosen. At the same time, two rocks from site 10 were moved to site 3 and labelled. During translocation, which took less than 30 min, rocks were maintained right side up in water collected at their site of origin. Unfortunately, rocks moved from site 10 to site 3 were removed by persons unknown. In October 2015 (T0 in the translocation experiment), epilithon was sampled by scraping three replicate patches (5 × 5 cm each) per rock. Half of the total surface of the two rocks was then scrubbed clean and the rocks were moved to site 10. After 30 days, in November 2015 (T1 in the translocation experiment), the epilithic associations were re-sampled from the same two rocks moved to site 10 (three patches per rock), taking samples from both the unscraped surfaces (the epilithon left intact at T0 and hence belonging to site 3) and from the regrown surfaces (the epilithon that had regrown on the surfaces scraped bare at T0, and hence belonging to site 10). Moreover, at T1, three epilithic replicates from three rocks in site 10 were sampled to obtain a reference $\delta^{15}N$ value for epilithon growing naturally at that site. The samples were stored in plastic Petri dishes and conserved on ice for transport to the laboratory.

2.3. Laboratory procedures

Epilithic samples were conserved at −80 °C before being freeze-dried for 24 h and ground to a fine homogeneous powder using a ball mill (Fritsch Mini-Mill Pulverisette 23 with a zirconium oxide ball).

For each epilithic sample, two replicates (2.0 ± 0.2 mg) were sub-sampled and pressed into ultra-pure tin capsules and analysed using an Elementar Vario Micro-Cube elemental analyser (Elementar Analysensysteme GmbH, Germany) coupled with an IsoPrime100 isotope mass ratio spectrometer (Isoprime Ltd., Cheadle Hulme, UK). The obtained nitrogen (N) stable isotope ratios (^{15}N:^{14}N) were expressed in δ units, i.e. parts per thousand deviations from international standards (atmospheric N_2), in accordance with the following equations: $\delta R(‰) = [(R_{SAMPLE} - R_{STANDARD})/R_{STANDARD}] *10^3$ (Ponsard and Arditi, 2000), where R is the heavy-to-light isotope ratio of the element. The internal laboratory standard was IAEA-600 Caffeine. Measurement errors were found to be typically smaller than ± 0.05‰. In accordance with Cole et al. (2004), Derse et al. (2007) and Kendall et al. (2007), four impact classes, based on observed epilithic $\delta^{15}N$ signatures, were identified: 'inorganic input' ($\delta^{15}N < 3‰$), 'non-impacted' ($3‰ \leq \delta^{15}N \leq 6‰$), 'moderate organic input' ($6‰ < \delta^{15}N \leq 9‰$) and 'high organic input' ($\delta^{15}N > 9‰$).

2.4. Statistical analysis

The entire data-set includes 287 $\delta^{15}N$ observations.

Two-way ANOVA was applied to test for the effect of impact class (i.e. non-impacted, moderate organic, highly organic, inorganic) and season on epilithic $\delta^{15}N$ values. In addition, the Kruskal-Wallis test for repeated measures was used in order to test for differences between mean values of impact classes for the study period as a whole. In this case, sampling times were considered to be repeated measures. Each site was assigned to a certain impact class in accordance with its $\delta^{15}N$ value at the early summer 2 sampling time. This sampling occurred after a public holiday for the entire Municipality of Rome (the nearest major city to the lake, 4.3 million inhabitants), and was the time when tourism pressure and the mean and range of nitrogen isotopic signatures across sites were all at their highest.

Due to the presence of non-linear relationships among $\delta^{15}N$ signatures and/or sampling sites/time, we used a Generalised Additive Model (GAM) (significance level $\alpha = 0.05$). GAM can be considered a semi-parametric generalisation of linear regression and Generalised Linear Models. It is able to deal with non-linear relationships (Zuur et al., 2007, 2009) and to take account of space-time variability (Ciannelli et al., 2008; Colloca et al., 2014; Pastor et al., 2014). Therefore we considered the observed $\delta^{15}N$ epilithic signatures as a function of space (longitude and latitude coordinates of the sampling site) and time (sampling time). This allowed us to interpolate the $\delta^{15}N$ values along the entire lake perimeter at each sampling time. A Mantel test was applied to determine whether differences in $\delta^{15}N$ (in ‰) between site pairs were related to the distance (in km) between sites.

