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Nitrogen – 15 signals of anthropogenic nutrient loading in *Anemonia sulcata* as a possible indicator of human sewage impacts on marine coastal ecosystems: a case study of Pirovac Bay and the Murter Sea (Central Adriatic)

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The results of the present research study indicate that the isotopic composition of nitrogen in the sea anemone *Anemonia sulcata* may be an excellent parameter for detecting and monitoring human sewage input into the marine coastal ecosystems of the Adriatic Sea if caution is used in selecting unpolluted reference sites. The ^{15}N signal of *Anemonia sulcata* tissue was significantly higher at more or less polluted sites of the semi-enclosed Pirovac Bay and in the coastal part of the Murter Sea (Central Adriatic) than in the reference location. ^{15}N enrichment was as high as 7.7 ‰ and is larger that would be expected from natural variation alone. Furthermore, the results also enabled us to create a map of $\delta\ ^{15}N$ values, which could be useful in monitoring the influence of human sewage impacts in marine coastal ecosystems.

INTRODUCTION

Stable isotopes have been used effectively to trace wastewater nutrients derived from animal wastes, septic systems and sewage treatment plants as they physically and biologically move through ecosystems.[1,2] Benthic and benthic feeding animals, as well as other organisms from sewage-impacted areas have shown $\delta\ ^{15}N$ values distinct from those collected at reference sites or from areas unaffected by human sewage.[3–8] The stable nitrogen isotopes can thus be used to distinguish between natural and anthropogenic sources of nitrate. Furthermore, they could also be an organic matter source indicator.[7,9,10] As the various sources of nitrogen pollution to coastal ecosystems often have a distinguishable nitrogen isotopic composition, the $\delta\ ^{15}N$ signal could thereby be used to identify the source of pollution.[11] For example, $\delta\ ^{15}N$ values of nitrate of commercial fertilizer typically range from –2.5 to +2.0 ‰, organic soil nitrate ranges from –2 to +9 ‰, and human and animal wastes range from +10 to +22 ‰.[11–14] Generally, $\delta\ ^{15}N$ values of NO_3 > +10 ‰ are regarded as being indicative that at least some of the nitrogen present in the sample is of faecal origin.[14] Treated sewage is also enriched in ^{15}N and shows $\delta\ ^{15}N$ values around +10 ‰.[11] On the contrary, sewage particulate organic matter and sewage sludge are depleted in ^{15}N relative to background organic matter

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and exhibit δ ^{15}N values mostly in the range between –1.1 and +7.2 ‰.[3] Similar low δ ^{15}N values for sewage, which fell within the same range as the sewage particulate organic matter and sewage sludge δ ^{15}N values measured by Van Dover *et al.,*[3] were also reported by Sweeney *et al.,*[15], Sweeney and Kaplan[16] and Spies *et al.*[4] Due to their low δ ^{15}N signal, sewage particulate organic matter (POM) and sewage sludge can also be recognized as a part of the diet of various organisms.[7,15–17]

Stable nitrogen isotopes are also useful in tracing organic matter through food webs. Animals raised on diets with a known nitrogen composition were found to prefer ^{15}N to ^{14}N, producing protein that is enriched in ^{15}N relative to the food source.[18,19] In general δ ^{15}N values in consumers thus reflect those of their food, after allowing for 1–4 ‰ enrichment due to trophic fractionation.[20] A large number of studies have shown that δ ^{15}N values of animal tissues are generally 3 ‰ more positive than those of their dietary source N, irrespective of habitat, growth rate or form of nitrogen excretion.[18,19,21–24]

This study was designed to: 1) test the hypothesis that the ^{15}N content of *Anemonia sulcata* tissue collected near-shore and offshore along the coastal part of the semi-enclosed Pirovac Bay and Murter Sea (Central Adriatic) is a reliable indicator of anthropogenic nitrogen impact arising from mostly untreated domestic and industrial wastes discharged into the coastal marine ecosystems of this part of the Central Adriatic, and 2) to create a map of δ ^{15}N values, which would enable us to determine the geographical extent of such anthropogenic impact on the adjacent area of the Murter Sea arising from transport by currents.

