D3.5.1
Preliminary study for detection of anthropogenic nutrients in marine coastal areas

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1. INTRODUCTION

Mass tourism is a recent phenomenon that may lead to considerably high density of visitors within restricted spaces and time periods. A prime example of this is represented by beaches that constitute attractive holiday destinations and have worldwide become very popular. Tourism brings economic benefits to countries, but there are usually substantial environmental threats and costs associated with it (Davenport and Davenport, 2006). Nevertheless, the overall understanding of the interaction between tourism and the environment, especially in coastal areas is quite poor. One of the major effects of tourism on coastal areas is due to the organic and nutrient load linked especially to the abrupt increase of population during the high-season tourist peaks. Indeed, during summer, many tourist destinations frequently experience deterioration of coastal water quality due to the high organic and nutrient load. Besides human waste, septic tanks or inadequate sewage systems at beach bars and resorts, or fertiliser run-off from golf courses may substantially affect marine coastal systems. The introduction of excess nutrients into the sea has a number of impacts, first of all the eutrophication, the increase in organic material that may be followed by secondary effects such as hypoxia (Nixon, 1995).

Therefore, one of the aims of the BLUEISLANDS project is to evaluate the effects of tourism, as a source of anthropogenic nutrients, on coastal waters, and to develop strategies for minimizing these negative effects. A suitable approach to track the excess of nutrients and organic matter of anthropic origin in coastal waters is the use of marine macroalgae, which are proper biological indicators, due to their high uptake and assimilation rate of bioavailable nitrogen from seawater (Hurd et al., 2014), together with their wide distribution, abundance and long life (Cole et al., 2005).

In this context, a suitable method to assess the presence and extent of anthropic nutrients in macroalgae exposed to nutrient-enriched seawater is the analysis of nitrogen stable isotope ratio ($\delta^{15}N$). Stable isotopes of nitrogen ($^{15}N/^{14}N$ or $\delta^{15}N$) and carbon ($^{13}C/^{12}C$ or $\delta^{13}C$) represent powerful and highly informative tools that have been widely used in environmental studies and food web ecology to trace the origin of nutrients and organic matter in natural systems (Castro et al., 2007; Vizzini and Mazzola, 2006a, 2004). In particular, $\delta^{15}N$ is useful in the identification of nitrogen sources entering marine ecosystems (e.g., atmospheric deposition, wastewater, fertilizers), as marine, terrestrial and anthropic-derived organic matter have different $\delta^{15}N$ signatures (Castro et al., 2007; Cole et al., 2005; Olsen et al., 2010). In this context, macroalgae are able to integrate spatial and temporal variability of $\delta^{15}N$ of dissolved nitrogen and their isotopic composition provides information on the origin of nitrogen exploited (natural *vs*. anthropogenic) (Cole et al., 2005, 2004; Costanzo et al., 2001). Indeed, anthropic-derived nitrogen typically has higher $\delta^{15}N$ than terrestrial and marine sources due to the isotopic fractionation that occurs during nitrogen transformations (e.g. ammonia volatilisation and denitrification) and leaves the residual nitrogen pool enriched in ^{15}N (Heaton, 1986). Therefore, marine macroalgae exposed to ^{15}N-enriched waters show a predictable enrichment in their $\delta^{15}N$ values, reflecting the nitrogen input typology and contribution from land to water bodies (Cole et al., 2005).

If the nitrogen isotopic signature of marine macroalgae is yet broadly used to monitor anthropogenic nitrogen inputs into aquatic ecosystems (e.g. Derse et al., 2007; Lin et al., 2007; Morris et al., 2009; Savage et al., 2005), a further step beyond is represented by the "deployment approach" consisting in short-term macroalgae deployments in selected areas to assess the change of their $\delta^{15}N$ values over the deployment period, according to environmental conditions. The main advantage of this recent approach, compared with the standard monitoring of nutrients concentration in seawater, derives

from the ability of macroalgae in providing a time-integrated picture of the bioavailable nutrients combined with the efficacy of $\delta^{15}N$ in providing information about their source (marine *vs*. anthropogenic).
This approach was set up and successfully used in many areas worldwide, from Moreton Bay, Australia (Costanzo et al., 2005, 2001), to the southern Baltic Sea (Deutsch and Voss, 2006), the coral reef on Maui, Hawaii, USA (Dailer et al., 2012) and the Mediterranean Gulf of Gaeta (Orlandi et al., 2014; Rossi et al., 2018). For its effectiveness, this approach was adopted in the BLUEISLANDS project with the aim to detect the occurrence and temporal variation of anthropogenic nutrients in coastal waters in relation to tourist flows in three Mediterranean islands.

2. MATERIALS AND METHODS

2.1. Study sites and experimental design

The experimental activities aimed at evaluating the occurrence and temporal variation of anthropogenic nutrients in coastal waters, consistent with tourist flows, were carried out in three Mediterranean islands: Cyprus, Sicily (Italy) and Rhodes (Greece) (Fig. 1). The approach used was based on short-term macroalgae deployments and was conducted in three periods: June (putatively before the tourist period), August (during the tourist peak) and October (putatively at the end of the tourist period), in three experimental sites per island. The sites were selected in order to compare a potential impacted site (e.g. featured by large tourist infrastructures, popular beaches, wastewater treatment plants) that experiences a sharp increase in population density during the tourist peak (hereafter Impact site), with two coastal sites where tourist activities and variation in population density throughout the year are negligible (hereafter Control 1 and Control 2 sites). The activities were carried out in 2017 in Cyprus and Sicily and in 2018 in Rhodes, due to technical delay in obtaining the required authorization from local authorities.
In each island, sites were selected with the collaboration of local partners. In Cyprus (Fig. 1a), the tourist Sunrise beach, which is located in the small town of Protaras, was selected as Impact site. It is an urban beach that provides many leisure activities to the beach users, such as lidos, watercrafts and water sports and is surrounded by many touristic facilities such as hotels, resorts and restaurants. The two Control sites were identified in the southernmost rocky area of Cavo Greco peninsula for their reference conditions. The peninsula hosts a protected National Forest Park and agricultural fields, hence no infrastructures are present; tourist attendance is very low during the high season or limited to local people, but small tourist cruise ships stop in the area during summer daily excursions.
In Sicily (Fig. 1b), the Impact site chosen was the urban beach of Giardini Naxos, located at the southern part of the Giardini Naxos Bay, which is delimited by a touristic harbour at the south-eastern side. The beach is characterised by several tourist facilities, such as lidos, kiosks and water sports, and is surrounded mainly by hotels, restaurants and bars, and private houses. The two Control sites were selected along the northernmost area of Fondaco Parrino, which is characterized by a long remote beach with no tourist infrastructures, and attended only by few locals in August.
Lastly, in Rhodes (Fig. 1c), the beach of the resort village of Faliraki was selected as Impact site. Mainly big resorts characterise the beach front, while restaurants, bars and tourist shops are abundant at the back of the beach. In the southernmost area of Afandou, two Control sites were chosen in two beaches where two small lidos are mostly attended by locals.

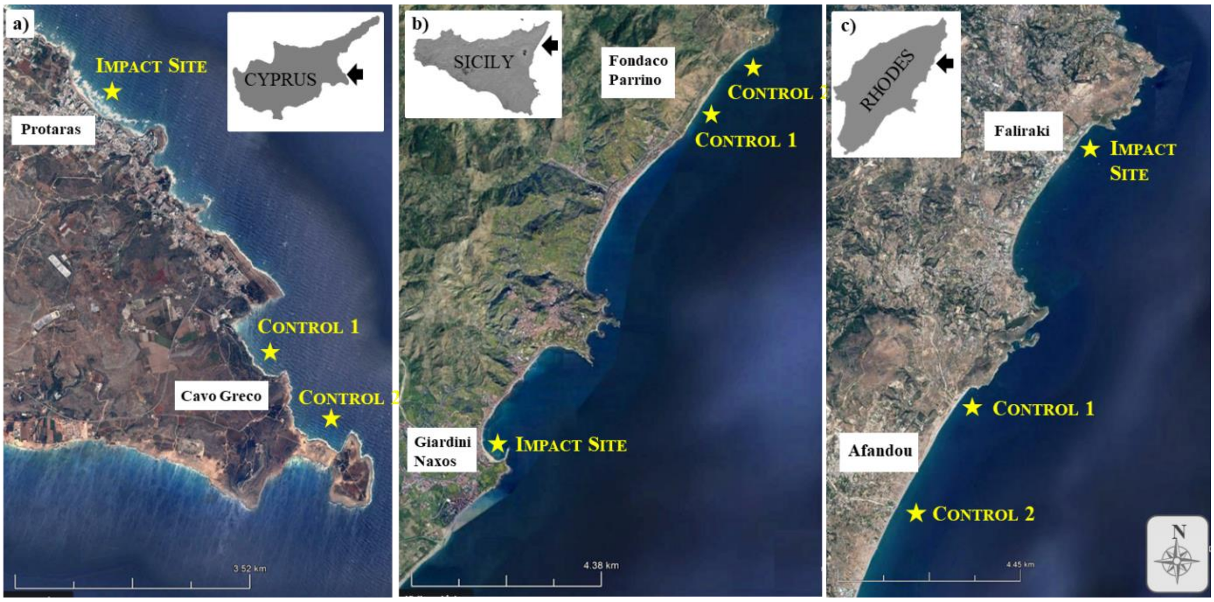

Figure 1. Study sites in a) Cyprus, b) Sicily (Italy) and c) Rhodes (Greece).

Prior to the field activity, authorizations were requested to local Authorities in the three countries to obtain the permission for carrying out the experiment in the selected sites. In Cyprus, the Department of Environment (LP) and the Department of Fisheries and Marine Research of the Ministry of Agricultural, Rural Development and Environment released the authorization for field activities. In Sicily, the Regional competent authority, namely the "Assessorato Territorio e Ambiente, Regione Siciliana", together with the Maritime Authority of Messina, gave the formal authorization to carry out the field activities. In Rhodes, the Marine Scientific Research Authorization Committee of the Ministry of Foreign Affairs and the Ephorate of Underwater Activities gave the permission for field activities through the General Direction for the European Union of the Ministry of Foreign Affairs and International Cooperation and the Italian Embassy in Greece. In all cases, the project was explained with its aims, outputs and potential repercussions for management of tourism and coastal marine areas.

Overall, the experimental fields were constituted by a georeferenced grid of 30 points at the Impact site and 21 points at each Control site. Points were distant 50 m each other, and distributed along three transects parallel to the coastline, according to the geomorphology of the study site (Fig. 2a). In Cyprus and Rhodes, the three transects were placed at 100, 200 and 300 m from the coastline, while in Sicily they were placed at 200, 250 and 300 m, as to limit any interference with bathing and boating activities according to the prescription of the Local Authority (Maritime Authority of Messina, Italy) which released the authorization for the field activities.

Each point of the experimental field represents the exact position where macroalgae were deployed within easily deployable and removable structures made of nylon net bags (20x10 cm). Each bag containing the macroalgae thallus was fixed to a rope that was anchored at the bottom with a ballast and kept straight in the water column with a buoy at a depth of 1.5 m, to ensure the optimal solar radiation for the macroalgae (Fig. 2b).

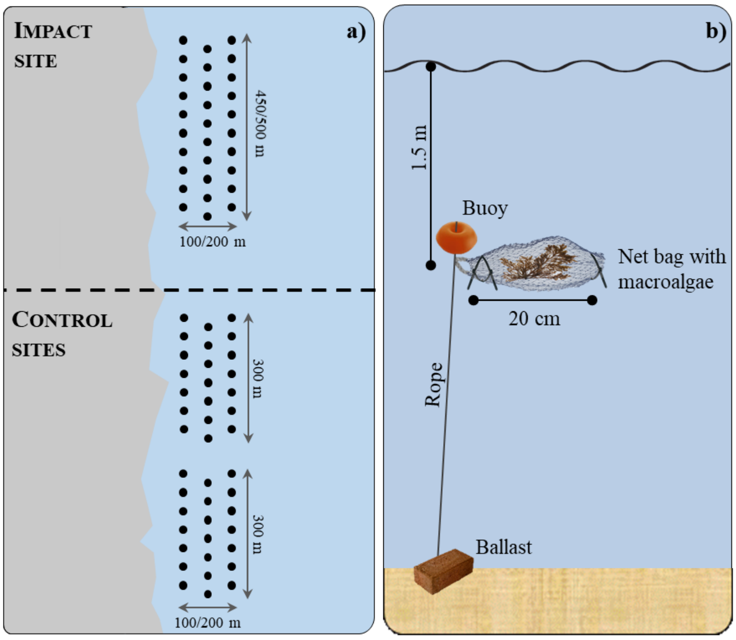

Figure 2. Scheme of (a) the experimental fields at both Impact and Control sites, where each point represents a macroalgae deployment point and distances indicate the whole area covered according to the different study sites; (b) the structure of a single macroalgae deployment.

In each island, the species of macroalga to be used in the experiments was selected based on specific features such as the perennial cycle and local abundance; these were two prerequisites to allow the replicability of the experiment using the same species across all the experimental periods. For this purpose, brown macroalgae of the genus *Cystoseira* were suitable to be used in all the study areas: *Cystoseira humilis* was used in Cyprus, *C. amentacea* in Sicily and *C. compressa* in Rhodes.

In the three islands, macroalgae thalli of the species selected were sampled before the onset of the experiment from a pristine shore located in the same study area, mentioned hereafter as “Collection site” known to host the species throughout all the experimental periods.