All statistical analyses were performed using open-source R software.

3. Results

3.1. Field sampling

Nitrogen isotope statistics are shown in Table 2 and physicochemical parameters are shown in Table S1 in the online supplementary material. Epilithic $\delta^{15}N$ was not found to be related to physicochemical parameters (pH: $R^2 = 0.04$, $p = 0.51$; T: $R^2 = 0.00$, $p = 0.99$; TDS: $R^2 = 0.14$, $p = 0.18$; O_2 mg/l: $R^2 = 0.01$, $p = 0.71$; O_2%: $R^2 = 0.01$, $p = 0.68$). Similarly, we found no significant correlation between $\delta^{15}N$ and%N, either at individual sampling times or considering the study period as a whole (p always > 0.05). Distance between site pairs explained less than 5% of the observed epilithic isotopic variation between sites (Mantel Test, $R^2 = 0.04$, $t = 2.53$, $p > 0.05$). As an example, sites 1 and 13 were 8.8 km apart but their $\delta^{15}N$ signatures differed by only 0.3‰. At the opposite extreme, sites 15 and 16 were only 0.9 km apart but their $\delta^{15}N$ signatures differed by 3.3‰. The lowest average $\delta^{15}N$ value was observed at site 16, near greenhouses and crops. The highest value was observed at site 10, near the tourism lakeside of Bracciano town. The range (i.e. max-min) of $\delta^{15}N$ values across sites was greatest in early summer 2 and lowest in winter (Table 2). Based on the observed $\delta^{15}N$ values, 9 sites were classed as non-impacted, 7 as moderate organic, 2 as high organic, and 2 as inorganic (Table 1)

Considering the mean value of all sites within each class, epilithic $\delta^{15}N$ varied among both impact classes and sampling times (Two-way ANOVA, impact class: $F = 5.4$, $p < 0.001$; sampling time: $F = 20.6$, $p < 0.0001$; interaction between impact class and sampling time: $F = 0.8$, $p > 0.05$). When considering the sampling times as repeated observations, all pairwise comparisons between impact classes showed significant differences (Kruskal-Wallis test for repeated measures, $Hc = 18.9$, $p < 0.001$; Mann-Whitney pairwise comparisons between impact classes, p always < 0.05) (Fig. 2). Generalised additive models (GAM) made it possible to observe significant differences in $\delta^{15}N$ between sites at all times ($p < 0.05$) (Table S2 in the online supplementary material). GAM also made it possible to include latitudinal and longitudinal variations in $\delta^{15}N$ signatures, to interpolate $\delta^{15}N$ across sites, and hence to produce an isotopic map of the lake littoral belt (Fig. 3). In early summer 1, there were diffuse moderate organic inputs along all the western side of the lake, whereas high

Table 2
Epilithic $\delta^{15}N$ signatures for each site (rows) and sampling time (columns). In each cell, the average isotopic signature and its standard error are reported. The last column shows the average isotopic signature, standard error and range (Δ, as max–min) for each sampling site. The last row shows the range (max–min across all sites) at each sampling time.