EXPERIMENTAL

The individuals of *Anemonia sulcata* analysed in this study were collected by scuba diving from the sea at depths of approximately 2–5 m in the semi-enclosed Pirovac Bay and Murter Sea (Figure 1). This species, one of the most abundant sea anemones in the Adriatic Sea, was collected as a part of a larger study in the framework of a Slovenian-Croatian Bilateral Project on the use of different organisms as bioindicators of marine pollution. All *Anemonia sulcata* individuals were collected from the same depth range (2–5 metres) along the coastal part of Pirovac Bay, as well as of various inshore and offshore islands of the Murter Sea, and on shallow reef flats to check for possible variations in nitrogen isotopic composition with depth. Additionally, all *Anemonia sulcata* individuals were size-matched (basal diameter 3–4 cm; tentacles extending 10–15 cm) and weighed (fresh weight: 40–50 g; dry weight after lyophilisation: 8–10 g) to avoid possible isotope effects caused by ontogenetic dietary shifts[19,25] or differences in age, which could also affect the nitrogen isotopic composition.[26] For this study only pale green coloured individuals with purple tips at the end of their tentacles were chosen. Pale green coloured anemones can often be found in coastal

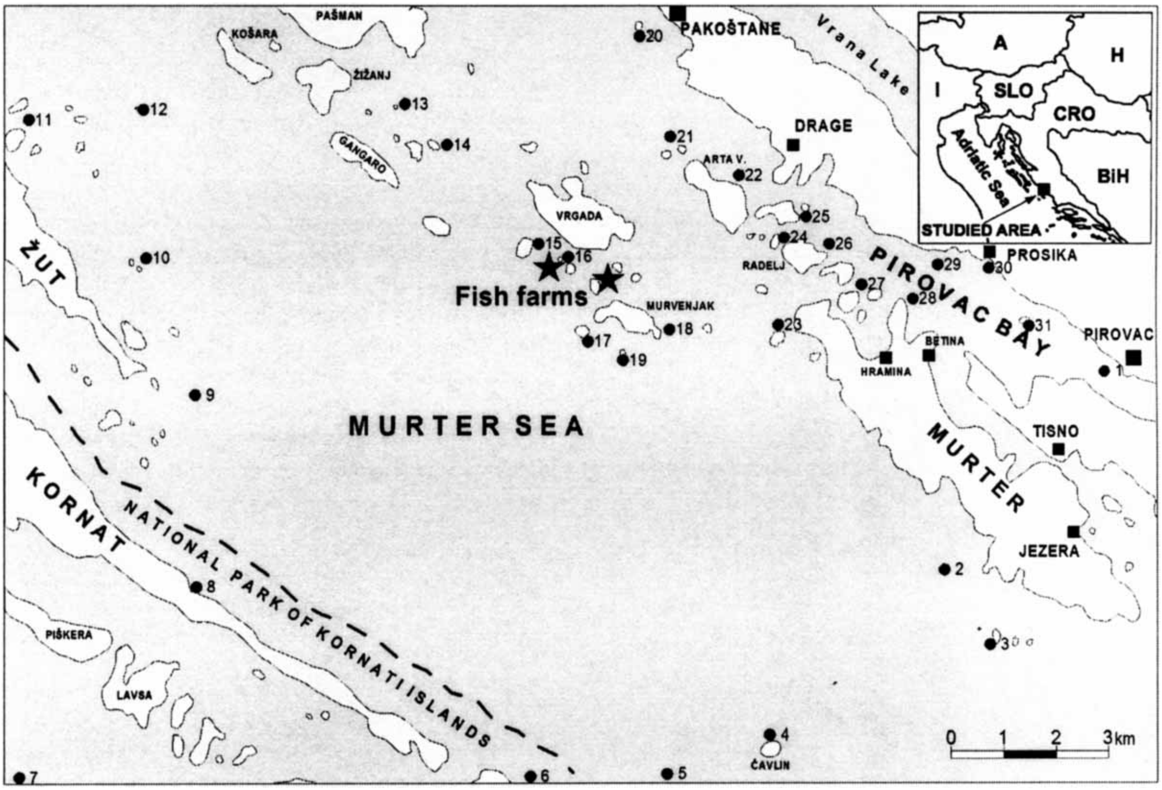

Fig. 1. Map of the study area in the Murter Sea and Pirovac Bay (Central Adriatic) showing sites of *Anemonia sulcata* sampling.

areas, as well as on the reef flats in the open sea. In contrast, dull gray or brown coloured individuals mostly occur in the coastal area, while on the reef flats in the open sea they are very scarce or even absent. The green colour is given by chlorophyll, produced by zooxanthellae endosymbiotic algae. When the numbers of algae diminish the anemone may appear dull gray or brown in colour.

To avoid the small seasonal differences in δ ^{15}N values of anemone tissue observed during a preliminary study,[27] as well as to get the strongest possible δ ^{15}N signal, we limited our analyses in this study to *Anemonia sulcata* individuals collected during the summer period at the peak of the tourist season (the last two weeks in August 2002) when the primary production is high because nutrients are plentiful and the light intensity is high and the input of untreated human sewage is also maximal. Fresh *Anemonia