The macroalgae collected were:

i) analysed for $\delta^{15}N$ to record the isotopic signature at the onset of the experiment (Collection day: Day 0), representing the isotopic baseline to which compare the $\delta^{15}N$ of the deployed macroalgae at the end of the experiment;
ii) deployed in the Collection site using the same type of structure and for the same duration as in the Impact and Control sites, with the purpose to check any effect of the experimental procedure (procedural control) on the macroalgae performance, by comparing their $\delta^{15}N$ signature (Collection day: Day 3-Control *in situ*) with that of further samples contextually collected in the Collection site (Collection day: Day 3);
iii) deployed in the Impact and Control sites following the experimental design illustrated above.

To do this, thalli were carefully and individually placed within the nylon bags and deployed in the experimental points for three days in each period: June, August and October. At the end of the 3 day-deployment, each structure was removed from the coastal sites, the macroalgae were carefully removed from the net bag, rinsed and stored in the cold until the arrival to the laboratory, where they were frozen at -20°C.

Furthermore, main physicochemical parameters of seawater (temperature and salinity) were also recorded using a multiparameter probe, and surface seawater samples were collected in triplicate (10 l each) to obtain the background isotopic signature of the particulate organic matter (POM). In Sicily and Rhodes, additional water samples were also collected from the local Wastewater Treatment Plant, situated well south (Sicily) and north (Rhodes) from the experimental sites, just to assess the isotopic value of anthropogenic organic matter sources.

2.2. Sample processing and laboratory analysis

Macroalgae samples were processed in the laboratory and only the apical portion of the frond of each thallus, corresponding to the new grown tips, was selected for the isotopic analysis, as apical tips of perennial macroalgae integrate nutrient concentration and isotopic values from seawater nutrients during their growing period (Viana and Bode, 2013). Then, apical tips were quickly rinsed with distilled water to remove any external material and gently scraped to remove epiphytes, when necessary. Water samples were filtered on precombusted (450°C, 4h) filters (GF/F Whatman, pore size 0.45 µm) and rinsed with distilled water. Macroalgae subsamples and POM filters were then oven dried at 60°C for 24/48 hours and subsequently ground to a fine powder using a micro-mill.

An aliquot of each ground sample was packed in tin capsules and analysed for $\delta^{15}N$ at the Laboratory of Isotopic Ecology of the Operational Unit of CoNISMa at University of Palermo, using an Isotope Ratio Mass Spectrometer (Thermo Delta IRMS Plus XP) coupled to an Elemental Analyser (Thermo Flash EA1112). Nitrogen stable isotope ratio was expressed in δ unit notation, as parts per mil deviation from the international standard (atmospheric N_2) and determined as follows: $\delta^{15}N = [(^{15}N/^{14}N_{sample})/(^{15}N/^{14}N_{standard}) - 1] \times 10^3$. Minimum analytical precision based on the standard deviation of replicates of internal standards (International Atomic Energy Agency IAEA-CH-6) was 0.1‰.

2.3. Data analysis

Data obtained were analysed separately for the three islands. $\delta^{15}N$ values of the macroalgae collected at the Collection site were compared between Periods and Collection days through PERMANOVA (software PRIMER 6 v6.1.10 & PERMANOVA+ β20; Anderson et al., 2008) on Euclidean distance matrices obtained from untransformed data. In particular, we were interested in checking for isotopic variation i) naturally occurring across the same 3-days of the experiment (by comparing Collection Day 0 and 3), and ii) due to the experimental procedure (i.e. net effect) on the macroalgae performance (by comparing the procedural control Collection Day 3-Control *in situ* and the Collection Day 3). To do this, a two-factor design (factor Period with three levels: June, August, October; factor Collection Day with three levels: Day 0, Day 3 and Day 3–Control *in situ*; fixed and orthogonal) was set.

PERMANOVA was also used to test for isotopic differences between Periods and Sites for the suspended particulate organic matter (POM) samples. In this case, a two-factor design (factor Period with three levels: June, August, October; factor Site with three levels: Impact, Control 1, Control 2; fixed and orthogonal) was set. The significance of the models was assessed using the Monte Carlo permutation-based test when permutations were < 100.

The influence of tourist activities on the occurrence of anthropogenic nutrients in the coastal waters of the Mediterranean islands studied through the macroalgae deployments was assessed by comparing first, the nitrogen isotopic values ($\delta^{15}N$) of the deployed macroalgae in each Period, Site and Distance (from the coastline), with the correspondent baseline (i.e. the mean $\delta^{15}N$ value of the macroalgae from the Collection site at Day 0 in each period). To do this, a t-test between independent groups was performed using the software STATISTICA v.10. Homogeneity of variance was previously checked through Levene test. Afterwards, the isotopic variation at the end of the deployment period (i.e. the difference between the $\delta^{15}N$ of the deployed macroalgae and the $\delta^{15}N$ of the baseline) was calculated for each deployed sample (across periods, sites and distances from the coast) and expressed as $\Delta\delta^{15}N$. In more detail, $\Delta\delta^{15}N$ indicates the extent and the direction of the isotopic variation of the macroalgae tissues at the end of the experiment: isotopic enrichments (positive $\Delta\delta^{15}N$ data) and depletions (negative $\Delta\delta^{15}N$ data) are expected when the macroalgae were exposed respectively to more ^{15}N-enriched or more ^{15}N-depleted nutrients than those at the Collection site.

Temporal (among periods) and spatial (among sites and distances) differences of $\Delta\delta^{15}N$ values were tested through PERMANOVA. A three-factor design (factor Period with three levels: June, August, October; factor Site with three levels: Impact, Control 1, Control 2; factor Distance with three levels: 100 m, 200 m, 300 m in Cyprus and Rhodes; 200 m, 250 m, 300 m in Sicily; fixed and orthogonal) was used. This experimental design was conceived to assess the variability of the isotopic variation of the macroalgae during the deployment period depending on the seasonal tourist flow, on the people attendance on the beach, on the direct input of anthropogenic nutrients form the coast and the interaction of all the three factors. Pairwise tests were performed whenever the differences tested were significant (p-value < 0.05).

The analysis of spatial patterns in the variation of the isotopic values of macroalgae ($\Delta\delta^{15}N$) was conducted also through the Geographic Information System (GIS) Quantum GIS (QGIS version 2.18.7). The Inverse distance weighted (IDW) interpolation was the technique selected to map the spatial distribution of $\Delta\delta^{15}N$. IDW simply allows to determine cell values using a linearly weighed combination of a set of sample points where the weight is a function of inverse distance (Philip and Watson, 1982; Watson and Philip, 1985). $\Delta\delta^{15}N$ values were ranked into 10 classes (corresponding to different colours) across all study areas, sites and periods using an Equal Interval Classification, so that the contours of these categories indicate the occurrence and the extent of plumes of anthropogenic (positive values; warm colour scale) *vs*. marine (negative values; cold colour scale) nutrients in the coastal sites over the experimental periods (*sensu* Costanzo et al., 2001).

3. Results

3.1. Macroalgae deployment in Cyprus

3.1.1. Main physicochemical features of the study sites and experiment procedural control

Main physicochemical variables characterizing the experimental sites in Cyprus followed a temporal trend. Seawater temperature at the Impact and Control sites was quite homogenous in all the experimental periods, varying across the three sites between 21.9 and 22.7°C in June, then reaching the highest values in August, comprised between 28.8 and 29.3°C, and lastly decreasing in October with values between 25.0 and 26.1°C. With a similar temporal trend, salinity ranged between 38.0 and 38.2 psu in June across all the sites, reached the peak, comprised between 41.0 and 41.4 psu, in August, and then was steadily 41.0 psu in October.

Mean $\delta^{15}N$ signature of the macroalga *Cystoseira humilis* collected at the Collection site at Day 0, Day 3 and the procedural control (Day 3 – Control *in situ*) varied across all the experimental periods between -0.36 and 1.22‰ (Fig. 3). The low $\delta^{15}N$ values might reflect the influence of N derived from the inland as characterised by agricultural fields. Indeed, terrestrial nutrients and especially most fertilizers have $\delta^{15}N$ of about 0‰ and the marine coastal areas affected by continental and fertilizer nitrogen input are expected to exhibit $\delta^{15}N$ values between -4 and +4‰ (Derse et al., 2007). This is confirmed by the sharp $\delta^{15}N$ decrease recorded in October at Day 3, after rainy days occurred during the implementation of the experiment, as a possible result of abundant terrestrial runoff. PERMANOVA highlighted significant differences for the interaction of the factors Period x Collection day (Table 1a). The $\delta^{15}N$ signature of the macroalgae collected at Day 0 was higher in June than in August and October (Fig. 3, Table 1b), likely due to the influence that environmental conditions, such as seawater temperature and light, exerts on stable isotope ratios of macroalgae (e.g. Hyndes et al, 2013; Ochoa-Izaguirre & Soto-Jiménez, 2015). The comparison between the $\delta^{15}N$ signature of the macroalgae collected at Day 3 and those deployed as procedural control (Day 3 – Control in situ) did not reveal any significant difference within periods (Table 1c), but showed a high variability among periods, as $\delta^{15}N$ in October dropped to negative values (Fig. 3; Table 1b) as discussed above due to terrestrial runoff from surrounding agricultural fields. This finding allowed to exclude any effect of the experimental procedure (e.g. cut, handling, deployment) on the macroalgae performance.

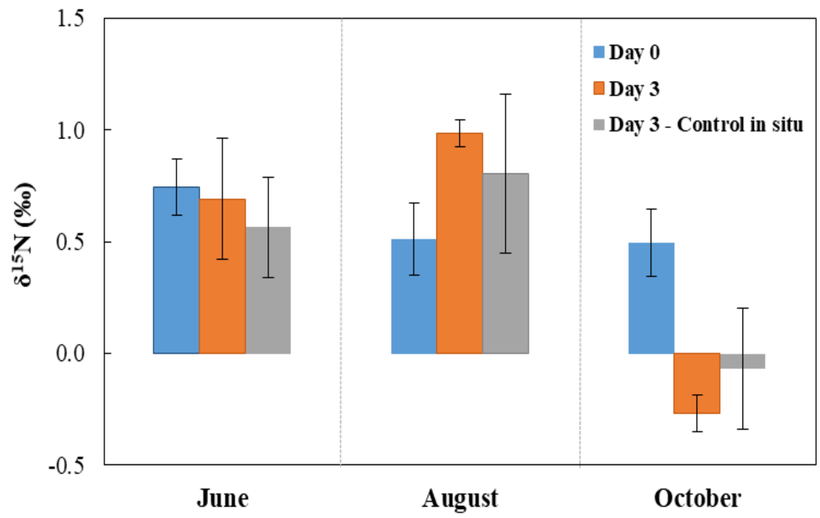

Figure 3. $\delta^{15}N$ (mean ± standard deviation) of *Cystoseira humilis* collected in the Collection site at Day 0 (same sampling day as the thalli used for the experiment), Day 3 and Day 3 – Control in situ (procedural control).

Table 1. Results of PERMANOVA (main test and pairwise tests) testing for differences in $\delta^{15}N$ values of *C. humilis* for the factors Period and Collection day.

a) Main test	**Source of variation**	**df**	**MS**	**Pseudo-F**	**P(perm)**	**Perms**
	Period	2	2.185	48.326	**0.001**	998
	Collection day	2	0.092	2.027	0.130	996
	Period x Collection day	4	0.491	10.853	**0.001**	999
	Residuals	35	0.045			
b) Pair-wise tests	**Between Periods within Collection days**			**t**	**P(perm)**	**Perms**
Day 0	June vs August			2.550	**0.010**	105
	June vs October			2.837	**0.023**	113
	August vs October			0.156	0.858	120
Day 3	June vs August			2.088	0.079	126
	June vs October			7.558	**0.009**	126
	August vs October			25.188	**0.015**	126
Day 3 - Ctrl situ	June vs August			1.277	0.226	126
	June vs October			4.049	**0.007**	126
	August vs October			4.362	**0.010**	126
c) Pair-wise tests	**Between Collection days within Periods**			**t**	**P(perm)**	**Perms**
June	Day 0 vs Day 3			0.394	0.719	126
	Day 0 vs Day 3 - Ctrl situ			1.570	0.185	126
	Day 3 vs Day 3 - Ctrl situ			0.811	0.487	126
August	Day 0 vs Day 3			5.539	**0.006**	123
	Day 0 vs Day 3 - Ctrl situ			1.676	0.156	123
	Day 3 vs Day 3 - Ctrl situ			0.979	0.353	126
October	Day 0 vs Day 3			10.020	**0.005**	126
	Day 0 vs Day 3 - Ctrl situ			4.100	**0.013**	112
	Day 3 vs Day 3 - Ctrl situ			1.570	0.181	123

3.1.2. Macroalgae experiment

$\delta^{15}N$ of primary producers is known to be useful to indicate the presence of anthropogenic nutrients well before that main ecological changes become observable (Schubert et al., 2013). In fact, despite crystal-clear waters characterized the experimental sites in Cyprus across the experimental periods, $\delta^{15}N$ in deployed macroalgae showed spatial and temporal patterns that provide indication of the presence of anthropogenic nitrogen input and its extent.