Site	early summer 1	early summer 2	summer	autumn	winter	spring	mean ± S.E. (Δ)
01	4.67 ± 0.32	4.66 ± 0.29	4.37 ± 1.17	3.76 ± 0.52	4.17 ± 0.94	6.52 ± 0.28	4.69 ± 0.32 (2.76)
02	5.23 ± 0.22	5.48 ± 0.36	3.36 ± 0.33	2.68 ± 0.42	3.10 ± 0.47	2.67 ± 0.05	3.75 ± 0.52 (2.81)
03	5.28 ± 0.33	6.23 ± 0.14	6.86 ± 0.67	5.44 ± 0.63	3.19 ± 0.03	2.46 ± 0.32	4.91 ± 0.41 (4.4)
04	6.06 ± 0.29	5.35 ± 1.05	4.30 ± 0.45	4.04 ± 0.38	6.32 ± 0.32	3.70 ± 0.52	4.96 ± 0.31 (2.62)
05	6.29 ± 0.51	6.57 ± 0.51	7.29 ± 0.63	10.08 ± 0.61	5.49 ± 1.02	4.30 ± 0.97	6.67 ± 0.50 (5.78)
06	6.22 ± 0.26	6.28 ± 0.27	3.66 ± 0.02	2.41 ± 0.06	3.69 ± 0.18	1.65 ± 0.39	3.98 ± 0.43 (4.63)
07	7.58 ± 0.23	–	7.67 ± 1.48	5.99 ± 0.21	4.12 ± 0.23	4.59 ± 0.52	5.99 ± 0.48 (3.55)
08	7.08 ± 0.46	6.88 ± 0.16	1.88 ± 0.32	7.99 ± 1.04	6.20 ± 0.11	4.30 ± 0.29	5.72 ± 0.51 (6.11)
09	8.32 ± 0.15	8.48 ± 0.19	8.49 ± 0.31	7.71 ± 0.53	7.19 ± 0.23	7.37 ± 0.52	7.93 ± 0.18 (1.3)
10	10.86 ± 0.56	13.33 ± 0.42	11.35 ± 1.20	8.74 ± 0.27	7.60 ± 1.02	8.55 ± 0.17	10.07 ± 0.53 (5.73)
11	–	–	6.85 ± 0.46	–	–	–	–
12	8.46 ± 0.74	11.31 ± 1.63	9.75 ± 0.72	6.33 ± 0.45	4.70 ± 0.46	6.15 ± 0.05	7.78 ± 0.62 (6.61)
13	5.59 ± 0.75	8.05 ± 0.04	4.32 ± 0.27	3.37 ± 0.17	3.52 ± 0.59	–	4.97 ± 0.49 (4.68)
14	7.43 ± 0.42	6.19 ± 0.12	3.87 ± 1.48	6.15 ± 0.38	6.70 ± 1.68	4.56 ± 0.41	5.82 ± 0.44 (3.56)
15	–	7.38 ± 0.50	4.77 ± 0.48	3.90 ± 0.89	–	7.00 ± 0.53	5.76 ± 0.52 (3.48)
16	2.15 ± 0.20	1.67 ± 0.09	2.73 ± 1.37	1.35 ± 0.89	4.42 ± 0.25	2.37 ± 0.56	2.45 ± 0.34 (3.07)
17	–	5.14 ± 0.10	–	3.57 ± 0.81	–	–	4.35 ± 0.51 (1.57)
18	–	–	–	2.17 ± 0.36	–	–	–
19	–	5.14 ± 0.10	1.72 ± 0.39	3.57 ± 0.81	7.25 ± 1.94	2.24 ± 0.33	3.98 ± 0.65 (5.53)
20	–	6.4 ± 0.23	–	–	–	3.26 ± 0.82	4.58 ± 0.87 (3.14)
mean	6.52 ± 0.32	6.73 ± 0.37	5.48 ± 0.44	4.96 ± 0.35	5.18 ± 0.29	4.48 ± 0.52	5.48 ± 0.42
Δ (max–min)	8.71	11.66	9.63	8.73	4.5	6.9	7.62

organic impact was detected only at site 10, near the tourism lakeside of Bracciano town. Site 10 retained its high organic impact status in early summer 2 and summer, and then decreased to moderate organic between autumn and spring. Inorganic N input was evident in the East-North East (near greenhouses and crops) in early summer 1, autumn and spring, while it was reduced in early summer and summer and was absent in winter. Regardless of season, sites near the towns of Trevignano Romano and Anguillara Sabazia had isotopic signatures of 3‰ to 6‰.

Earnings from parking meters near tourism structures and the lake shore increased from May to August, whereas earnings from parking meters further away from tourism structures and the lake shore were lower and did not vary in this period (Fig. 4 and Fig. S1 in the online supplementary material). Rainfall in the drainage basin of the lake varied between sampling times, being highest in autumn and lowest in winter (Fig. 4). The mean $\delta^{15}N$ of sites assigned to the 'high organic impact' class was directly related to earnings from parking meters near tourism structures (Fig. 4) but was not related to rainfall ($R^2 = 0.001$, $p > 0.05$). In non-impacted sites it was related to neither earnings from parking meters nor rainfall (Fig. 4). Similarly, in moderate organic and inorganic impacted sites it was not related to earnings from parking meters (p always > 0.05). On the other hand, in inorganic impacted sites $\delta^{15}N$ decreased with increasing rainfall (Fig. 4) and was not related to earnings from parking meters ($R^2 = 0.01$, $p > 0.05$). Rainfall was not related to $\delta^{15}N$ in non-impacted, moderate and high organic impacted sites (p always > 0.05). Independently by the impact class, $\delta^{15}N$ was never related with earnings from parking meters away from the shore (p always > 0.05). Based on the linear regression between earnings from parking and $\delta^{15}N$ in impacted sites (Table S3), we were able to determine thresholds in terms of the number of tourists and g of N per day that are expected to increase pollution levels in tourism sites from non-impacted to moderate organic and high organic impact (Fig. S2, on-line supplementary material).