sulcata* samples were placed in plastic bags and stored at –20 ºC until ready for use in the laboratory. Each sample was weighed prior to use in subsequent experiments. In the laboratory samples were freeze-dried with a minimum duration of 72 h. Then they were crushed and homogenized by grinding in an agate mortar to avoid heavy metal contamination. Lyophilized samples were preserved in desiccators at room temperature until the analyses were carried out.

Samples analyzed for nitrogen isotopic composition were untreated. They represent the combined host tissue and zooxanthellae of the whole single animal. However, to obtain further insight into the nitrogen isotopic variability inside a single individual, selected parts of animals such as tips of tentacles, tentacles and colons were also analyzed. Nitrogen isotopic composition was measured using a Europa 20–20 mass spectrometer with an ANCA SL preparation module (PDZ Europa Ltd., U.K.). The results were expressed in the standard δ ^{15}N notation expressed in per mille (‰) relative to atmospheric (AIR) nitrogen (δ ^{15}N = 0 ‰). The analytical precision (1 standard deviation) of triplicate analyses of IAEA N-1 and N-2 standards was better than ±0.16 ‰. Precision (1 standard deviation) of duplicate isotopic analyses of samples was within ±0.2 ‰.

From δ ^{15}N values obtained at each sampling point (as δ ^{15}N values of a homogenized powder of a whole single animal), a contour map was obtained by kriging[28,29] using the SURFER 8 computer package. This technique has already been successfully used to obtain shoot density maps of *Posidonia oceanica,* as well as for detecting and mapping sewage impacts (to create maps of δ ^{15}N values) in plant tissue.[8,30]

RESULTS

The results of δ^{15}N determinations in *Amonia sulcata* are listed in Table I, while the regional distribution pattern of their δ ^{15}N values is shown in Figure 2. Data presented in Table I represent the average nitrogen isotopic composition of a whole single animal, while those marked with ([(a)]) refer to selected parts of an animal. δ ^{15}N of *Anemonia sulcata* tissue ranged from +4.2 to +11.9 ‰ (Table I). From Table I it is clearly evident that the δ ^{15}N values of various animal tissues do not dif-

Table I. δ ^{15}N values of *Anemonia sulcata* individuals collected in the Murter Sea and Pirovac Bay – Central Adriatic

