Use of stable nitrogen ($\delta^{15}N$) isotopes in food web of the Adriatic Sea, Croatia

Uporaba stabilnega dušikovega izotopa ($\delta^{15}N$) v prehranjevalni verigi Jadranskega morja, Hrvaška

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Abstract: This study presents the first attempt to classify organisms in the food web of the Adriatic Sea into trophic levels using a nitrogen ($\delta^{15}N$) isotope tracer. Three main trophic levels were identified with significantly different nitrogen values, ranging from primary producers to higher consumers. TL-1 represents plankton and POM samples, TL-2 mostly benthic organisms and TL-3 fishes. The results also indicate the influence of anthropogenic pollution, which significantly increases nitrogen values.

Izvleček: Raziskava je prvi poskus klasifikacije organizmov v prehranjevalni verigi Jadranskega morja v trofične nivoje na podlagi izotopske sestave dušika $\delta^{15}N$. Organizmi so bili generalno razvrščeni v tri trofične nivoje, ki vključujejo primerke od primarnih producentov do višjih konzumentov. TL-1 pomeni plankton in POM, TL-2 večinoma bentoške organizme in TL-3 ribe. Na izotopsko sestavo dušika pomembno vpliva antropogeno onesnaževanje, neobdelane komunalne in industrijske odplake, kot tudi marikulturne dejavnosti, ki občutno povišajo vrednosti dušikovega izotopa $\delta^{15}N$.

Original scientific paper

Key words: food web, trophic levels, nitrogen ($\delta^{15}N$) isotope composition, Adriatic Sea.
Ključne besede: prehranjevalna veriga, trofični nivoji, izotopska sestava dušika, Jadransko morje

Introduction

In ecological studies, stable isotopes, mostly carbon and nitrogen, are commonly used to trace food webs and distinguish between natural and anthropogenic sources. Using stable isotopes the sources and manner of feeding in marine ecosystems can be determined. The isotopic distribution in animals reflects the isotopic composition of their food and their position in the food web. The nitrogen ($\delta^{15}N$) values in animal bodies are usually more positive than those in their food (DeNiro & Epenstein, 1981; Minagawa & Wada, 1984; Fry, 1988). Heavy isotope enrichment for nitrogen is about 1.3–5.3 ‰ per trophic level and is more useful for studying the relations between trophic levels than that for carbon, for which enrichment is estimated to be about 1 ‰ per trophic level (Minagawa & Wada, 1984 Peterson & Fry, 1987; Fry, 1988). On the basis of stable isotope ($\delta^{15}N$, $\delta^{13}C$) composition, many researchers have tried to classify organisms into groups (trophic levels) in different aquatic and terrestrial ecosystems (Corbisier et al., 2006; Fry, 1988; Hobson et al., 2001; Iken et al., 2005; Wada et al., 1993).

The stable nitrogen isotopes also reflect unusual anthropogenic food sources that can increase or decrease nitrogen values (Fry, 1988). For this reason the interpretation should consider natural and also anthropogenic factors. Stable nitrogen isotopes can be used to track untreated communal and industrial sewage as well as animal excrement. Many papers have shown the negative influences of untreated municipal and industrial sewage (Constanzo et al., 2001; Dolenec & Vokal, 2002; Dolenec et al., 2005, 2006b, 2006c, 2007; Rogan et al., 2007) and fish farm activities on marine ecosystems (Dolenec et al., 2006a, 2007; La Rosa et al., 2001; Mirto et al., 2002; Pergent et al., 1999; Rogan et al., 2007; Ruiz et al., 2001; Sara et al., 2004). In addition, variations in stable nitrogen ($\delta^{15}N$) within the same species could be the result of seasonal effects (Constanzo et al., 2001), size and age effects (Minagawa & Wada, 1984; Wada et al., 1993), or depth effects (Saino & Hatory, 1980; Muscatine & Kaplan, 1994).

The aim of this paper is to present the relationships between stable nitrogen ($\delta^{15}N$) values in organisms in the

Adriatic Sea and their nutrition and position in the food web. For this purpose selected organisms, from primary consumers to higher consumers, were studied, and on the basis of nitrogen ($\delta^{15}N$) values were classified into different trophic levels.