The comparison between the $\delta^{15}N$ values of the macroalgae deployed at increasing distance from the coastline in each study site and period, and the baseline $\delta^{15}N$ (i.e. the mean $\delta^{15}N$ value of the macroalgae from the Collection site at Day 0 in each period) allowed to detect the presence of anthropogenic nutrients in coastal waters (Fig. 4). In particular, in June, putatively before the tourist peak, only the macroalgae deployed within 200 m from the coastline of the Impact site showed $\delta^{15}N$ values significantly higher than the baseline (t-test p-value < 0.001), with mean values respectively of 1.49±0.21‰ at 100 m and 1.21±0.12‰ at 200 m *vs*. 0.74±0.13‰ of the baseline. After that, in August, during the high tourist peak, $\delta^{15}N$ of macroalgae deployed over the whole Impact site resulted significantly higher than the baseline (t-test p-value < 0.001 at 100 and 200 m, and < 0.01 at 300 m), with mean values respectively of 1.28±0.29‰ at 100 m, 0.99±0.32‰ at 200 m, and 0.87±0.24‰ at 300 m *vs*. 0.51±0.16‰ of the August baseline. This seems to be associated to a higher availability of anthropogenic dissolved nutrients, as macroalgae typically uptake heavier isotopes (^{15}N) when they are more available, quickly integrating them into their tissues (Cole et al., 2005; Costanzo et al., 2001;

Fernandes et al., 2012). On the contrary, in the two Control sites, the δ^{15}N signature registered in the macroalgae tissues was not significantly higher than that of the baseline in June and August, with the exception of macroalgae deployed at 300 m distance from the coastline of Control 2 site in August. In October, at the end of the tourist period, no significant differences emerged between the δ^{15}N of the macroalgae deployed at 100 m at the Impact site and that of the baseline, but δ^{15}N of the macroalgae deployed at 200 m and 300 m, and also at both Control sites significantly reduced (t-test p-value < 0.05 and <0.01, see Fig. 4). This can be due to a lesser presence of anthropogenic nitrogen in seawater, as expected at the end of the touristic season, but can have been also influenced by the adverse meteorological conditions that characterized the last days of the experiment implementation. In fact, in shallow marine areas interested by strong mixing of waters, local environmental conditions, especially in terms of increased turbidity and reduction of light intensity, can change profoundly the performance of macroalgae, including nutrient uptake and assimilation (Barr et al., 2013; Celis-Plá et al., 2014).

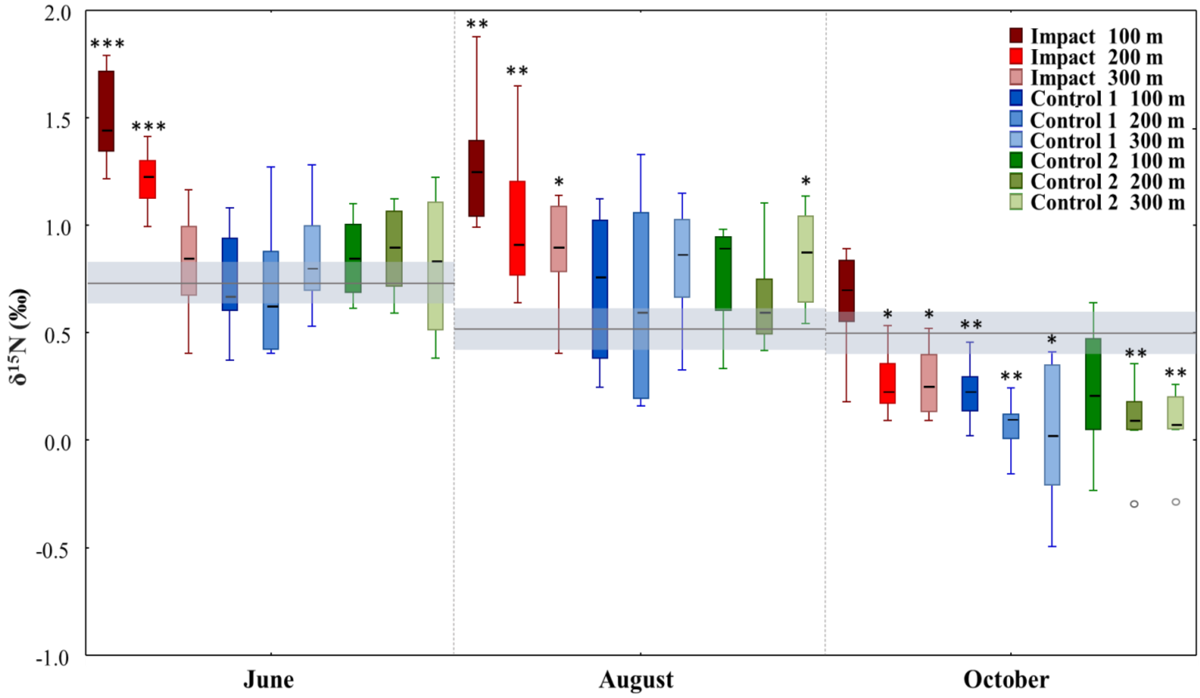

Figure 4. Boxplot of δ^{15}N values of the macroalgae deployed at different distance from the coastline (100, 200, 300 m) in the study sites: Impact (Sunrise beach), Control 1 and 2 (Cavo Greco) in June (before the tourist peak), August (during the tourist peak) and October (after the tourist peak). Each box contains 50% of the data, the thick horizontal line indicates the median; lower and upper whiskers represent respectively the first and fourth quartiles of the total range and circles represent outliers of the distribution. Asterisks indicate the significance level of the differences between the δ^{15}N values of the deployed macroalgae and the baseline, according to t-test. *p*-values: * = *p*-value< 0.05, ** = *p*-value< 0.01, *** = *p*-value< 0.001. Horizontal lines with shadow areas overlaying the boxplots at each period indicate the mean of the specific baseline and the related standard deviation.

These findings are in accordance with the δ^{15}N signature of the suspended particulate organic matter (POM) recorded in the experimental sites (Fig. 5). Despite POM is not a direct proxy for nutrients used by macroalgae, which are instead included into DIN (dissolved inorganic nitrogen in the form of ammonia, nitrate and nitrite), its δ^{15}N signature is widely recognized to give indication of the trophic condition of the environment, including the presence of anthropogenic inputs or events of eutrophication (Cole et al., 2005; Vizzini and Mazzola, 2006). The suspended POM in coastal waters is generally composed by a mixture of detrital material and living phytoplankton, which are known to quickly uptake dissolved nutrients, and then respond to the nutrient load and typology with different $\delta^{15}N$.

As expected, at the study sites, POM $\delta^{15}N$ signature showed the same temporal trend as the macroalgae, and statistical analysis highlighted highly significant differences for the interaction of the factors Period x Site and, in particular, between periods both at the Impact and Control 2 sites, and between Impact and both Control sites in both periods (Table 2). In fact, the mean value registered at the Impact site during the tourist peak (August) was more than 2-fold higher than in October in the same site and in the Control sites in both periods. This corroborates the isotopic enrichment of macroalgae at the Impact site, although the values were within the range recorded in other Mediterranean oligotrophic waters (around 2-5‰ e.g. Vizzini and Mazzola, 2006b, 2005).

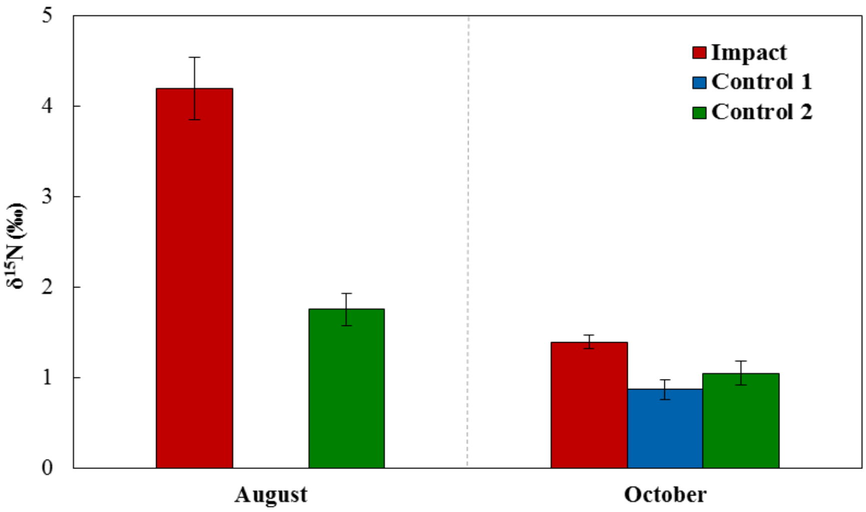

Figure 5. $\delta^{15}N$ (mean ± standard deviation) of suspended particulate organic matter (POM) collected in the Impact site (Sunrise beach) and the two Control sites (Cavo Greco).

Table 2. Results of PERMANOVA (main test and pairwise tests) testing for differences between $\delta^{15}N$ signature of POM (suspended particulate organic matter) collected in the Impact (Sunrise beach) and Control sites (Cavo Greco) during the experiments of August and October.

a) Main test		Source of variation	df	MS	Pseudo-F	P(perm)	Perms
		Period	1	9.314	158.080	**0.001**	997
		Site	2	2.993	50.805	**0.001**	999
		Period x Site	1	3.232	54.860	**0.001**	997
		Residuals	10	0.059			
b) Pair-wise tests		**Between Periods within Sites**			**t**	**P(MC)**	**Perms**
	Impact	August vs October			13.701	**0.001**	10
	Control 2	August vs October			3.154	**0.035**	10
c) Pair-wise tests		**Between Sites within Periods**			**t**	**P(MC)**	**Perms**
	August	Impact vs Control 2			8.228	**0.002**	10
	October	Impact vs Control 1			6.928	**0.004**	10
		Impact vs Control 2			3.880	**0.018**	10
		Control 1 vs Control 2			1.859	0.142	10

The analysis of macroalgae $\delta^{15}N$ data standardized to the seasonal baseline allowed to quantify the isotopic variation ($\Delta\delta^{15}N$) occurred in the macroalgae tissues over the experiment, and to highlight spatial and temporal trends, both by means of boxplot graphs (Fig. 6) and of georeferenced maps (Fig. 7). Overall, small variations were recorded. $\Delta\delta^{15}N$ ranged from -0.99 to +1.36‰, with the same order of magnitude found in similar studies using species of the same genus. Orlandi et al. (2014), for instance, found that $\Delta\delta^{15}N$ of *C. amentacea* and *Ulva lactuca* incubated for 48 hours in a strongly urbanised coastal area impacted by polluted waters ranged on average from +1.04±0.04‰ to +1.6±0.72‰ and from +1.95±0.21‰ to +2.86±0.58‰ respectively. Authors attributed the lower variation of *C. amentacea* to the lower turnover and uptake rates of this species selected, which is a perennial and slow-growing compared to the opportunistic fast-growing *U. lactuca*. The $\Delta\delta^{15}N$ found in this study overlaps the low limit of the range reported by Orlandi et al. (2014) and is indicative of the presence of ^{15}N-enriched nutrient although they do not reach high levels.
Significant differences among Periods and in the interaction of the factors Site x Distance (Table 3a). All the three periods were significantly different each other, overall showing higher $\Delta\delta^{15}N$ in August, followed by June and then October (Table 3b). As regards the interaction of the factors Site x Distance, $\Delta\delta^{15}N$ at 100 and 200 m from the coastline of the Impact site was significantly higher than $\Delta\delta^{15}N$ in both Control sites (Table 3c), and decreased significantly from the landward line (100 m) to the seaward line (300 m) only at the Impact site (Tab 3d).

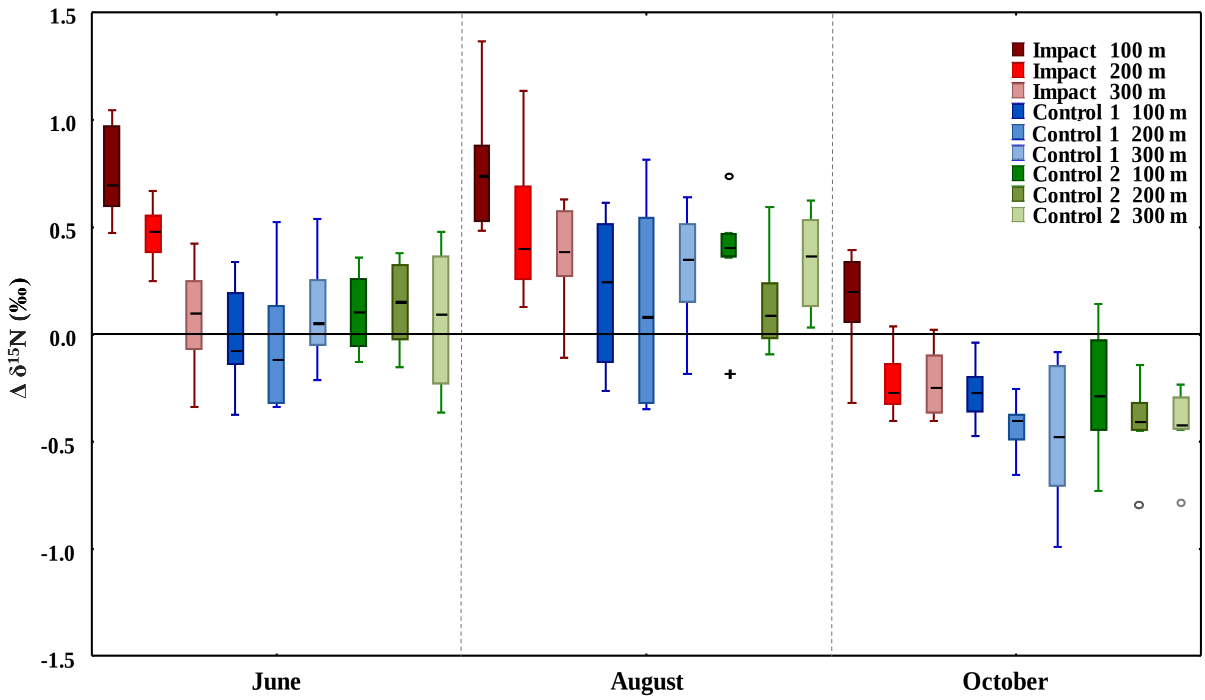

Fig. 6. Boxplot of $\Delta\delta^{15}N$ values (variation of $\delta^{15}N$ compared to the baseline) of the macroalga *C. humilis* deployed at different distance from the coastline (100, 200, 300 m) in the study sites: Impact (Sunrise beach), Control 1 and 2 (Cavo Greco) sites during the experimental periods: June (before the tourist peak), August (during the tourist peak) and October (after the tourist peak). Each box contains 50% of the data, the thick horizontal line indicates the median; lower and upper whiskers represent respectively the first and fourth quartiles of the total range and circles represent outliers of the distribution. Black horizontal line overlaying the graph indicates the baseline; data above and below the baseline indicate ^{15}N-enrichment and ^{15}N-depletion of macroalgae respectively.