Sample No.	Sample type	Sampling site	δ ^{15}N / ‰
1	*A. sulcata* (whole single animal)	Pirovac (coast)	11.9
2	*A. sulcata* (whole single animal)	Murter Island SE	6.4
3	*A. sulcata* (whole single animal)	Kukuljari Islands	6.1
4	*A. sulcata* (whole single animal)	Reef flat Čavlin	5.8
5	*A. sulcata* (whole single animal)	Nozdra Island	5.0
6	*A. sulcata* (whole single animal)	Reef flat Puh	4.7
7	*A. sulcata* (whole single animal)	Reef flat Lumbarda[(b)]	4.2
8	*A. sulcata* (whole single animal)	Bikarijca (coast)	5.1
9	*A. sulcata* (whole single animal)	Reef flat Kamenjar	5.4
10	*A. sulcata* (whole single animal)	Dinarići Islands	5.6
11	*A. sulcata* (whole single animal)	Gustac Island	5.1
12	*A. sulcata* (whole single animal)	Reef flat Galijolica	5.8
13	*A. sulcata* (whole single animal)	Ošljak Island	5.2
14	*A. sulcata* (whole single animal)	R. Kotula Island	5.3
15	*A. sulcata* (whole single animal)	Špinata Island	6.7
16	*A. sulcata* (whole single animal)	Rakita Island	6.3
17	*A. sulcata* (whole single animal)	Gira Island	6.1
18	*A. sulcata* (whole single animal)	Murvenjak Island	5.8
19	*A. sulcata* (whole single animal)	Vrtlić Island	5.4
20	*A. sulcata* (whole single animal)	Žavinac Island	7.3

Sample No.	Sample type	Sampling site	δ ^{15}N / ‰
21	*A. sulcata* (whole single animal)	Sestrice Islands	7.1
22	*A. sulcata* (whole single animal)	Arta V. Island	7.1
23	*A. sulcata* (whole single animal)	Prišnjak V. Island	6.3
24	*A. sulcata* (whole single animal)	Prišnjak M. Island	7.8
25	*A. sulcata* (whole single animal)	Arta M. Island	7.6
26	*A. sulcata* (whole single animal)	Radelj Island	7.3
27	*A. sulcata* (whole single animal)	Vinik Island	9.8
28	*A. sulcata* (whole single animal)	Gradina (coast)	9.5
29	*A. sulcata* (whole single animal)	Spličak (coast)	10.1
30	*A. sulcata* (whole single animal)	Prosika (coast)	10.8
31	*A. sulcata* (whole single animal)	Sustipanac Island	11.8
5/2a	*A. sulcata* (green tentacle)[a]	Nozdra Island	4.8
5/2b	*A. sulcata* (purple tip)[a]	Nozdra Island	4.9
5/2c	*A. sulcata* (colon)[a]	Nozdra Island	5.2
12/2a	*A. sulcata* (green tentacle)[a]	Reef flat Galijolica	6.1
12/2b	*A. sulcata* (purple tip)[a]	Reef flat Galijolica	6.0
12/2c	*A. sulcata* (colon)[a]	Reef flat Galijolica	5.7
15/2a	*A. sulcata* (green tentacle)[a]	Špinata Island	6.4
15/2b	*A. sulcata* (purple tip)[a]	Špinata Island	6.6
15/2c	*A. sulcata* (colon)[a]	Špinata Island	6.8

[a] selected tissue
[b] reference site

fer appreciably. The variation in δ ^{15}N values within the same individual is less than 0.5 ‰.