Materials and methods

Study area

The study area included mostly coastal areas of the Northern and Central Adriatic Sea in Croatia. Samples were collected at several locations along the coast of Piran bay and Istra Peninsula (from Savudrija to Pula), and inshore and offshore areas of Murter Sea, the semi-enclosed Pirovac Bay, and sites around the Kornati Islands and Korčula Island (Figure 1). Some sampling locations were highly polluted by human sewage and industrial effluents, especially coastal areas in the vicinity of larger cities, marinas and ports, as well as by fish farming activities that produce N-rich waste of fish excrement and uneaten food. Sampling was also carried out at relatively unaffected sites to obtain information about the natural variability of stable nitrogen ($\delta^{15}N$) isotope composition in the Adriatic Sea.

Sample collection and analysis

Several marine organisms as well as particulate organic matter (POM) were sampled for isotopic analyses. Samples were collected from spring to autumn (May to September) from 2000 to 2008, but the seasonal and annual variations are not the topic of this study.

Water for particulate organic matter (POM) was collected at about 1 m depth at different sampling sites with different amounts of anthropogenic pollution. 5 L to 10 L of water was filtered through glass fibre microfilters (GF/C, Whatman). Filters were freeze-dried in liofilizator and prepared for isotopic analyses.

Plankton samples were collected with a plankton net at 50–0 m depth and other organisms (anemones, sponges, molluscs, sea urchins, crustaceans, fishes) by scuba diving from about 25 m depth. For this study average values of soft parts of tissues were presented. All samples were stored in plastic capsules or bags and refrigerated immediately after collection. In the laboratory, samples were rinsed with deionized water and freeze-dried for at least 72 h. Dried samples were homogenized and crushed to a fine powder by grinding in an agate mortar. Powdered samples were packed into tin capsules and were preserved in desiccators at room temperature until the analyses were carried out.

Nitrogen ($\delta^{15}N$) isotopic composition was measured using a Europa 20–20 mass spectrometer with an ANCA

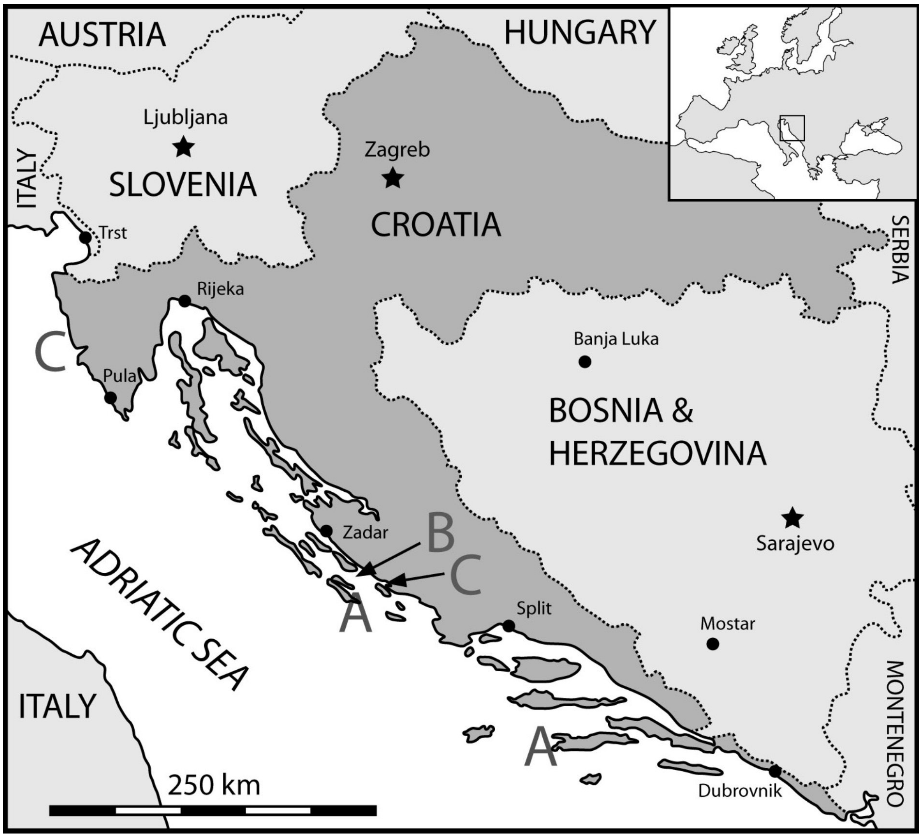

Figure 1. Map of the study area in the Adriatic Sea (Croatia) and locations of groups A, B and C with different amounts of anthropogenic pollution