3.2. Translocation experiment

The $\delta^{15}N$ of epilithon collected on rocks moved from site 3 (a non-impacted site) to site 10 (high-organic impact) varied during the 30 days of the experiment (one-way ANOVA and Tukey post-hoc comparisons, Rock 1: $F = 18.8$, $p < 0.001$; Rock 2: $F = 72.5$, $p < 0.001$) (Fig. 5). For both rocks, the $\delta^{15}N$ of epilithon that grew back on the scraped patches (R) did not differ from that of epilithon

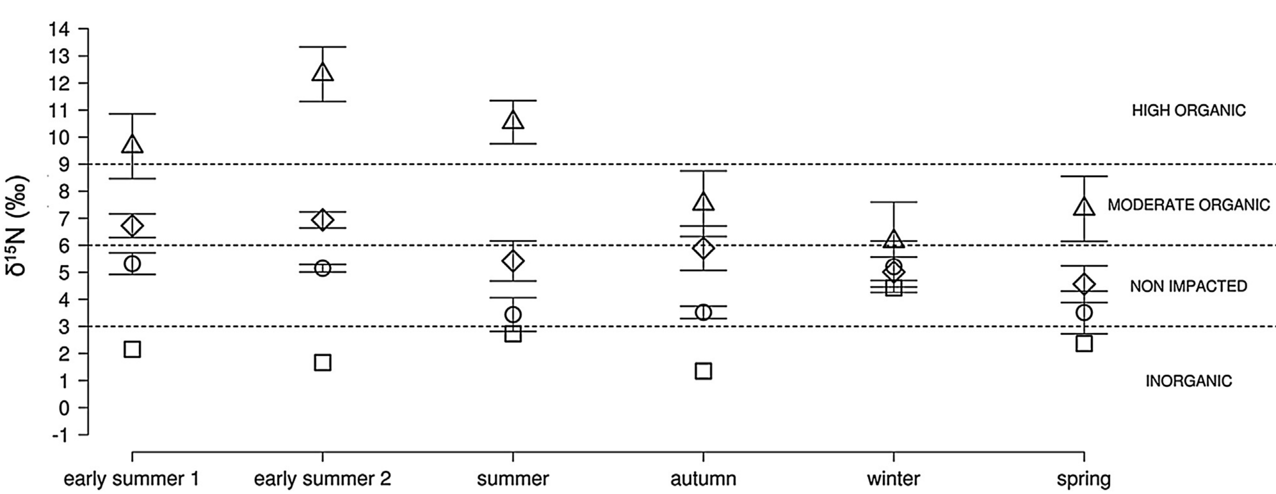

Fig. 2. Plot of the four impact class averages (± S.E.) at each sampling time. Legend: triangle = high organic signature, rhombus = moderate organic signature, circle = non-impacted signature, square = inorganic signature.