The results indicate that δ ^{15}N of *Anemonia sulcata* tissue was significantly higher at the anthropogenically more or less affected sampling sites along the coast of the semi-enclosed Pirovac Bay and the rocky shores of various islands close to Murter Island compared with *Anemonia sulcata* tissue from the selected unaffected reference site on the shallow approximately 3 m deep reef flat of Lumbarda in the open sea, and that from small uninhabited offshore islands. Individuals living on the offshore reef flats or on rocky shores of small isolated islands of the Murter Sea, as well as along the coastal part of the Kornati Islands and small, uninhabited islands around the Island of Žut (sample points 4 to 12) had consistently lower animal tissue values (average: +5.2 ‰; range: +4.2 to +5.8 ‰) than individuals living on rocky surfaces of the coastal parts of the islands closer to the coast (sample points 13 to 19). Their δ ^{15}N values had an average of +5.8 ‰ and a range from +5.2 to +6.7 ‰. Slightly enriched δ ^{15}N values (up to 2.5 ‰ relative to the reference site) in this region were found in anemones from the small islands around fish farms (sample points 15, 16 and 17). Considerably higher δ ^{15}N values (average +6.9 ‰; range +6.1 to +7.8 ‰) were measured along the islands which separate Pirovac Bay from the Murter Sea (sample points 21, 22, 24, and 25), as well as along the coastal part of Murter Island (sample points 2 and 23) and the Islands of Kukuljari (sample point 3). However, the highest δ ^{15}N values (average +10.2 ‰; range +7.3 to +11.9 ‰) were found in *Anemonia sulcata* tissues from the inner part of Pirovac Bay (sample points 1 and from 26 to 31). The ^{15}N values plotted in Figure 2 clearly show that the ^{15}N enrichment signal decreases with distance from the coast to the open sea.

DISCUSSION

δ ^{15}N values for marine organisms generally range from about –3 to about +20 ‰.[26] However, some organisms from hydrothermal vents or hydrocarbon seeps, as well as those possessing endosymbiotic bacteria, exhibit values as low as –12 ‰.[17] Even lower δ ^{15}N values of about –16 ‰ were measured in mussels at the vent fields of Menez Gwen and Lucky Strike at the Mid-Atlantic Ridge.[31] Animals such as anemones, scallops, mussels, brittle stars, benthic amphipods, small polychaetes and copepods, which represent the TL-2 trophic level had δ ^{15}N values mostly in the range between +5 and +7 ‰.[32] The lowest trophic level TL-1 consists of phytoplankton with δ ^{15}N values of about +5 ‰.[32] δ ^{15}N values of *Anemonia sulcata* analyzed during this study ranged from +4.2 ‰ at the reference site (the relatively »clean« reef