Table 3. Results of PERMANOVA (main test and pairwise tests) testing the differences between the $\Delta\delta^{15}N$ values of the macroalga *C. humilis* deployed at different distance from the coastline (100, 200, 300 m) of the three study sites (Impact, Control 1 and 2) across the periods (June, August and October).

a)	Main test	Source of variation	df	MS	Pseudo-F	P(perm)	Perms
		Period	2	6.697	105.370	**0.001**	998
		Site	2	2.373	37.335	**0.001**	999
		Distance	2	0.531	8.357	**0.002**	997
		Period x Site	4	0.029	0.456	0.757	998
		Period x Distance	4	0.061	0.959	0.429	999
		Site x Distance	4	0.408	6.422	**0.001**	999
		Period x Site x Distance	8	0.060	0.941	0.461	999
		Residuals	168	0.064			
b)	**Pair-wise tests**	**Between Periods**			**t**	**P(perm)**	**Perms**
		June vs August			3.176	**0.003**	998
		June vs October			12.354	**0.001**	995
		August vs October			13.163	**0.001**	998
c)	**Pair-wise tests**	**Between Sites within Distances**			**t**	**P(perm)**	**Perms**
	100 m	Impact vs Control 1			7.880	**0.001**	999
		Impact vs Control 2			6.845	**0.001**	996
		Control 1 vs Control 2			0.616	0.532	997
	200 m	Impact vs Control 1			4.661	**0.001**	995
		Impact vs Control 2			3.882	**0.001**	998
		Control 1 vs Control 2			1.075	0.307	997
	300 m	Impact vs Control 1			1.247	0.188	998
		Impact vs Control 2			1.131	0.264	996
		Control 1 vs Control 2			0.191	0.857	998
d)	**Pair-wise tests**	**Between Distances within Sites**			**t**	**P(perm)**	**Perms**
	Impact	100 vs 200 m			5.215	**0.001**	996
		100 vs 300 m			7.092	**0.001**	997
		200 vs 300 m			2.626	**0.011**	998
	Control 1	100 vs 200 m			1.006	0.323	995
		100 vs 300 m			0.031	0.975	997
		200 vs 300 m			0.957	0.332	996
	Control 2	100 vs 200 m			0.547	0.592	997
		100 vs 300 m			0.377	0.708	998
		200 vs 300 m			0.160	0.890	998

Temporal and spatial trends of $\Delta\delta^{15}N$ are easily readable in the georeferenced maps, in which dark orange contours are concentrated in the landward line (100 m from the coastline) suggesting the presence of ^{15}N-enriched (i.e. anthropogenic) nutrients at the Impact site in June, when the tourist period started, with a decline at increasing distance due to the dilution effect of seawater (Fig. 7). Then in August, during the summer tourist peak, darker orange contours clearly spread up to 300 m, while in October, the presence of ^{15}N- enriched nutrients seems to drop in the whole area, as evident from the dominance of light green contours that reflect the exposure of macroalgae to marine nitrogen. Unlike the Impact site, at both Control sites, there was only a very low isotopic variation of macroalgae, with very low positive or negative $\Delta\delta^{15}N$ values, indicating overall the occurrence of natural ^{15}N fluctuation (Fig. 7).

Concluding, these findings give a clear clue of the origin and the timing of anthropogenic nutrient spread, that, as expected, is strictly associated to coastal activities. Sources of impact characterizing such tourist destination, in fact, can be attributed to the proximity of numerous holiday villas, hotel infrastructures, and many leisure activities including bathing, boating and water sports. The sum of all of these elements evidently exerts a detectable effect, although minor, on the coastal zone, which is clear from the beginning of the tourist periods, reaches higher levels during tourist peak and then declines at the end of the tourist season.

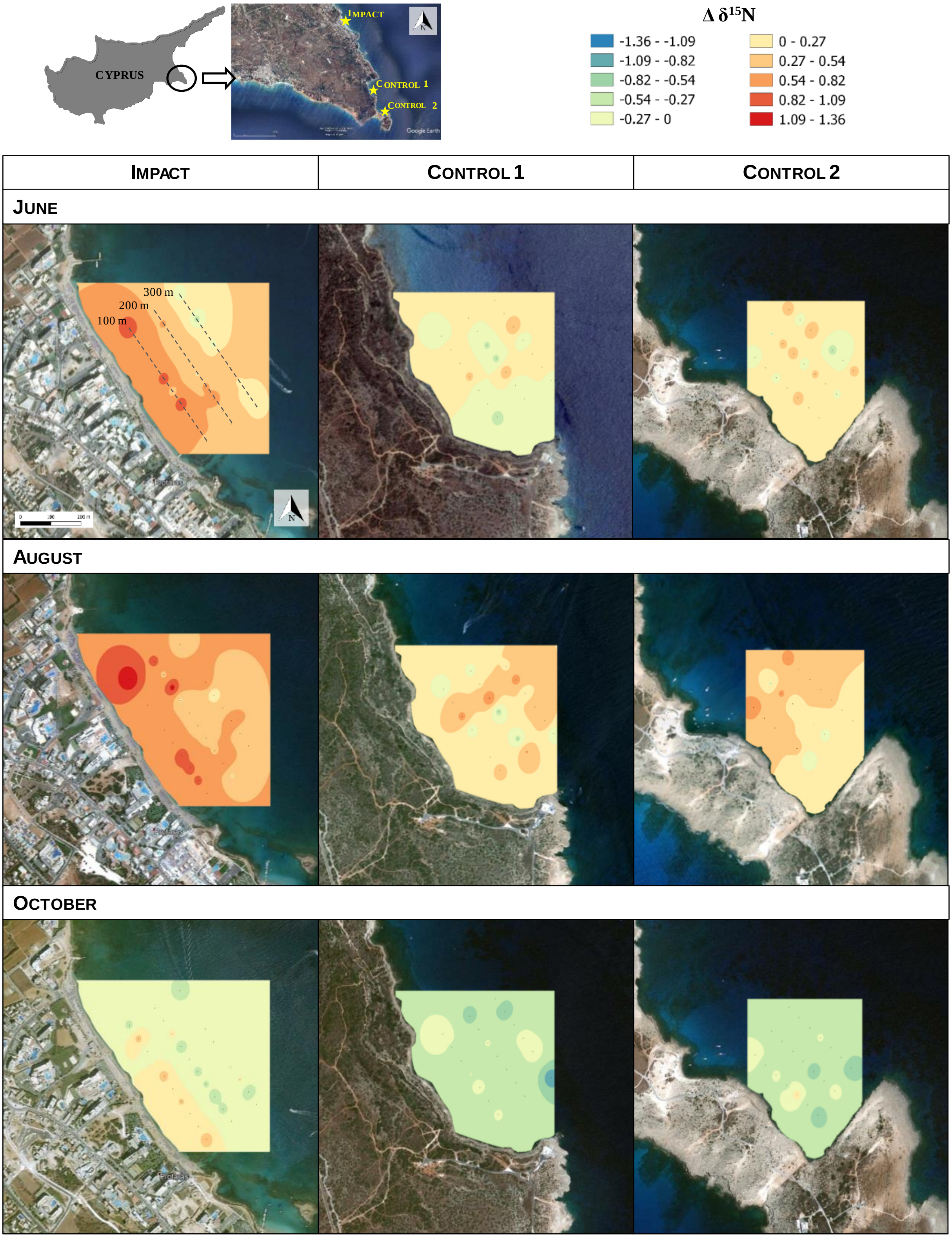

Figure 7. Georeferenced maps of $\Delta\delta^{15}N$ values in June, August and October 2017 at the Impact site (Sunrise beach in Protaras) and Control sites (Cavo Greco) of Cyprus. Dashed lines (superimposed only to the first panel for the sake of simplicity) indicate the distance from the coastline (100, 200 and 300 m) where macroalgae were deployed.

3.2. Macroalgae deployment in Sicily

3.2.1. Main physicochemical features of the study sites and experiment procedural control

Temperature and salinity of surface seawater showed a clear temporal trend in the coastal area of Giardini Naxos. In June, temperature varied between 21.3 and 22.9°C across the experimental sites, while in August higher values were recorded in all the three sites, ranging from 25.5 to 26.7°C, and then, in October, temperature decreased in the whole area, showing values comprised between 20.8 and 22.7°C. Salinity values were more homogeneous across periods and sites: they varied between 37.2 and 38.7 psu in June, 38.7 and 38.9 psu in August and 38.3 and 38.6 psu in October.

Mean $\delta^{15}N$ values of the macroalga *Cystoseira amentacea* collected from the Collection site varied the interaction of the factors Period and Collection Day (Table 4a). In more detail, the macroalgae collected at Day 0 showed comparable values in the first two periods (June and August), which were lower than in the third one (October) (Table 4b, Figure 8). This variability reflects temporal variations in local environmental conditions, being stable isotope ratios of benthic macrophytes influenced by light, types and concentrations of nutrients, and seawater temperature (e.g. Hyndes et al., 2013; Ochoa-Izaguirre and Soto-Jiménez, 2015). Moreover, no significant differences were highlighted in any of the periods between $\delta^{15}N$ of the macroalgae taken at Day 3 and the procedural control (Day 3 – Control *in situ*) (Table 4c), suggesting that the experimental procedures (cut, handling and deployment) did not influence the $\delta^{15}N$ signature of the macroalgae.

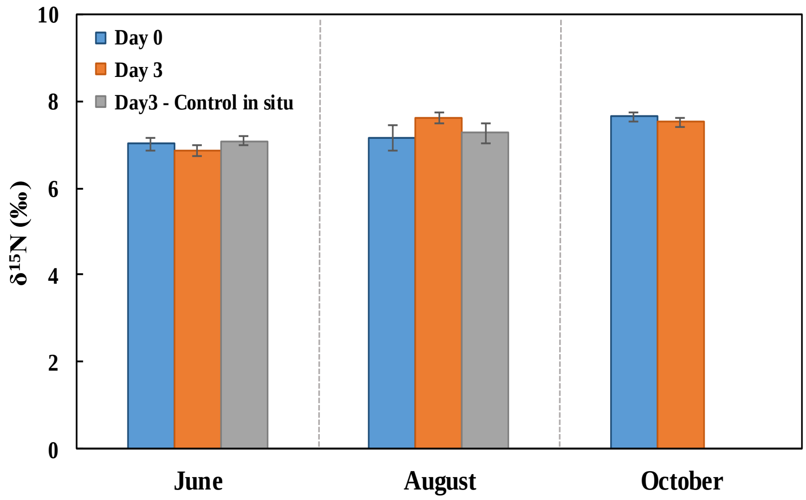

Figure 8. $\delta^{15}N$ (mean ± standard deviation) of *Cystoseira amentacea* collected in the Collection site at Day 0 (same sampling day as the thalli used for the experiment), Day 3 and Day 3 – Control in situ (procedural control).