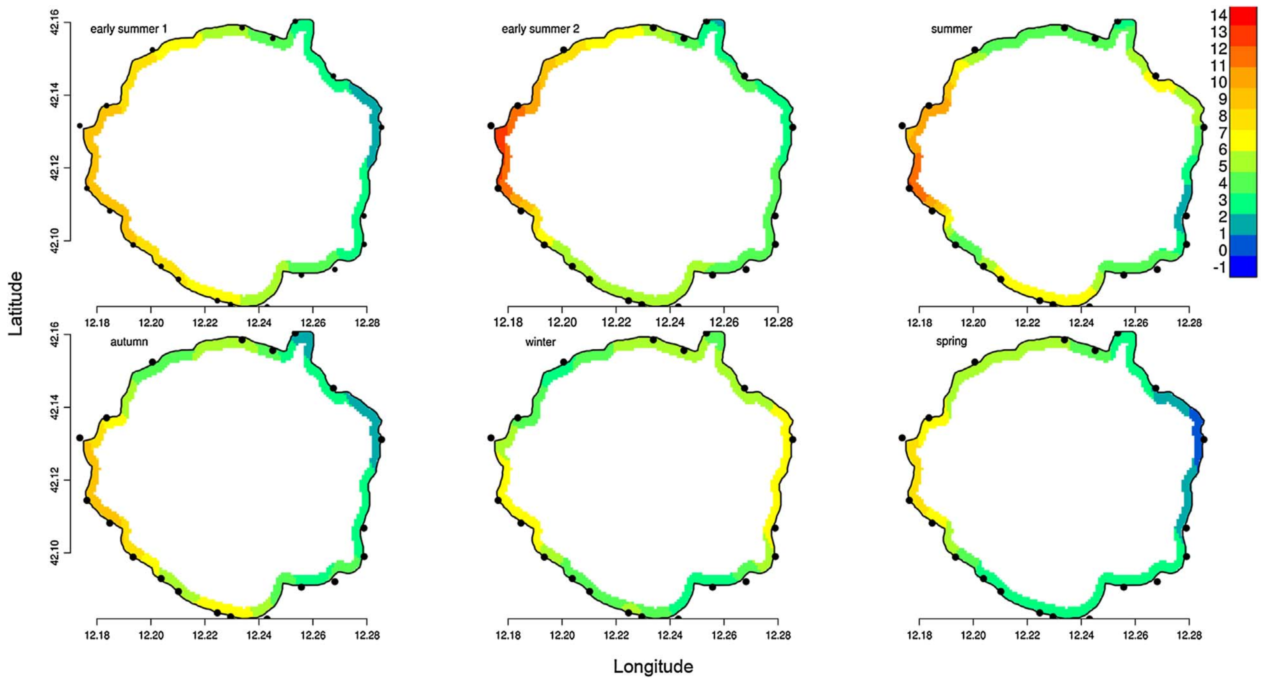

Fig. 3. Plot of GAM-predicted epilithic $\delta^{15}N$ signatures along the littoral belt at all sampling times. Inorganic input range (blue) $\delta^{15}N < 3‰$, Non-impacted input range (green) $3 \le \delta^{15}N \le 6‰$, Moderate organic impacted range (yellow) $6 < \delta^{15}N \le 9‰$, High organic impacted range (red) $\delta^{15}N > 9‰$.

growing on rocks naturally present at site 10 (R vs. site 10, Rock 1: $p = 0.22$; Rock 2: $p = 0.11$), while it was higher than epilithic $\delta^{15}N$ measured at site 3 before translocation (T0) (R vs. T0, Rock 1: $p = 0.02$; Rock 2: $p < 0.001$). The $\delta^{15}N$ of the unscraped epilithon taken from site 3 but left exposed to site 10 for one month (T1) also increased after translocation (T0 vs. T1, Rock 1: $p = 0.03$; Rock 2: $p < 0.001$) and was similar to the $\delta^{15}N$ of epilithon that grew back on scraped patches (R vs. T1, Rock 1: $p = 0.08$; Rock 2: $p = 0.14$). Lastly, there was no difference between the $\delta^{15}N$ of the epilithon that grew back on the two translocated rocks ($p = 0.35$).

4. Discussion

Our results indicate that epilithic $\delta^{15}N$ signature represents a powerful tool in lacustrine environmental monitoring due to its ability to detect and record different types of anthropogenic nitrogen input in the water body. Indeed, consistent with our expectations and with previous research into macroalgal and epilithic $\delta^{15}N$ signatures in several non-lacustrine aquatic ecosystems (Bentivoglio et al., 2016; Cejudo et al., 2014; Dailer et al., 2010; Jona-Lasinio et al., 2015; Pastor et al., 2014), we found that the magnitude and type of input reaching the lake littoral zone varied strongly in space and time. Accordingly, the $\delta^{15}N$ values associated with these inputs were found to vary across sites and seasonally. It was shown how, for a lake affected by a variety of anthropic activities, especially tourism and agriculture, epilithic $\delta^{15}N$ was able to describe both pulsed and more persistent inputs to the coastal waters. Based on $\delta^{15}N$, we were able to distinguish between N loadings of organic origin associated with short-term and seasonal pressures generated by tourism (on public holidays and in summer) and those of inorganic origin resulting from agricultural practices, which where dependent on runoff from agricultural areas.