SL preparation module (PDZ Europa Ltd., U. K.). The results were expressed in the standard $\delta^{15}N$ notation as parts per mille (‰) relative to the nitrogen standard (atmospheric nitrogen, $\delta^{15}N = 0$ ‰). The analytical precision (1 standard deviation) of triplicate analyses of IAEA N-1 and N-2 standards was better than ±0.16 ‰. The precision (1 standard deviation) of duplicate isotopic analyses of samples was within ±0.2 ‰.

Statistical analysis

Statistical analysis of presented data was performed by use of the statistical software program Statistica 6.0. The differences between the groups A, B, C of TL-1 and TL-2 and groups I, II, III of TL-3 were tested with t-test (Table 5).

Results and discussion

In order to understand the energy flow through the complex marine food web in the Adriatic Sea different organisms, from primary producers to upper trophic level consumers, were collected. Sampling was carried out in three areas with different amounts of anthropogenic pollution: (A) no or low anthropogenic impact around the Kornati Islands and Korčula Island, (B) inputs of fish faeces and uneaten food around fish farms near Vrgada Island and (C) municipal, industrial and agricultural inputs in coastal areas around Murter Island, the semi-enclosed Pirovac Bay and the Istra Peninsula (Figure 1).

The lowest nitrogen ($\delta^{15}N$) values were generally observed in plankton and POM samples. Plankton values in group A were between +2.5 ‰ and +4.4 ‰, and in the more polluted group C values increased up to +8.0 ‰. The mean $\delta^{15}N$ value of all plankton samples was 5.16 ‰ (Table 1, Figure 2). Similar values but with slightly higher variation were measured in POM, which generally contains a mixture of detritic material, phyto- and zooplankton, bacteria and particles from different sources. The nitrogen values varied between –1.27 ‰ and +13.79 ‰ with a mean value of 4.84 ‰ (Table 2, Figure 3). Extreme values were detected in areas that were highly influenced by human activity (group C) in the high tourist season, and low values were found in unaffected areas of group A. The relatively large variations in nitrogen ($\delta^{15}N$) values of POM and plankton samples could also be related to the rapid movement of floating material owing to the wind, current circulation and tides. Although POM and plankton samples showed notable variations in nitrogen values, they still exhibited the lowest values and were thus classified as trophic level 1 (TL-1).

The second trophic level (TL-2) contained mostly benthic organisms, including anemones (*Anemonia sulcata*), sponges (*Aplysina aerophoba*), barnacles (*Balanus perforatus*), molluscs (*Mytilus galloprovincialis, Arca neae, Patella sp., snails*), sea urchins (*Abracia lixula*) and crustaceans (*Squilla mantis, Nephrops norvegicus*). These organisms are sessile or have a very limited habitat and reflect local environmental conditions. The food for these organisms is generally from the lower trophic level TL-1 at the base of the food web. Nitrogen ($\delta^{15}N$) values varied between –1.30 ‰ up to +11.87 ‰ with a mean value of +4.74 ‰ (Table 3, Figure 4). Division into three groups regarding the amount of pollution was also evident in this group. Low values were observed in the non-affected areas (A), medium values around fish farms (B) and the highest values near the larger sources of pollution (C).