Table 4: Results of PERMANOVA (main test and pairwise tests) testing for differences in $\delta^{15}N$ values of *C. amentacea* from the Collection site.

a) Main test	Source of variation	df	MS	Pseudo-F	P(perm)	Perms
	Period	2	0.009	0.336	0.716	999
	Collection day	2	0.639	23.553	**0.001**	998
	Period x Collection day	3	0.139	5.128	**0.019**	998
	Residuals	16	0.027			
b) Pair-wise tests	**Between Periods within Collection days**			**t**	**P(MC)**	**Perms**
Day 0	June vs August			0.704	0.521	10
	June vs October			6.545	**0.003**	10
	August vs October			2.828	**0.040**	10
Day 3	June vs August			6.962	**0.003**	10
	June vs October			6.651	**0.004**	10
	August vs October			0.829	0.465	10
Day 3 - Ctrl situ	June vs August			1.268	0.294	10
c) Pair-wise tests	**Between Collection days within Periods**			**t**	**P(MC)**	**Perms**
June	Day 0 vs Day 3			1.396	0.223	10
	Day 0 vs Day 3 - Ctrl situ			0.704	0.534	10
	Day 3 vs Day 3 - Ctrl situ			2.322	0.077	10
August	Day 0 vs Day 3			2.514	0.069	10
	Day 0 vs Day 3 - Ctrl situ			0.560	0.630	9
	Day 3 vs Day 3 - Ctrl situ			2.255	0.094	10
October	Day 0 vs Day 3			1.431	0.228	10

3.2.2. Macroalgae experiment

Comparison of the $\delta^{15}N$ of *Cystoseira amentacea* deployed in the study sites with the specific baseline $\delta^{15}N$ (i.e. the mean $\delta^{15}N$ value of the macroalgae from the Collection site at Day 0 in each period) revealed different patterns across periods and sites, indicating different behaviour of the macroalgae throughout the experiment (Fig. 9). In more detail, in June, overall most macroalgae deployed in the three sites had lower $\delta^{15}N$ than the baseline (7.02±0.14‰), and in particular, those collected at 200 m (6.67±0.24‰) and 300 m (6.51±0.04‰) from the coastline of Control 1 (t-test p-value < 0.05 and < 0.01 respectively) (Fig. 9). In August, the mean value of the macroalgae deployed at 200 and 250 m from the coastline of the Impact site was significantly higher than the baseline (7.15±0.29‰), with mean values respectively of 7.74±0.39‰ and 7.46±0.18‰ (t-test p-value < 0.05) (Fig. 9). In contrast, no significant differences resulted from the comparison between the macroalgae from both Control sites, as the upper range of the data distribution always overlapped the baseline range (Fig. 9). Lastly, in October, the macroalgae from all the three sites showed a comparable trend, being all partially overlapped to the baseline range, and no significant differences with the baseline were highlighted (Table 9).

Although the slow uptake and growth rate of perennial macroalgae (Martínez et al., 2011), the contrasting behaviour of deployed *C. amentacea* between Impact and Control sites, particularly evident during the tourist peak (August), suggests the uptake of ^{15}N-enriched nutrients in the coastal waters in the Impact Site. Indeed, anthropogenic nitrogen loads into water bodies are associated with higher δ^{15}N values of dissolved inorganic nitrogen (DIN), particulate organic matter (POM) and macroalgae than in pristine sites (Heaton, 1986).

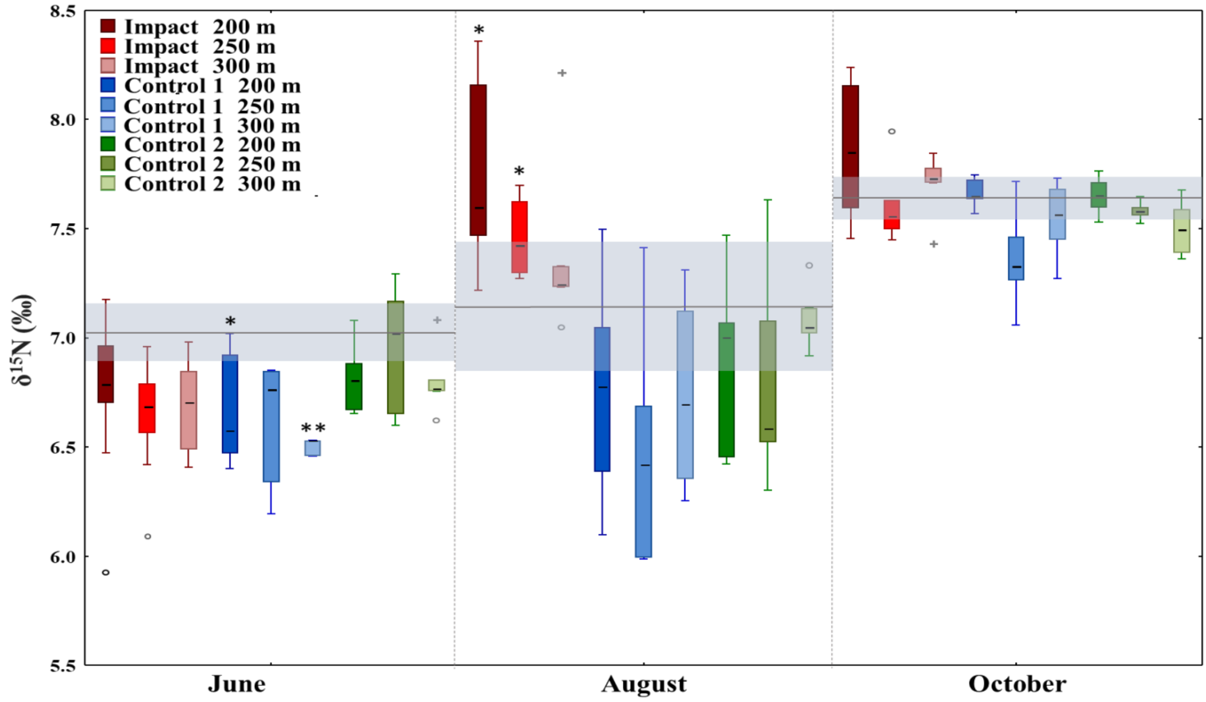

Figure 9: Boxplot of δ^{15}N values of the macroalga *C. amentacea* deployed at different distance from the coastline (200, 250, 300 m) in the study sites: Impact (Giardini Naxos Bay), Control 1 and 2 (Fondaco Parrino beach) across the experimental periods: June (before the tourist peak), August (during the tourist peak) and October (after the tourist peak). Each box contains 50% of the data, the thick horizontal line indicates the median; lower and upper whiskers represent respectively the first and fourth quartiles of the total range and circles represent the outliers of the distribution. Asterisks indicate the significance level of the differences between the δ^{15}N values of the deployed macroalgae and the baseline according to t-test. *p*-values: * = *p*-value< 0.05, ** = *p*-value< 0.01, *** = *p*-value< 0.001. The horizontal lines and the shadow areas overlaying the boxplots at each period indicate respectively the mean δ^{15}N value of the baseline and the relative standard deviation.

Suspended particulate organic matter (POM) from all the study sites showed a clear temporal trend, consistent with that of macroalgae, with the highest δ^{15}N values in August and the lowest in October, and a sharp increase from June to August, which was particularly evident in the Impact site (Fig. 10). This is because POM is generally composed by plankton, which is known to quickly take up dissolved nutrients, hence it is a good proxy for the origin of nutrients available (Cole et al., 2005; Vizzini and Mazzola, 2006).

Statistical analysis revealed highly significant differences of POM δ^{15}N for the interaction Period x Site (Table 5a). In particular, POM from the Impact site was significantly more ^{15}N-enriched in August than in the other periods, suggesting the presence of anthropogenic nitrogen, which typically shows high δ^{15}N values. Significant differences among periods were found also in Control 1 and Control 2, although they were more limited. Spatial differences of the isotopic signature of POM, which were significant between the Impact and both Control sites only in August (Table 5b), provide evidence on different trophic conditions in the Impact site across periods. Indeed, the August δ^{15}N peak recorded in the Impact site (7.9±0.3‰) reflects an increase in ^{15}N-increased organic matter likely attributable to the numerous tourist activities present along the coast.

The effluent of the Wastewater Treatment Plant (WTP) of Giardini Naxos (located south of the experimental sites) was also analysed for the $\delta^{15}N$ signature of POM, which showed values in the range 4.35-7.94‰, resulting slightly more ^{15}N-enriched than that of marine POM with the exception of August. Overall, the isotopic values were similar to data found in secondary treated effluents (like the Giardini Naxos WTP) from other geographical areas (e.g., Australia, Piola et al., 2006) because nitrification and denitrification associated with secondary treatment tend to result in the accumulation of the heavier nitrogen isotope, ^{15}N (Heaton, 1986).

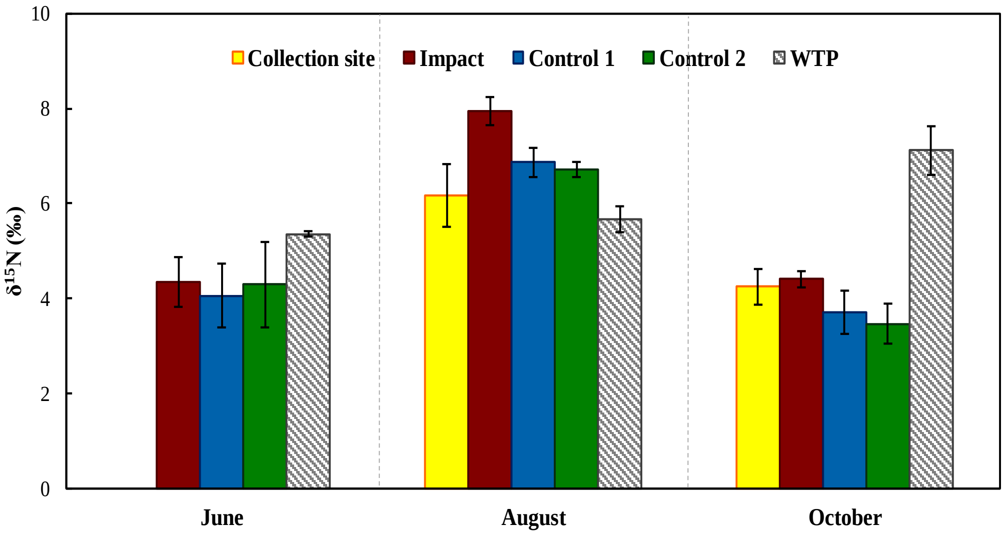

Figure 10: $\delta^{15}N$ (mean ± standard deviation) of suspended particulate organic matter (POM) in the Collection site, in the Impact site (Giardini Naxos Bay) and the two Control sites (Fondaco Parrino beach), and also in the Wastewater Treatment Plant (WTP).

Table 5: Results of PERMANOVA (main test and pairwise tests) testing the differences between δ¹⁵N signature of POM (suspended particulate organic matter) collected at Impact and Control sites during the experimental campaign of June, August and October.

a) Main test	Source of variation	df	MS	Pseudo-F	P(perm)	Perms
	Period	2	16.916	69.485	**0.001**	999
	Site	2	3.664	15.052	**0.001**	999
	Period x Site	4	1.267	5.206	**0.011**	999
	Residuals	18	0.243			
b) Pair-wise tests	**Between Periods within Sites**			**t**	**P(MC)**	**Perms**
Impact	June vs August			10.385	**0.001**	10
	June vs October			0.169	0.887	10
	August vs October			18.345	**0.001**	10
Control 1	June vs August			3.319	**0.024**	10
	June vs October			0.745	0.487	10
	August vs October			5.466	**0.010**	10
Control 2	June vs August			2.699	0.052	10
	June vs October			1.447	0.223	10
	August vs October			8.638	**0.002**	10
c) Pair-wise tests	**Between Sites within Periods**			**t**	**P(MC)**	**Perms**
June	Impact vs Control 1			0.590	0.596	10
	Impact vs Control 2			0.093	0.939	10
	Control 1 vs Control 2			0.363	0.731	7
August	Impact vs Control 1			8.926	**0.002**	10
	Impact vs Control 2			11.601	**0.002**	10
	Control 1 vs Control 2			0.800	0.451	10
October	Impact vs Control 1			2.526	0.054	10
	Impact vs Control 2			3.592	**0.031**	10
	Control 1 vs Control 2			0.691	0.504	10

After assessing the differences between the raw isotopic data of the deployed macroalgae compared with the specific baseline, we focused on the extent of the variation in δ¹⁵N compared to the baseline (hereafter Δδ¹⁵N), by means of boxplot graphs (Fig. 11) and georeferenced maps (Fig. 12). Both approaches allowed to quantify the ¹⁵N enrichment/depletion of the macroalgae in the experimental sites across the periods. Although *Cystoseira amentacea* is a perennial macroalgae with longer tissue turnover rates than opportunistic macroalgae which were used for deployments in other studies (e.g. *Ulva* spp., Orlandi et al., 2014), it was able to respond to the three-day incubation experiment, showing an overall narrow Δδ¹⁵N range, from -1.16 to +1.21‰, which was indicative of low anthropogenic nutrient input in the study area. Nevertheless, relevant Δδ¹⁵N patterns turned out (Fig. 11) allowing to infer about the influence of the tourist activities on the Sicilian coastal waters.

Statistical analysis highlighted significant Δδ¹⁵N differences among Distances and for the interaction Period x Site (Table 6a). As regards distances, pairwise tests revealed significant differences between the Δδ¹⁵N of the macroalgae deployed at 200 and those deployed at the other two distances (250 m and 300 m) (Table 6b). Despite statistics did not highlight any effect of the interaction of the factors Distance x Site, the Δδ¹⁵N decreasing gradient from the coast to the offshore was clearly more evident in the Impact site (Fig. 11).

As regards the interaction of the factors Period x Site, Δδ¹⁵N of the macroalgae deployed in the Impact site showed a clear temporal trend, with significantly higher values in August, followed by October and then June. Moreover, $\Delta\delta^{15}N$ of the macroalgae deployed in the Impact site was significantly higher than that observed in the macroalgae from both Control sites in August and in October, while there was no ^{15}N-enrichment in June, when the tourist period was not yet started. In particular, although limited (up to ~ 1.3‰), the highest ^{15}N-enrichment values were recorded at 200 m from the coastline of the Giardini Naxos Bay in August, when the beach is typically highly frequented, and then gradually decreased at 250 and 300 m, with a certain overlap between these two distances. Moreover, unlike the Impact site, a tendency to ^{15}N-depletion was highlighted across the periods in the Control sites, indicating the exposure to low-^{15}N nutrients compared to the Collection sites where the macroalgae were sampled at the beginning of the experiment.