Our sampling design, with six sampling times and a dense sampling grid with sites along the whole of the lake perimeter, enabled us to identify and describe potential sources of nitrogen in a space-time framework. Early summer was the period of greatest pressure from tourism, with agricultural inputs also being detected. This produced a broad range of epilithic $\delta^{15}N$ signatures across sites. In the period of lowest human presence (winter), it was possible to recognise a decrease in the $\delta^{15}N$ values of tourism-impacted sites and in the variability of $\delta^{15}N$ along the lake perimeter. Indeed, tourism sites belonging to the high-organic impact class in early summer and summer approached non-impacted or moderate-organic impacted conditions in autumn and winter. This suggests a meta-stable dynamics of tourism-related N pollution in the lake and the ability of the lake to recover on a seasonal temporal scale. In addition, the results obtained at site 10 in early summer 2 emphasise the presence of hidden inputs of nutrients. Inputs of nitrogen and phosphorous were also found in this part of the lake by Catalani et al. (2006). As in studies of benthic macroalgal $\delta^{15}N$ (Orlandi et al., 2014) and river epilithic $\delta^{15}N$ (Schiller et al., 2009), our study also had fine temporal resolution. Indeed, in sites with high organic impact, $\delta^{15}N$ increased by 2.6‰ between early summer 1 and 2, in association with a 40% increase in tourism pressure (as measured by earnings from parking meters), suggesting that even short-term, pulsed inputs of anthropogenic N can be detected by means of isotopic analysis of epilithon. As expected with an efficient basin-wide wastewater collection system, the towns of Anguillara Sabazia in the South and Trevignano Romano (except after the public holiday) in the North remained in the non-impacted range. Lastly, anthropogenic inorganic N inputs were observed in the East-North East near greenhouses and crops. These inputs were evident in early summer 1, autumn and spring, suggesting that N inputs from agriculture are of a highly pulsed nature linked to discharge of fertiliser-derived N after rainfall. As for organic inputs, inorganic loadings were "absorbed" by the lake over a seasonal temporal scale and were completely absent in winter, when the agricultural activity in the lake basin drops.

The absence of a linear correlation between epilithic $\delta^{15}N$ and nitrogen content was explained by the contrasting types of nitrogen input to the water body. Indeed, we would expect a positive linear correlation between $\delta^{15}N$ and%N if the sole nitrogen input was organic, and a negative linear correlation if the sole nitrogen input was inorganic. Due to the fact that Lake Bracciano received two distinct kinds of input there was no evidence, at any of our sampling times, that high/low isotopic signatures were reflected in high/low epilithic nitrogen content.

Another advantage of epilithic $\delta^{15}N$ signature, which arises from our translocation experiment, is the independence of isotopic values