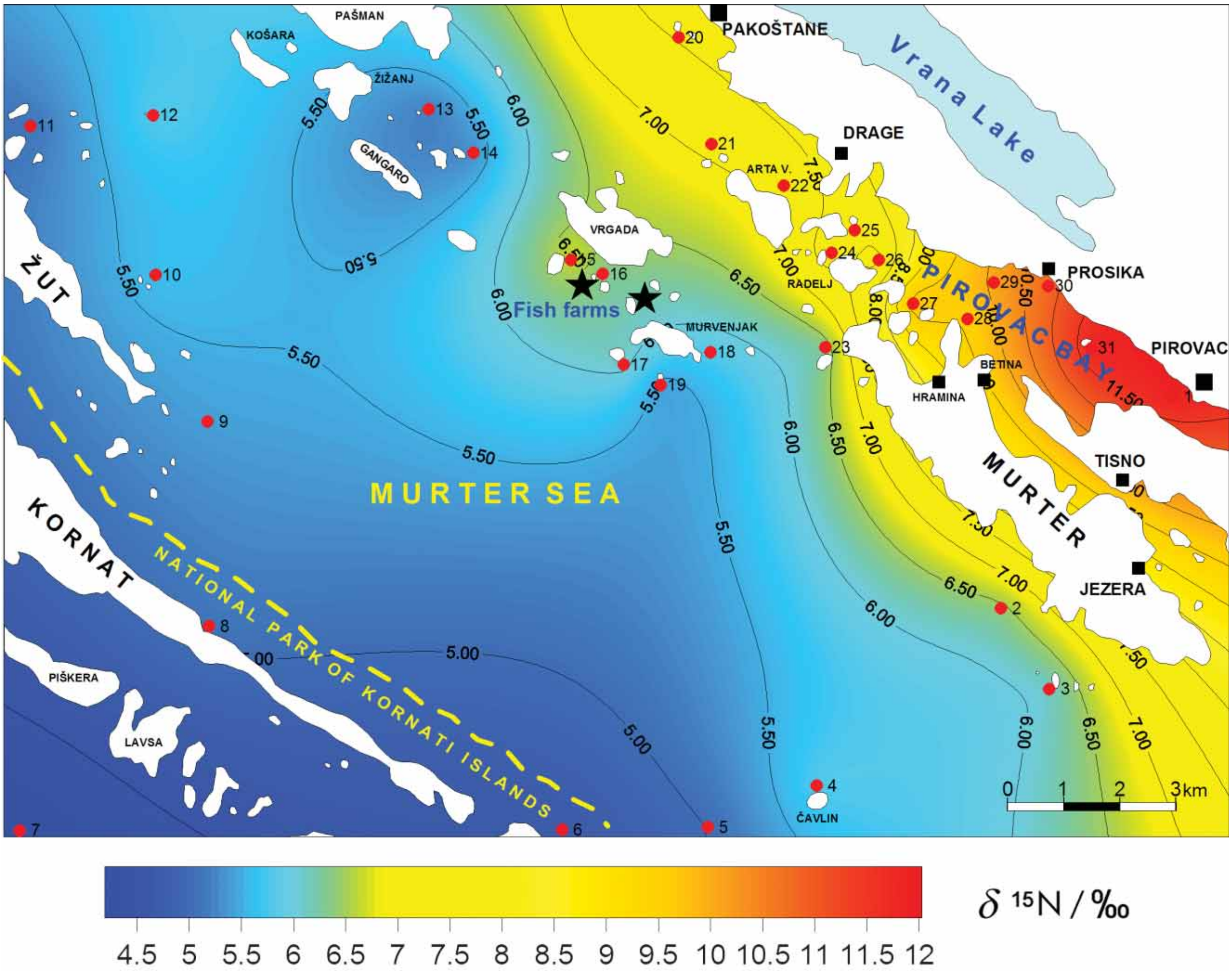

Fig 2. Spatial distribution pattern of δ ^{15}N values of *Anemonia sulcata* throughout Pirovac Bay and the Murter Sea (Central Adriatic). Contoured geochemical map for the isotopic composition of nitrogen was produced by kriging using the Surfer 8 software package.

flat of Lumbarda) to +11.8 ‰ in the human sewage-impacted semi-enclosed Pirovac Bay. δ ^{15}N enrichment was as high as 7.5 ‰ and is larger that would be expected from natural variation for a single species of invertebrate.[1] Variations in δ ^{15}N values in individuals of the same species could be related to size and age effects caused either by ontogenetic dietary shifts,[18,24] a depth effect,[25,33] seasonal effects,[8,9] a light availability/photosynthesis effect in the case of reef corals[25,34,35] and differences due to varying levels of sewage input.[8,17] The isotopic composition of organisms feeding in either clean or polluted environments should also depend upon their position in the food chain. This follows from suggestions that there is a trophic level effect on the δ ^{15}N values of animals.[22] Furthermore, ecosystems with different levels of sewage input should exhibit differences in the δ ^{15}N signal at each trophic level. Identical trophic structures, in our case *Anemonia sucata* individuals, utilizing nitrogen sources with different nitrogen isotopic composition should, according to Wada *et al.,*[24] (1991), clearly reflect these differences.