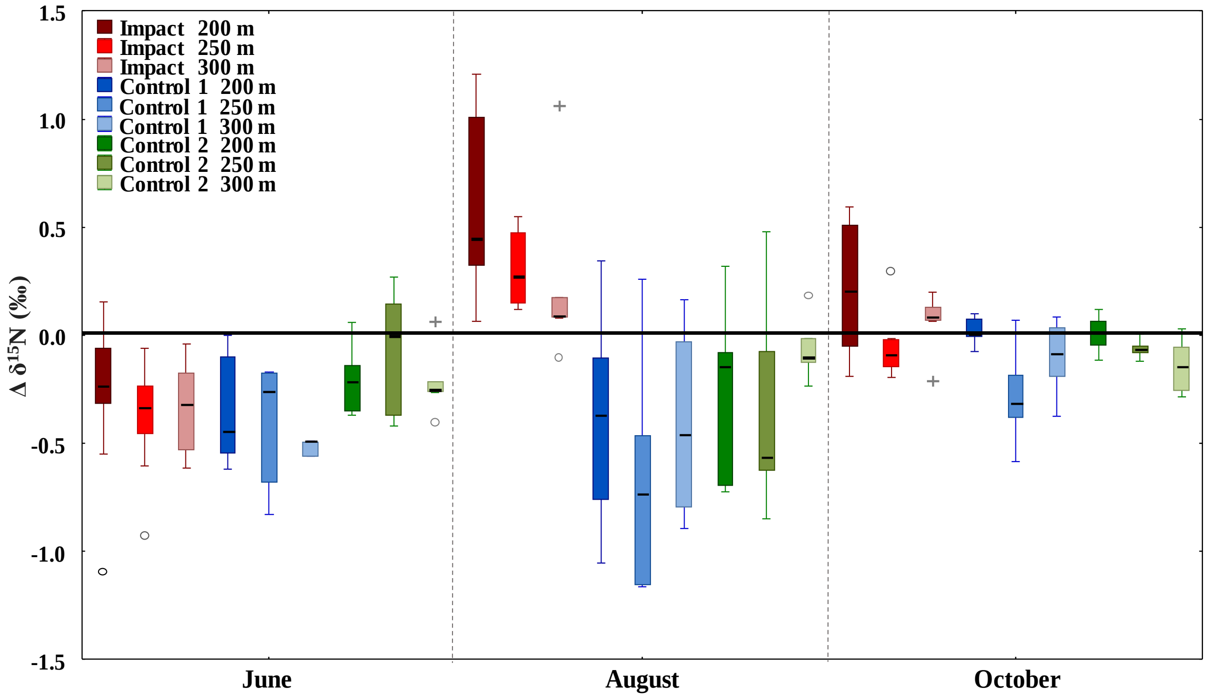

Figure 11: Boxplot of Δδ15N values (variation of δ15N compared to the baseline) of the macroalga C. amentacea deployed at different distance from the coastline (200, 250, 300 m) in the study sites: Impact (Giardini Naxos Bay), Control 1 and 2 (Fondaco Parrino beach) across the experimental periods: June (before the tourist peak), August (during the tourist peak) and October (after the tourist peak). Each box contains 50% of the data, the thick horizontal line indicates the median; lower and upper whiskers represent respectively the first and fourth quartiles of the total range and circles represent outliers of the distribution. Black horizontal line overlaying the graph indicates the baseline; data above and below the baseline indicate 15N-enrichment and 15N-depletion of macroalgae respectively.

Table 6: Results of PERMANOVA (main test and pairwise tests) testing the differences between the Δδ15N values of the macroalga *C. amentacea* deployed at different distance from the coastline (200, 250, 300 m) in the three study sites (Impact, Control 1 and 2) across the periods (June, August and October).

a) Main test	**Source of variation**	**df**	**MS**	**Pseudo-F**	**P(perm)**	**Perms**
	Period	2	0.821	10.763	**0.001**	998
	Site	2	1.855	24.335	**0.001**	999
	Distance	2	0.369	4.845	**0.014**	999
	Period x Site	4	0.812	10.650	**0.001**	998
	Period x Distance	4	0.077	1.006	0.419	999
	Site x Distance	4	0.117	1.540	0.166	999
	Period x Site x Distance	8	0.068	0.892	0.526	999
	Residuals	142	0.076			
b) Pair-wise tests	**Between Distances**			**t**	**P(perm)**	**Perms**
	200 m vs 250 m			2.794	**0.010**	995
	200 m vs 300 m			2.139	**0.034**	997
	250 m vs 300 m			0.743	0.441	995
c) Pair-wise tests	**Between Periods within Sites**			**t**	**P(perm)**	**Perms**
Impact	June vs August			8.770	**0.001**	995
	June vs October			5.086	**0.001**	993
	August vs October			3.763	**0.002**	998
Control 1	June vs August			0.511	0.648	996
	June vs October			3.640	**0.003**	998
	August vs October			2.660	**0.015**	995
Control 2	June vs August			0.503	0.636	997
	June vs October			1.669	0.111	998
	August vs October			1.489	0.153	998
d) Pair-wise tests	**Between Sites within Periods**			**t**	**P(perm)**	**Perms**
June	Impact vs Control 1			1.121	0.274	995
	Impact vs Control 2			2.014	**0.043**	996
	Control 1 vs Control 2			3.306	**0.004**	995
August	Impact vs Control 1			5.950	**0.001**	998
	Impact vs Control 2			4.999	**0.001**	994
	Control 1 vs Control 2			1.665	0.098	999
October	Impact vs Control 1			2.769	**0.013**	996
	Impact vs Control 2			2.359	**0.027**	997
	Control 1 vs Control 2			1.068	0.302	998

Georeferenced $\Delta\delta^{15}N$ maps obtained through the spatial analysis conducted in the Geographic Information System (GIS) gave a graphical overview of the influence of anthropogenic nitrogen in the coastal areas of Sicily across the experimental periods. In June, the diffused green/blue contours indicate an overall decrease of the isotopic signal of the deployed macroalgae, as an effect of the exposure to low-^{15}N nutrients. In August, the maps clearly show a change in the Impact site, where the contours vary from orange to red indicating an isotopic enrichment of almost all the deployed macroalgae as an effect of the exposure to more ^{15}N-enriched nutrients than in the Controls. In particular, the closest to the coastline are the points, the most intense orange/red colour are the contours, showing very clearly that the coastal activities represent the source of anthropogenic nutrients. The higher human waste linked to the drastic increase of tourists and bathers along the beach of Giardini Naxos in August, coupled with the presence of a touristic harbour and several recreational water activities (boating, water sports) and tourist facilities with potentially inadequate sewage systems, may be responsible for this small-spatial scale pattern. In contrast, the situation recorded in June in the Control sites persisted in August, except for a few sparse macroalgae. Afterwards, in October, the macroalgae collected from the Impact site, after the short-term deployment, were slightly isotopically enriched, notably those deployed in the southern part of the bay, suggesting a localised higher availability of ^{15}N-enriched nutrients, possibly linked to the presence of the touristic port and breakwaters, which reduce the water exchange in that area. This clear temporal pattern was not observed in the Control sites, and therefore in the Impact site it is likely attributable to the influence of tourist flows. Indeed, only a few points revealed a small enrichment of macroalgae in both Control sites.

Concluding, being the isotopic signature of marine macroalgae the mirror of the typology of nutrients exploited, the observed spatial and temporal patterns indicate a different pressure across the sites and periods with an increase of ^{15}N-enriched nutrients consistent with the tourist flows. In more detail, a touristic harbour, along with other tourist infrastructures (hotels, restaurants, bars, shops, beach lidos) and private houses feature all the coastline just behind the beach. All these activities are clearly more frequented in August when many local and foreign tourists spend their vacations in there, and therefore exert an effect on the adjacent coastal zone.

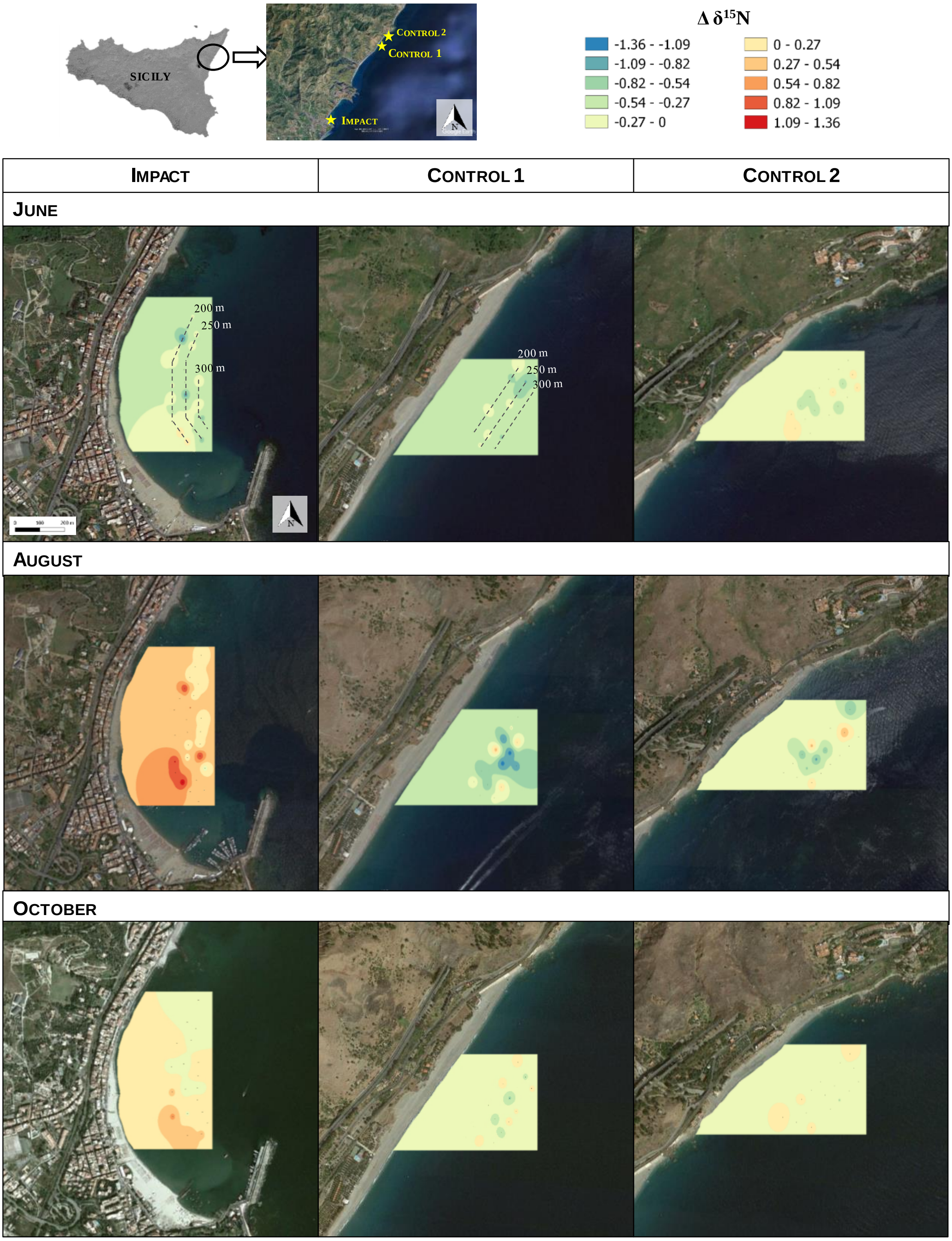

Figure 12. Georeferenced maps of $\Delta\delta^{15}N$ values in June, August and October 2017 at the Impact site (Giardini Naxos Bay) and Control sites (Fondaco Parrino beach) in Sicily. Dashed lines (superimposed only to the first two panels for the sake of simplicity) indicate the distance from the coastline (200, 250 and 300 m) where macroalgae were deployed.

3.3. Macroalgae deployment in Rhodes

3.3.1. Main physicochemical features of the study sites and experiment procedural control

Temperature of seawater at the three experimental sites in Rhodes (Impact, Control 1 and 2) followed a clear temporal trend, varying similarly across the three sites, with mean values between 25.8 and 26.5°C in June, then rising in August with values comprised between 29.3 and 30.6°C, and lastly decreasing in October with values ranging from 27.8 to 29.1°C. Salinity was measured only in October because of technical problems encountered with the multiprobe and was 40.6 ± 0.3 psu.

Mean $\delta^{15}N$ measured in the macroalga *Cystoseira compressa* collected from the Collection site at Day 0, Day 3 and the procedural control (Day 3 – Control *in situ*) ranged from 1.9 to 3.0‰ across the experimental periods, showing overall the highest values in October (Fig. 13). PERMANOVA revealed significant differences both among Periods and for the interaction of the factors Period x Collection day (Table 7a). In more detail, $\delta^{15}N$ of the macroalgae collected at Day 0 was significantly higher in October, followed by June and August (Fig. 13, Table 7b). Moreover, in October, $\delta^{15}N$ of *C. compressa* was significantly higher at Day 0, than at Day 3 and Day 3 – Control *in situ* (Fig. 13, Table 7c). This slightly higher values recorded in October at Day 0 are probably due to adverse meteorological conditions occurred the day before (e.g., water column mixing, freshwater input), which were registered by the macroalgae (e.g. Hyndes et al, 2013; Ochoa-Izaguirre & Soto-Jiménez, 2015).
In all periods, the comparison between the $\delta^{15}N$ values of the macroalgae collected at Day 3 and the procedural control (Day 3 – *control in situ*) showed no significant differences (Table 7c), suggesting that the experimental procedures (cut, handling and deployment) did not influence the $\delta^{15}N$ signature of the macroalgae.