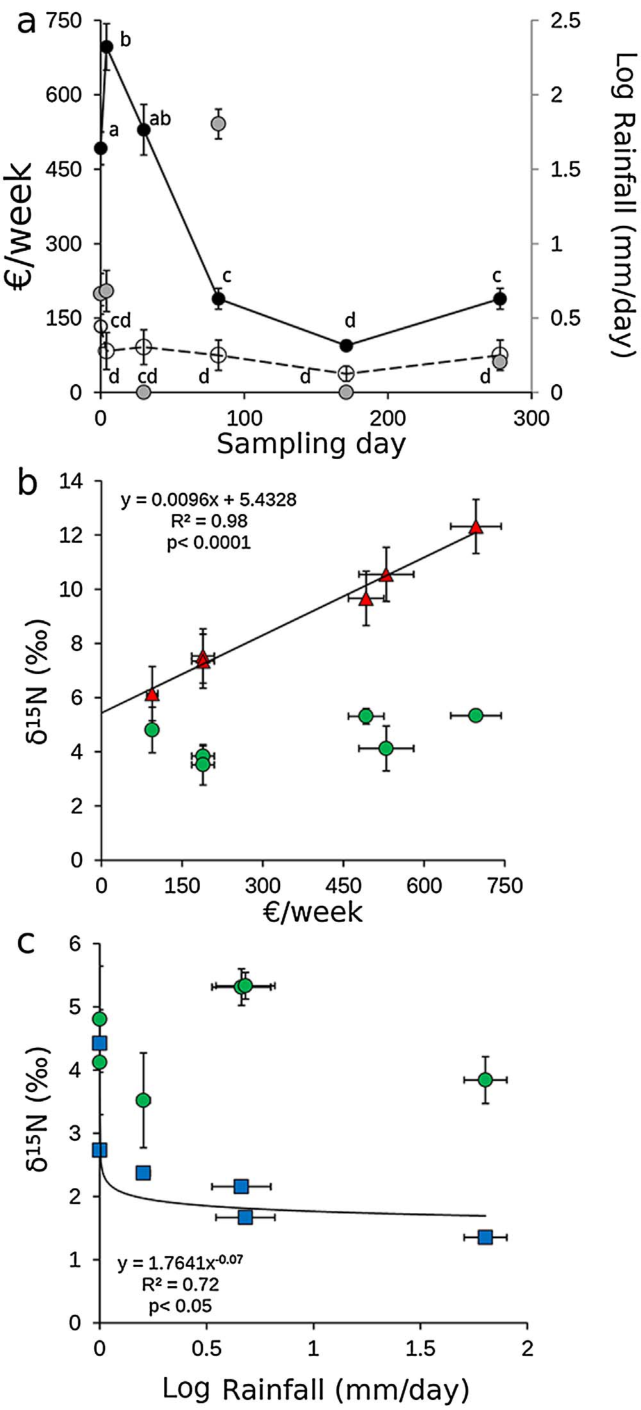

Fig. 4. Anthropogenic pressure, rainfall and δ^{15}N in epilithon. **a:** Rainfall (as mean mm/day during the week preceding each sampling date, grey symbols), earnings from parking meters close to tourism structures on the lake shore (black symbols) and away from the lake shore (empty symbols). All parking meters are for non-residents. Different letters indicate a significant difference between sampling times (from day 0: early summer 1, to day 278: spring) and/or parking meter locations (two-way ANOVA and Tukey post-hoc comparisons, $p < 0.05$). **b:** Relationship between earnings from parking meters close to tourism structures on the lake shore and δ^{15}N in sites with high-organic impact (triangles) and in non-impacted sites (circles). **c:** Relationship between rainfall and δ^{15}N in sites with inorganic impact (squares) and in non-impacted sites (circles). Linear regression is not shown when not significant ($p > 0.05$).

from the site-specific composition of the epilithon (MacLeod and Barton, 1998), meaning that the signatures were purely a function of local N inputs affecting the sampling site at different sampling times. While our translocation experiment was based on only two sites, this result is supported by the independence of δ^{15}N from physicochemical parameters and distance between site pairs. It is also consistent with previous observations by MacLeod and Barton (1998), who found no effect of epilithic composition on its δ^{15}N signature. This suggests that using epilithon to monitor N pollution does not necessarily require its taxonomic identification, resulting in a less time-consuming and more practical technique.

5. Conclusions

Freshwater ecosystems provide indispensable ecosystem services (Page et al., 2012; Pimentel et al., 2004; Smith, 2003) but these same services expose them to various forms of anthropic disturbance, especially nitrogen loads derived from agriculture and sewage (Dodds et al., 1989; Matson et al., 1997; Vitousek et al., 1997). There is a strong need therefore for an accurate, practical and fast environmental monitoring tool. In marine, coastal, transitional water and lotic ecosystems, macroalgal and epilithic δ^{15}N signatures have proved to be successful in determining the types and space-time variability of nitrogen loads (Bentivoglio et al., 2016; Cejudo et al., 2014; Cole et al., 2004; Dailer et al., 2010; Gartner et al., 2002; Jona-Lasinio et al., 2015; Orlandi et al., 2014, Pastor et al., 2013, 2014; Peipoch et al., 2012; Schiller et al., 2009; Titlyanov et al., 2011; Vizzini et al., 2005), while in lacustrine ecosystems there is a lack of knowledge concerning epilithic δ^{15}N signatures.