Regarding the dietary habits of *Anemonia sulcata*, Taylor[36] suggests that the excreted photosynthates of simbionts are of value only as a supplement to the host's metabolism, and that the primary source of nourishment comes from an exogenous food supply. Anemones generally ingest larger food items, both dead and alive, *e.g.* small fishes, just moulted palaemonid prawns, as well as gastropods and all sorts of crustaceans, which form the bulk of the diet in limited studies. According to trophic fractionation, δ ^{15}N values of *Anemonia sulcata* tissue should become on average 3 ‰ more positive than their food. As size, depth, season and colour of the anemones were taken into account during the sampling procedure, the δ ^{15}N variability of the individuals analyzed appears to indicate variations of the δ ^{15}N sewage enrichment signal of their diet. Although we have no data on the δ ^{15}N variability of the food sources of *Anemonia sulcata*, it is reasonable to assume that in ecosystems impacted by inputs of human faecal matter the sewage-induced δ ^{15}N enrichment signal should be also incorporated into the whole food web structure. For the present discussion it is also important to note that the natural feeding habit, as well as the trophic level of *Anemonia sulcata* does not vary within their sampling sites. This is confirmed with the stable nitrogen isotope signature of other marine biota (mussels, sponges, tunicates, seagrass) from the same localities, which exhibit a similar enrichment in ^{15}N like anemones (Dolenec *et al.*, in preparation). However, interpretation of the absolute values of δ ^{15}N for anemones must await data on the δ ^{15}N values of the source food, which at present are unknown.

The ^{15}N enrichment signal in anemones from the semi-enclosed Pirovac Bay and coastal parts of Murter Island, as well as from the inshore islands that separate Pirovac Bay from the Murter Sea, indicates that their primary food source is more or less affected by heavy nitrogen, most probably due to local inputs of untreated human sewage effluents (human faecal matter), with corresponding by high δ ^{15}N values, into the coastal marine environment. Since sewage-derived wastewater DIN (dissolved inorganic nitrogen) is typically enriched in ^{15}N and exhibits δ ^{15}N values mostly in the range between +10 and +22 ‰,[11] this source term may also be responsible for the heavy nitrogen enrichment in our samples. Such enrichment in ^{15}N due to ground water DIN was found in both primary producers and consumers in estuarine settings of Cape Cod, Massachusetts.[37,38] ^{15}N enrichment has also been found in reef molluscs, stomatopods, fishes and corals, in settings exposed to anthropogenic nutrient pollution.[1,2,38–41] Zooplankton and reef particulate organic matter may have higher δ ^{15}N values at sewage-polluted sites if ^{15}N-enriched wastewater is utilized by phytoplankton at the base of the food chain.[6] Elevated δ ^{15}N values have also been measured in marine plants exposed to septic system contaminated ground water[35] and sewage effluents.[8,42,43] However, low δ ^{15}N values, due to particulate sewage inputs, have also been observed in marine ecosystems.[3]

Organisms from ecosystems further from cities, harbours and tourist centres with no appreciable sewage impact should show lower δ ^{15}N tissue values, while the lowest δ ^{15}N values are characteristic of the highly oligotrophic conditions of offshore reef flat ecosystems with minimal anthropogenic impact, where algal fixation of atmospheric nitrogen is the major source of nitrogen.[18] As a result, the δ ^{15}N signal at the base of the food webs in such oligotrophic ecosystems, as well as the ^{15}N enrichment of various organisms, is relatively low. This is also the case with anemones offshore in the Murter Sea which exibit the lowest δ ^{15}N values. The slightly higher δ ^{15}N values measured in *Anemonia sulcata* tissues from the coastal ecosystems of the small inhabited islands south of Vrgada Island in the Murter Sea could be attributed to impacts from the surrounding fish farms in this area. It is possible that faecal matter derived from these fish farms may have slightly enriched the seawater DIN. For example, δ ^{15}N values of fish food and settled faecal matter from two fish farms in the Huon estuary, Tasmania, ranged mostly from +9.6 to +13.2 ‰.[44] Fish farms enrich the surrounding waters and the underlying sediments with nutrients and organic matter, and these loadings can modify the sediment characteristics and also induce a substantial impact on the benthic communities directly under and adjacent to fish cages.[44]