Table 1. Basic statistics for nitrogen ($\delta^{15}N$) isotopic composition of plankton in the Adriatic Sea

Group	N	Min	Max	Median	Means	SD
A	12	2.50	4.40	3.12	3.35	0.61
B	14	3.50	7.30	4.45	4.60	0.85
C	16	4.40	8.00	7.20	7.00	0.87
All groups	42	2.50	8.00	4.55	5.16	1.73

Table 2. Basic statistics for nitrogen ($\delta^{15}N$) isotopic composition of particulate organic mater (POM) in the Adriatic Sea

Group	N	Min	Max	Median	Means	SD
A	32	1.50	7.80	3.15	3.55	1.50
B	101	1.40	6.80	4.00	3.96	1.03
C	166	–1.27	13.79	5.70	5.61	1.91
All groups	299	–1.27	13.79	4.80	4.84	1.84

Table 3. Basic statistics for nitrogen ($\delta^{15}N$) isotopic composition of TL-2 in the Adriatic Sea

Group	N	Min	Max	Median	Means	SD
A	100	–1.30	10.60	2.40	2.60	2.13
B	146	1.10	8.80	4.60	4.63	1.43
C	115	0.50	11.87	6.70	6.73	2.39
All groups	361	–1.30	11.87	4.70	4.74	2.53

Table 4. Basic statistics for nitrogen ($\delta^{15}N$) isotopic composition of fishes in the Adriatic Sea

Groups	N	Min	Max	Median	Means	SD
I	8	6.50	13.45	8.12	9.16	2.48
II	14	6.90	12.70	9.67	9.93	1.79
III	31	7.80	14.70	11.20	11.22	1.90
All groups	53	6.50	14.70	10.60	10.57	2.09

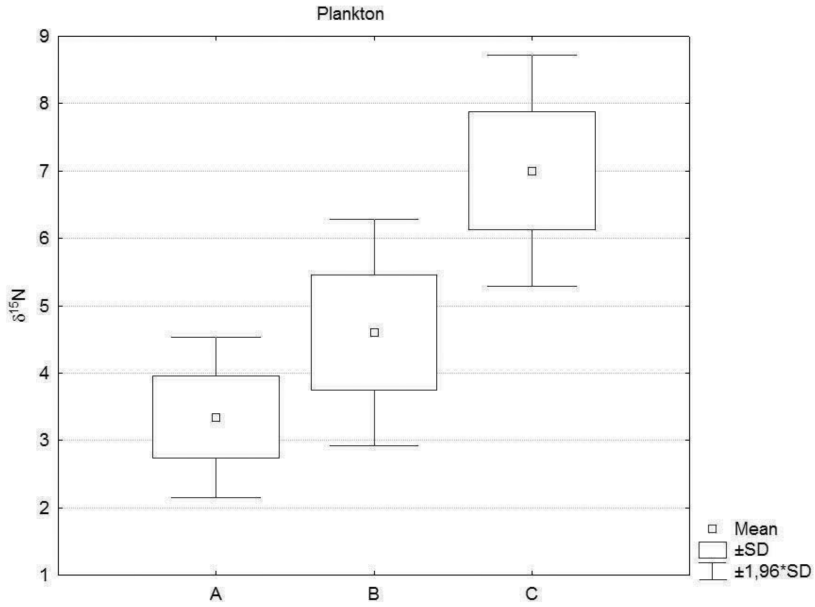

Figure 2. Whisker plots of nitrogen ($\delta^{15}N$) values of plankton in locations with different amounts of pollution