Our study demonstrates that epilithic δ^{15}N signatures fully satisfy the requirements for nitrogen input monitoring. Specifically, epilithic δ^{15}N signatures can detect the source of the inputs, their nature (inorganic vs. organic, reflecting anthropic activities) and their temporal variation, with a temporal resolution of as little as four days. In greater detail, epilithic δ^{15}N identified the critical season for each type of input, together with the main drivers linking N loading to anthropic activities in the lake basin. We were able to recognise the greatest and most persistent loads of organic nitrogen (from tourism) and inorganic nitrogen (from greenhouses and crops, transported by run-off) affecting the lake, although nutrient concentrations in water are below legal limits and the lake is classified as oligo-mesotrophic (Ferrara et al., 2002). This suggests that isotopic signatures are able to detect anthropogenic N inputs even at low concentrations, providing a monitoring tool that can detect early signs of ecological risk affecting the lake ecosystem. The independence of δ^{15}N from epilithic composition and water physicochemical parameters, together with the widespread presence of epilithon in freshwater ecosystems, suggests that the proposed method is of potentially broad application. Therefore, even in cases where epilithon is absent, impacted or potentially impacted sites can still be monitored by using samples obtained elsewhere.

The results of our experiment suggest that epilithic isotopic signals represent a highly efficient and rapid method of monitoring a lake's nitrogen loads and that, when isotopic variation can be quantitatively related to anthropogenic pressure, as measured here with reference to earnings from parking meters, isotopic thresholds can be directly translated into practical management information for decision makers. The results of this study indicate the need for improvement of sewage treatment facilities (with an emphasis on organic nitrogen removal), implementation of wastewater re-use protocols, and the conservation or recovery of healthy littoral belts of the lake ecosystem that are able to absorb agricultural N inputs after rainy periods.

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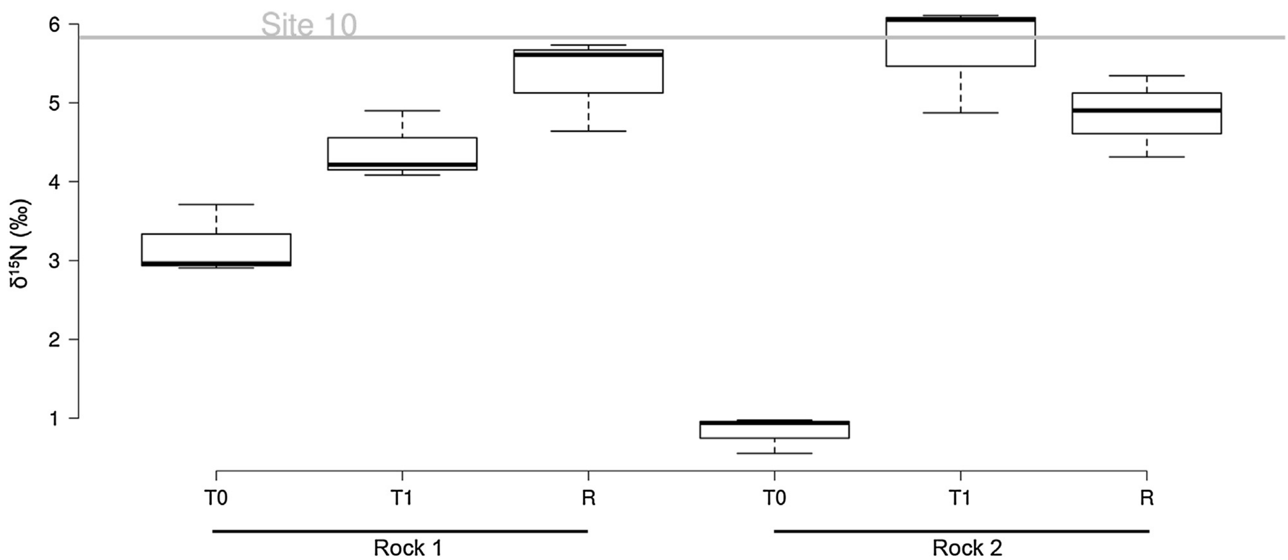

Fig. 5. Boxplot of transplant experiment. T0 = first sampling time, October, before the shift from site 3 to site 10. T1 = second sampling time, November, 30 days after the shift. R = regrown (on scraped patch) epilithic community. Rock 1 = first epilithic community. Rock 2 = second epilithic community. The grey line represents the autochthonous epilithic $\delta^{15}N$ signature of site 10.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecolind.2017.04.007.

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