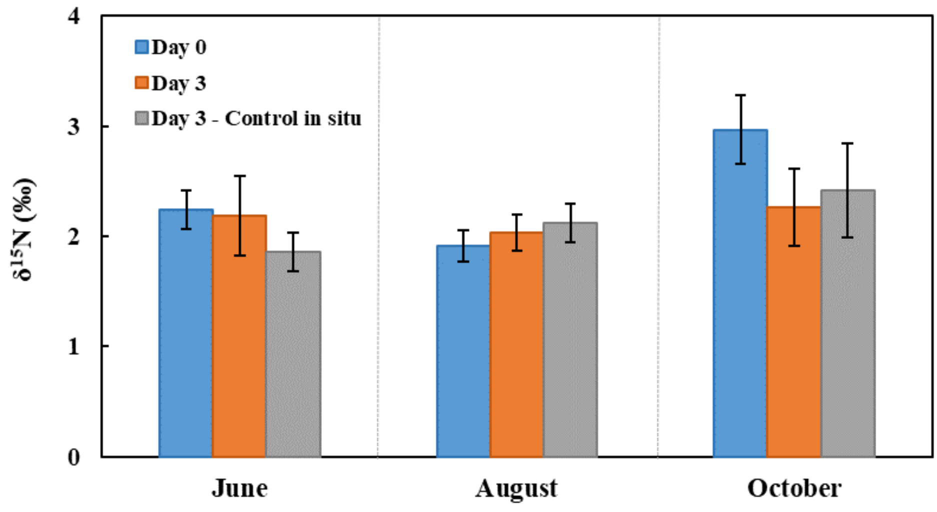

Figure 13. $\delta^{15}N$ (mean ± standard deviation) of *Cystoseira compressa* collected in the Collection site at Day 0 (same sampling day as the thalli used for the experiment), Day 3 and Day 3 – Control *in situ* (procedural control).

Table 7. Results of PERMANOVA (main test and pairwise tests) testing for differences in $\delta^{15}N$ values of *C. compressa* from the Collection site.

a) Main test	Source of variation	df	MS	Pseudo-F	P(perm)	Perms
	Period	2	1.212	16.159	**0.001**	998
	Collection day	2	0.264	3.515	**0.028**	998
	Period x Collection day	4	0.352	4.692	**0.005**	999
	Residuals	36	0.075			
b) Pair-wise tests	**Between Periods within Collection days**			**t**	**P(perm)**	**Perms**
Day 0	June vs August			3.280	**0.020**	122
	June vs October			4.514	**0.014**	123
	August vs October			6.824	**0.010**	126
Day 3	June vs August			0.883	0.546	112
	June vs October			0.305	0.686	126
	August vs October			1.299	0.261	116
Day 3 - Ctrl situ	June vs August			2.344	0.068	123
	June vs October			2.699	**0.046**	123
	August vs October			1.429	0.170	126
c) Pair-wise tests	**Between Collection days within Periods**			**t**	**P(perm)**	**Perms**
June	Day 0 vs Day 3			0.286	0.802	122
	Day 0 vs Day 3 - Ctrl situ			3.456	**0.016**	107
	Day 3 vs Day 3 - Ctrl situ			1.850	0.063	122
August	Day 0 vs Day 3			1.236	0.279	119
	Day 0 vs Day 3 - Ctrl situ			2.053	0.075	117
	Day 3 vs Day 3 - Ctrl situ			0.797	0.458	109
October	Day 0 vs Day 3			3.362	**0.021**	126
	Day 0 vs Day 3 - Ctrl situ			2.341	0.071	125
	Day 3 vs Day 3 - Ctrl situ			0.628	0.577	116

3.3.2. Macroalgae experiment

Comparison of $\delta^{15}N$ of the macroalga *Cystoseira compressa* deployed at increasing distance from the coastline in each study site and period, against the baseline $\delta^{15}N$ (i.e. the mean $\delta^{15}N$ value of the macroalgae from the Collection site at Day 0 in each period), revealed different spatial and temporal patterns. (Fig. 14). In June, most macroalgae showed lower mean $\delta^{15}N$ values than the baseline (2.24±0.17‰) and, in particular, those collected at 300 m from the coastline of Control 1 had significant lower values (1.86±0.25‰) than the baseline (t-test p-value < 0.05). In August, during the tourist peak, only the macroalgae collected at 100 m from the coastline of the Impact site reported significantly higher $\delta^{15}N$ (2.13±0.30‰), compared with the baseline (1.91±0.14‰) (t-test p-value < 0.05), while at the two Control sites, $\delta^{15}N$ of macroalgae did not differ substantially from the baseline. In October, the macroalgae deployed across all sites showed overall higher $\delta^{15}N$ values and wider ranges, compared with the other periods, and mean $\delta^{15}N$ was overall lower than the baseline (2.97±0.32‰). In particular, those deployed at 100 m from the coast of the Impact site, at 200 m from the coast of Control 1 and at 300 m from the coast of Control 2 showed significantly lower $\delta^{15}N$ than the baseline (t-test p-value < 0.05, Fig. 14).

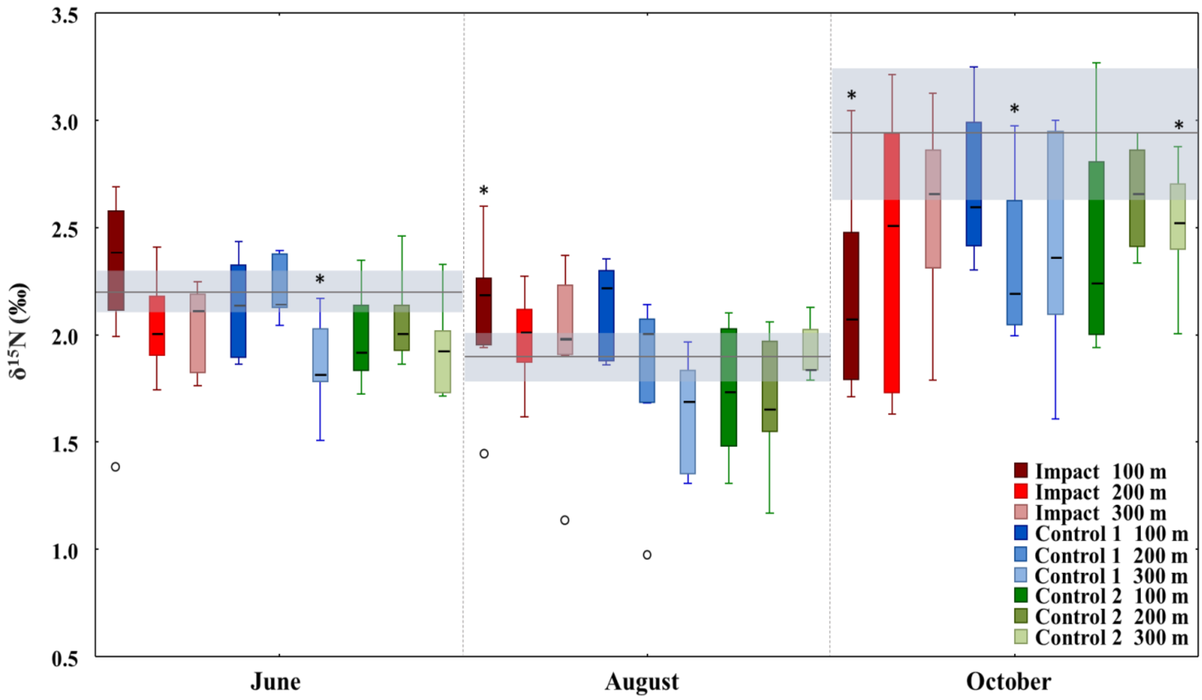

Figure 14. Boxplot of $\delta^{15}N$ values of the macroalgae deployed at different distance from the coastline (100, 200, 300 m) in the study sites: Impact (Faliraki beach), Control 1 and 2 (Afandou beach) in June (before the tourist peak), August (during the tourist peak) and October (after the tourist peak). Each box contains 50% of the data, the thick horizontal line indicates the median; lower and upper whiskers represent respectively the first and fourth quartiles of the total range and circles represent outliers of the distribution. Asterisks indicate the significance level of differences between the $\delta^{15}N$ values of the deployed macroalgae and the baseline according to t-test. p-values: * = *p*-value< 0.05. Horizontal lines with shadow areas overlaying the boxplots at each period indicate the mean of the specific baseline and the related standard deviation.

Nitrogen stable isotopic signature of particulate organic matter, POM, gives indication of the trophic condition of the environment where the macroalgae were incubated and can be an additional and useful variable to take into account when tracking the presence of anthropogenic nutrient input in coastal areas (Cole et al., 2005; Vizzini and Mazzola, 2006). At the study sites, mean $\delta^{15}N$ of the POM ranged between 1.40±0.38‰ and 3.37±0.17‰ and showed a similar temporal trend as the macroalgae, with overall lower values in August than in June and October at the Impact and Collection site, while comparable values were recorded across periods at the controls (Fig. 15). The higher values recorded in October are most probably due to the meteorological conditions preceding that sampling day, resulting in a higher terrestrial runoff that could have influenced the isotopic signal of the suspended particulate organic matter. In June, a high peak of temperature was experienced during the days preceding the sampling day, followed by production of mucilaginous material onto the shores spreading also in the water column. We think that these unpredicted phenomena can have potentially influenced the POM isotopic signature in the collection site because it is a very shallow in-shore site, while the different spatial patterns observed between Impact than Control sites are most probably linked to the different land-use of the two coastal zones. Statistical analysis run on POM isotopic signatures highlighted significant differences for the interaction of the factors Period x Site (Table 8a). Pairwise tests confirmed that a significant difference emerged at the Impact site between periods (Table 8b) with the values recorded in August significantly lower than the in other periods, similarly to the pattern observed in the Collection site. Moreover, in June and October, the POM signature at the Impact site was significantly higher than at the Control sites (only Control 1 in June).

As an additional information, the water collected from the last tank of the wastewater treatment process of the Wastewater Treatment Plant (WTP) of Rhodes (located at North of the experimental sites) was also analysed for the $\delta^{15}N$ signature. Overall, the nitrogen isotopic values were considerably higher than those registered at the three sites of the experiment in all periods, ranging from 3.77 ± 0.48‰ in August to 6.02 ± 0.01‰ in June. However, the outfall of the WTP is placed at about 400 m from the coastline and 100 m deep and, given the lower values of the POM $\delta^{15}N$ signature in the coastal sites, we can exclude that there could be a direct influence of such inputs on the coastal waters.

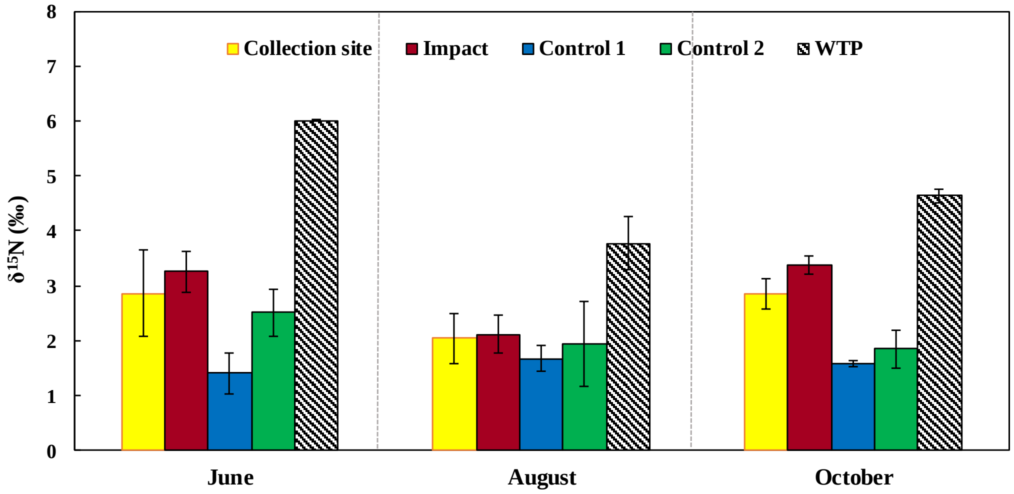

Figure 15. $\delta^{15}N$ (mean ± standard deviation) of suspended particulate organic matter (POM) at the Collection site, the Impact site (Faliraki beach) and the two Control sites (Afandou beach), and also in the Wastewater Treatment Plant (WTP).