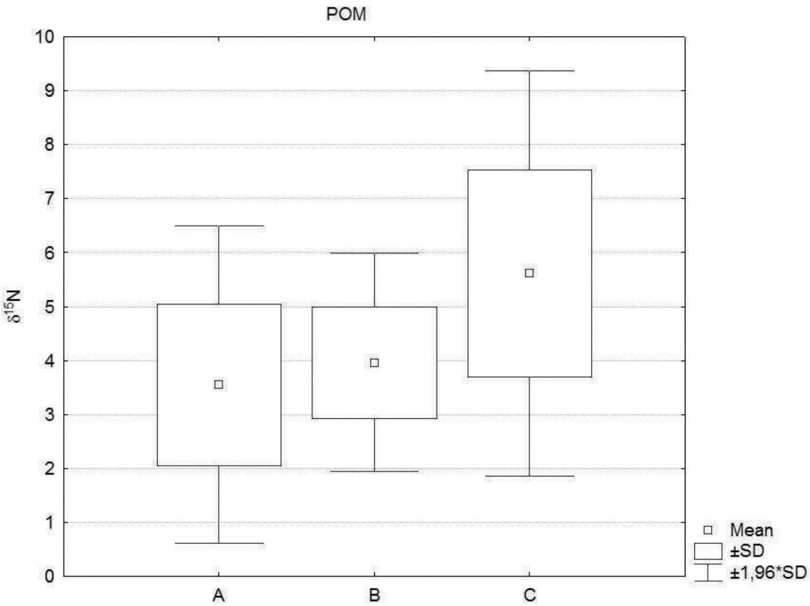

Figure 3. Whisker plots of nitrogen ($\delta^{15}N$) values of particulate organic matter (POM) in locations with different amounts of pollution

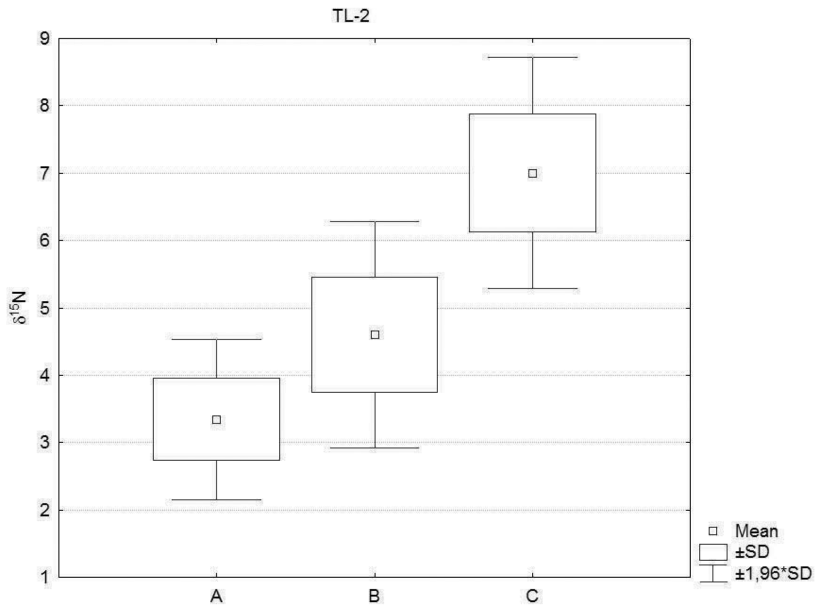

Figure 4. Whisker plots of nitrogen ($\delta^{15}N$) values of TL-2 in locations with different amounts of pollution

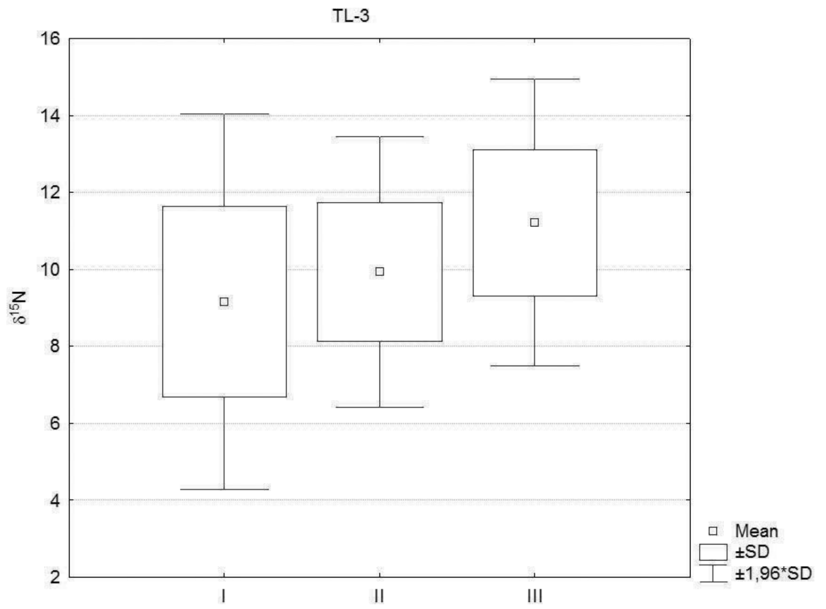

Figure 5. Whisker plots of nitrogen ($\delta^{15}N$) values of TL-3 fish tissues with different modes of nutrition