From Figure 2 it is evidently clear that the enrichment effect decreases with distance from the coast toward the open sea ecosystems. Such onshore to offshore δ ^{15}N variations most probably indicate that the sewage-induced ^{15}N enrichment signal is rapidly attenuated with distance from the sewage sources (within some kilometres for sewage from the inhabitated areas of Pirovac Bay and Murter Island). Similar inshore-offshore δ ^{15}N variations have also been observed in stomatopods from southwest Sulawesi[1] and corals from Indonesia, Zanzibar and the Maldives.[1,2]

As the most important characteristic of the Adriatic Sea is the general anti-clockwise circulation of seawater, the regional distribution pattern of sea anemone δ ^{15}N values also reflects this anti-clockwise current pollutant transport (Figure 2). The strongest δ ^{15}N signal is characteristic of more or less polluted coastal ecosystems, especially those of the semi-enclosed Pirovac Bay. Here ^{15}N enrichment undoubtedly resulted from wastewater nutrients derived mostly from septic systems in the surrounding villages and tourist centres of Pirovac Bay (Murter, Betina, Tisno, Jezera and Pirovac), and to a lesser extent from sewage impacted sea currents. The ^{15}N sewage signal of Pirovac Bay was evident up to nearly 8 km NW of the Bay, though it decreases with distance from the shore. It is supposed that mixing of sewage affected seawater from Pirovac Bay with less polluted southeast to northwest sea currents may have diluted the signal from the Bay.

Furthermore, the elevated δ ^{15}N signal in the coastal part of Murter Island also indicates that pollutants reach the coastal ecosystems of the Murter Sea by a prevalently current-derived mass transport from the southeast where such pollution sources are located (for example: Šibenik – a town and a shipping port, Vodice and Tribunj, – tourist centres and marinas).

CONCLUSIONS

Stable nitrogen isotopes enabled us to identify a sewage signal in *Anemonia sulcata* from the investigated part of the Adriatic Sea and suggested a higher contribution of sewage-derived material to the food web in coastal ecosystems. The results of this study, performed in the peak of the tourist season, further indicate that *Anemonia sulcata* from the most polluted sites are most probably consuming food with a significant sewage component. As size, depth and season of collection were allowed for in the sampling design, the observed variations in *Anemonia sulcata* δ ^{15}N values appear to be primarily explained by variation in the extent of domestic and industrial wastes which have been discharged into the coastal ecosystems of this part of the Central Adriatic. Enrichment in ^{15}N in *Anemonia sulcata* tissue thus appears to be a promising signal of sewage pollution in the Adriatic Sea. By using δ ^{15}N values of *Anemonia sulcata* the anthropogenic nitrogen inputs in many other marine coastal ecosystems could also be easily detected and mapped.

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SAŽETAK

Dušik-15 signali antropogenoga opterećenja hranjivim tvarima u *Anemonia sulcata* kao mogući indikator utjecaja komunalnoga otpada na morske obalne ekosustave: ispitivanje u Pirovačkome zaljevu i Murterskome moru (Središnji Jadran)

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Rezultati ove studije pokazuju da izotopni sastav dušika u morskoj anemoni *Anemonia sulcata* može biti izvrstan parametar za detekciju i praćenje unosa komunalnoga otpada u morske obalne ekosustave Jadranskoga mora, ako se pažljivo odaberu referentna nezagađena mjesta. Signal ^{15}N u tkivu *Anemonia sulcata* bio je značajno viši kod više ili manje zagađenih mjesta u poluzatvorenome Pirovačkome zaljevu i obalnome dijelu Murterskoga mora (središnji Jadran) nego na referentnim lokacijama. ^{15}N obogaćenje bilo je i do 7,7 ‰, što je veće nego očekivane prirodne varijacije. Rezultati su omogućili da se napravi mapa δ ^{15}N vrijednosti, koja bi se mogla rabiti za praćenje utjecaja komunalnoga otpada na morske obalne ekosustave.