Table 8. Results of PERMANOVA (main test and pairwise tests) testing for differences between δ^{15}N signature of POM (suspended particulate organic matter) collected at Impact and Control sites during the experimental campaign of June, August and October.

a) Main test		Source of variation	df	MS	Pseudo-F	P(perm)	Perms
		Period	2	0.550	3.579	**0.045**	999
		Site	2	4.232	27.521	**0.001**	999
		Period x Site	4	0.666	4.331	**0.019**	999
		Residuals	18	0.154			
b) Pair-wise tests		**Between Periods within Sites**			**t**	**P(MC)**	**Perms**
	Impact	June vs August			3.888	**0.012**	10
		June vs October			0.495	0.617	10
		August vs October			5.546	**0.005**	10
	Control 1	June vs August			1.054	0.376	10
		June vs October			0.778	0.499	10
		August vs October			0.710	0.542	10
	Control 2	June vs August			1.114	0.309	10
		June vs October			2.081	0.096	10
		August vs October			0.203	0.855	10
c) Pair-wise tests		**Between Sites within Periods**			**t**	**P(MC)**	**Perms**
	June	Impact vs Control 1			6.101	**0.006**	10
		Impact vs Control 2			2.264	0.090	10
		Control 1 vs Control 2			3.317	**0.027**	7
	August	Impact vs Control 1			1.790	0.160	10
		Impact vs Control 2			0.353	0.733	10
		Control 1 vs Control 2			0.582	0.572	10
	October	Impact vs Control 1			17.753	**0.001**	10
		Impact vs Control 2			6.971	**0.003**	10
		Control 1 vs Control 2			1.370	0.248	10

Looking at the temporal and spatial patterns of variation of the δ^{15}N values in the tissues of the deployed macroalgae with respect to the baseline (*i.e.* the Δδ^{15}N values), the overall mean values ranged from -0.75±0.50‰ to +0.23±0.20‰, values well below those found in similar studies (e.g. in Orlandi et al., 2014, Δδ^{15}N of *Cystoseira amentacea* deployed along an higlhy anthropized coastal area varied from +1.0 to +1.6‰). Although *Cystoseira compressa* has a lower turnover and uptake rate than opportunistic macroalgae, this finding are most likely the result of a very limited influence of inputs of anthropic origin in the study sites. Indeed, looking at the data recorded in June and August, when Rhodes was overrun with tourists, a depletion of the isotopic signature compared with the baseline occurred, except for the macroalgae deployed at100 m from the coastline of the Impact site in June, and up to 300 m in August. This pattern indicates that touristic flows and local activities cause the presence of ^{15}N-slight enriched nutrients only in the shallow coastal seawater of the Impact site, while the absence of tourist infrastructures and leisure activities in Control sites, seems to contribute to the lack of significant isotopic variation of the macroalgae.
Despite the small variation in δ^{15}N during the experiment, results of the statistical analysis on Δδ^{15}N values highlighted a significant effect of the interaction of the factors Period x Site and Site x Distance (Table 9a). The extent of the variation Δδ^{15}N at the Impact site in August was significantly higher than in June and in October (Table 9b) and, in the same period, it was significantly higher at the Impact Site than at both Control sites (Table 9c). However, there was no overall difference between distances

within sites (the only exception was between 100 and 300 m at Control 1) (Table 9d), therefore no evident spread of anthropogenic nutrients up to offshore areas was supported by the statistics.

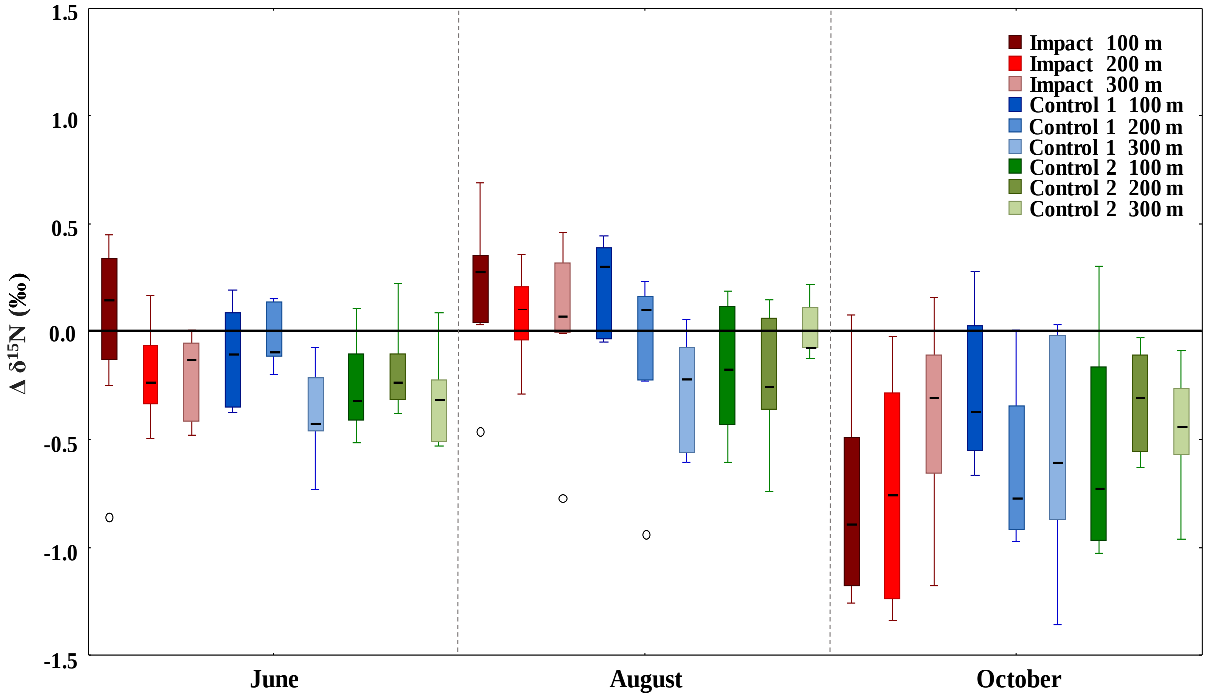

Fig. 16. Boxplot of $\Delta\delta^{15}N$ values (variation of $\delta^{15}N$ compared to the baseline) of the macroalga *C. compressa* at different distance from the coastline (100, 200, 300 m) in the study sites: Impact (Faliraki beach), Control 1 and 2 (Afandou beach) during the experimental periods: June (before the tourist peak), August (during the tourist peak) and October (after the tourist peak). Each box contains 50% of the data, the thick horizontal line indicates the median; lower and upper whiskers represent respectively the first and fourth quartiles of the total range and circles represent outliers of the distribution. Black horizontal line overlaying the graph indicates the baseline; data above and below the baseline indicate ^{15}N-enrichment and ^{15}N-depletion of macroalgae respectively.

Table 9. Results of PERMANOVA (main test and pairwise tests) testing the differences between the $\Delta\delta^{15}N$ values of the macroalgae *C. compressa* deployed at different distance from the coastline (100, 200, 300 m) in the three study sites (Impact, Control 1 and 2) across the periods (June, August and October).

a) Main test	Source of variation	df	MS	Pseudo-F	P(perm)	Perms
	Period	2	4.309	38.810	**0.001**	999
	Site	2	0.180	1.622	0.197	999
	Distance	2	0.148	1.333	0.279	998
	Period x Site	4	0.297	2.676	**0.027**	996
	Period x Distance	4	0.119	1.069	0.395	999
	Site x Distance	4	0.303	2.730	**0.033**	998
	Period x Site x Distance	8	0.156	1.403	0.205	997
	Residuals	142	0.111			
b) Pair-wise tests	**Between Periods within Sites**			**t**	**P(perm)**	**Perms**
Impact	June vs August			4.201	**0.001**	997
	June vs October			4.071	**0.001**	994
	August vs October			6.218	**0.001**	999
Control 1	June vs August			2.374	0.029	997
	June vs October			2.739	**0.010**	998
	August vs October			4.311	**0.002**	996
Control 2	June vs August			1.181	0.226	997
	June vs October			1.893	0.059	997
	August vs October			2.957	**0.008**	996
c) Pair-wise tests	**Between Sites within Periods**			**t**	**P(perm)**	**Perms**
June	Impact vs Control 1			1.146	0.231	995
	Impact vs Control 2			1.945	0.063	996
	Control 1 vs Control 2			0.820	0.410	995
August	Impact vs Control 1			2.498	**0.016**	998
	Impact vs Control 2			4.238	**0.001**	994
	Control 1 vs Control 2			1.697	0.096	999
October	Impact vs Control 1			0.739	0.463	996
	Impact vs Control 2			0.985	0.310	997
	Control 1 vs Control 2			0.263	0.797	998
d) Pair-wise tests	**Between Distances within Sites**			**t**	**P(perm)**	**Perms**
Impact	100 m vs 200 m			1.267	0.202	996
	100 m vs 300 m			0.168	0.876	996
	200 m vs 300 m			0.957	0.340	995
Control 1	100 m vs 200 m			1.548	0.126	998
	100 m vs 300 m			3.264	**0.002**	996
	200 m vs 300 m			1.960	0.056	996
Control 2	100 m vs 200 m			0.788	0.444	998
	100 m vs 300 m			0.739	0.492	996
	200 m vs 300 m			0.020	0.986	999
e) Pair-wise tests	**Between Sites within Distances**			**t**	**P(perm)**	**Perms**
100 m	Impact vs Control 1			0.725	0.473	997
	Impact vs Control 2			2.099	**0.044**	999
	Control 1 vs Control 2			2.717	**0.004**	997
200 m	Impact vs Control 1			0.537	0.597	997
	Impact vs Control 2			0.011	0.990	997
	Control 1 vs Control 2			0.683	0.489	997
300 m	Impact vs Control 1			2.247	**0.038**	999
	Impact vs Control 2			1.149	0.263	997
	Control 1 vs Control 2			1.394	0.171	996

Results of temporal and spatial trends of $\Delta\delta^{15}N$ are also reported in the georeferenced maps in Fig. 17. They clearly show that the yellow/orange contours, that indicate isotopic enrichment during the experiment, are darker only during the tourist peak of August at the Impact site, and somewhat spreading within 100 m off the Control 1 site (Fig. 17). During the other two periods investigated, only a low isotopic enrichment or even depletion of macroalgae occurred, as evident by the dominance of very light orange and green to blue contours in the map, that indicate small fluctuations of the nitrogen isotopic signature, consistent with the results of the particulate organic matter POM isotopic signature (Fig. 17).

Overall results obtained for the monitoring experiment carried out in Rhodes denounce a very limited influence of anthropogenic nutrients only in the shallower coastal area in the Impact site. Despite the presence of numerous resorts and horeca (hotels, restaurants, cafes) infrastructures along the coast, the sharp demographic increase and the many leisure activities including bathing, boating and water sports occurring during the tourist season, there was no an alarming anthropogenic influence in the coastal waters directly linked to the tourist flows.

Figure 17. Georeferenced maps of $\Delta\delta^{15}N$ values in June, August and October 2017 at the Impact site (Faliraki beach) and Control sites (Afandou beach) of Rhodes. Dashed lines (superimposed only to the first panel for the sake of simplicity) indicate the distance from the coastline (200, 250 and 300 m) where macroalgae were deployed.

4. Conclusions

Coastal tourism is one of the most fast growing sectors of the global economy, bringing important benefits to local economies and moving millions of people worldwide. However, together with tourism growth, increasing environmental impact affects coastal seawaters every year during summertime due to excess input of anthropogenic nutrients and organic matter.

Short-term macroalgae deployments worked as an effective system to detect the presence of ^{15}N-enriched nutrients as a proxy for anthropogenic nitrogen into coastal waters. In Cyprus, Sicily and Rhodes, the highly tourist sites investigated (respectively Protaras, Giardini Naxos and Faliraki) showed temporal trends of enriched nitrogen, with an increase in the high tourist season (i.e. August). A clear spatial gradient was also common to Cyprus and Sicily, which is clearly attributable to the release of anthropogenic nutrient from the coast.

In particular, the experiments carried out in Cyprus revealed the presence of ^{15}N-enriched nutrients in the tourist beach, although of not great concern, since the beginning of the tourist season (i.e. June) and then persisted in August. After the tourist peak, in October, the coastal system seems to return to the natural condition, facilitated by seawater mixing caused by autumn storms. ^{15}N-enriched nutrients, although of not great concern, were detected also in tourist beach of Sicily in August and October, especially in the shallower part, closer to the coast. In October, this enrichment was limited to the southern area as an effect of the touristic port and the scarce water renewal due to breakwaters, coupled with the tourist facilities along the beach. In Rhodes, a very low ^{15}N-enrichment was detected at the tourist beach, compared to the other two islands investigated. ^{15}N-enriched nutrients were detected in June and were more evident during the tourist peak (i.e. August). Similarly, to Cyprus, in October, at the end of the tourist season, an overall impoverishment in nutrients was evident across all sites, clearly indicating a substantial decrease in their load.

In conclusion, despite the spatial and temporal patterns highlighted and the low turnover and growth rates of the investigated macroalgae species, it must be pointed out that the variations in $\Delta\delta^{15}$N found among sites and periods were overall of low extent in all the islands (from -1.4 to +1.4‰), and especially in Rhodes Island. This suggests a limited influence of anthropogenic activities on nutrient input in coastal seawater. Although no dramatic extent of anthropogenic nutrient input was detected in the investigated sites (hence suggesting that management of wastewater seems to be efficient also during the tourist peak), specific strategies can be proposed and adopted in order to further limit input of anthropogenic nutrients in the marine coastal areas.

Increasing the awareness of island tourism stakeholders (from bathers and boaters to HORECA managers and policy-makers) about the importance of coastal systems, as well as the strong connection between the human behaviour and the system functioning, represents a fundamental challenge and a good starting point to promote sustainable practices advocated for the near future.

Finally, the approach adopted in this study can be used as an effective technique for the detection of anthropogenic nutrients in marine coastal areas, representing a smart early-warning system. Such system, and especially the final output consisting in easily readable georeferenced maps, could produce important information also within wider region's water quality monitoring programs, helping the decisional process of competent authorities in the eventual need to improve standards to face deterioration of water quality due to tourism impact.

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