Table 5. Significance levels of the t-test to evaluate the differences between groups with different amounts of anthropogenic pollution A, B, C of TL-1 and TL-2 and groups I, II and III of TL-3 (ns = not significant, * = $p \leq 0.05$; ** = $p \leq 0.01$)

TL1	**A**	**B**	**C**
A	–	**	**
B	**	–	**
C	**	**	–
TL2	**A**	**B**	**C**
A	–	**	**
B	**	–	**
C	**	**	–
TL3	**I**	**II**	**III**
I	–	ns	*
II	ns	–	*
III	*	*	–

TL-2 is the most heterogeneous, including a large number of different species. It could probably be further divided into sublevels but the ′state of the art′ data did not allow this. Sampling was carried out in different seasons and the number of individual species varied notably (from 5 up to 150 samples). Surprisingly, some species of TL-2 showed significantly lower nitrogen values than those in TL-1, such as *Aplysina aerophoba*, which has previously been observed and studied in detail by Rogan et al. (2007). Depleted nitrogen values have been interpreted by fractionation of nitrogen isotopes during uptake and assimilation by symbiotic bacteria (Dolenec et al., 2007). Otherwise, the nitrogen ($\delta^{15}N$) values of TL-2 organisms were generally enriched compared with those of TL-1.

The highest nitrogen ($\delta^{15}N$) values were measured in fishes, which comprised the highest trophic level (TL-3) of the samples studied. Within TL-3, fish samples were divided into three sublevels according to their mode of nutrition; I. planktivores and detritivores (*Liza spp., Boops boops, etc.*), II. herbivores (*Spondyliosoma cantharus, Diplodus vulgaris,* etc.), and III. piscivores (*Merluccius merluccius, Citharus linguatula, Phycis, phycis, Muraena helena, Dicentrarchus labrax, Seriola dumerili, Conger conger,* etc.). The mean nitrogen value increased from group I to III (Figure 5); +9.16 ‰ for group I, +9.93 ‰ for group II and +11.22 ‰ for group III (Table 4, Figure 5). The isotopic composition of fish tissues depends on their ′squeamishness′ and varies within the same group. Nitrogen ($\delta^{15}N$) values are also the result of anthropogenic inputs, which lead to fish migration and/or adjustment to polluted water (*Liza* spp.).

The results of the t-test of groups within trophic levels are presented in Table 5. T-tests between groups with different amount of pollution A, B, C (Figure 1) of trophic levels TL-1 and TL-2 all showed significant differences at confidence level 99 % ($p < 0.01$). Slightly lower significant differences were observed between I and III as well as II and III group of TL-3 (confidence level 95 %; $p < 0.05$), while t-tests between I and II group showed no significant differences.

Conclusion

The nitrogen ($\delta^{15}N$) isotope distribution of organisms in the Adriatic Sea can be used to follow the energy flow through the food web of the ecosystem and to classify individual species within trophic levels. Enrichment of nitrogen ($\delta^{15}N$) within the whole food web was more than +10 ‰, which enabled the assessment and interpretation of feeding habits and relationships between particular organisms. Organisms in the Adriatic Sea were classified into three trophic levels: TL-1 (plankton and POM), TL-2 (mostly benthic organisms; anemones, sponges, barnacles, molluscs, sea urchins and crustaceans) and TL-3 (fishes).

Apart from demonstrating the trophic level of different organisms, this study also showed significant variation in nitrogen ($\delta^{15}N$) values within individual species. These fluctuations were related to pollution of the sampling areas. Compared to the natural background, nitrogen ($\delta^{15}N$) enrichment was observed around cities, ports and tourist facilities in the coastal regions of Murter Sea, Pirovac Bay and Istra Peninsula. These areas are exposed to untreated municipal, industrial and agricultural pollution that increases the amount of nitrogen ($\delta^{15}N$) input into the marine ecosystem. Furthermore, nitrogen ($\delta^{15}N$) enrichment could also reflect intensive aquaculture with increased input from fish faeces and uneaten food.

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