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Is the Kornati National Park Still an Acceptable Reference Area for Environmental Studies?

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Abstract: The Kornati National Park (Croatia) is considered an environment with minimal anthropogenic input. The purpose of this study was to determine the isotopic characteristics of the sediment and muscle tissues of the banded dye-murex *Hexaplex trunculus*. We selected locations in the park according to their estimated risk of anthropogenic pollution (large, lower, and minimal). Isotopic analyses of the sedimentary organic carbon ($\delta^{13}\text{C}_{\text{org}}$ values) showed that the sedimentary organic matter in locations with *P. oceanica* meadows (Piškera, Vrulje) was enriched in ^{13}C compared to that of locations with the influx of terrestrial organic matter. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the muscle tissues of *H. trunculus* were the highest in the two locations with the highest possible anthropogenic impact (-14.47‰ and -15.66‰ for $\delta^{13}\text{C}_{\text{org}}$, $+8.87\text{‰}$ and $+10.4\text{‰}$ for $\delta^{15}\text{N}$). The high δ values may indicate the presence of the pigment indirubin ($\text{C}_{16}\text{H}_{10}\text{O}_2\text{N}_2$) and other derivatives that cause the purple coloration but are also elevated because of the discharge of untreated sewage from a nearby marina and village.

Keywords: Kornati National Park; marine sediment; banded dye-murex *Hexaplex trunculus*; stable isotopes; carbon and nitrogen

1. Introduction

The Kornati National Park (KNP) is usually considered an unpolluted reference area in environmental and ecological studies, but with the growth of tourism and a higher exposure to anthropogenic inputs, some parts of it are deteriorating into increasingly hazardous environments. The factors that contribute the most to the environmental risks are the fishing industry, chemical pollution and eutrophication, physical changes of the ecosystem, invasions of exotic species, and global climate changes [1].

Stable isotopes of carbon (C) and nitrogen (N) are commonly used to decipher the environmental conditions and trophic relations in ecosystems [2,3]. Stable isotope compositions of organisms are used to identify the biochemical processes and sources of the pollution, as they are mainly influenced by the isotopic ratios of diet, metabolic pathways, and fractionating processes [4,5]. With the isotopic analysis, it is also possible to determine the source of sedimentary organic matter (marine, terrestrial) and thus to assess the anthropogenic impacts on the observed area [6].

The isotopic composition of carbon ($\delta^{13}\text{C}$) in organisms is typically influenced by food source, type of environment (marine, terrestrial), distance from the shore, vegetation type, and seasonal and environmental changes, while the isotopic composition of nitrogen ($\delta^{15}\text{N}$) reflects the position of the organisms in the food web [3,7]. The enrichment of the consumers regarding their food source is on average about 3.4‰ per trophic level for $\delta^{15}\text{N}$ and about 1‰ for $\delta^{13}\text{C}$ [3,5,7]. The areas with a minimal anthropogenic input usually show relatively low $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values, while ecosystems and organisms found within more anthropogenically impacted regions show increased $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values as a consequence of the utilization of ^{15}N - and ^{13}C -enriched N and C [5,8].

The isotopic measurements in this research were focused on the banded dye-murex *Hexaplex trunculus* (Linne, 1758), which is a 4 to 10 cm-long mollusc and one of the most common organisms in the Adriatic Sea [9]. The molluscs are simple to sample and are an excellent biomarker of seawater pollution, as they are present in polluted as well as in unpolluted areas [10]. They mainly occur in rocky and muddy parts of the sublittoral zone, at depths between 1 m and 100 m [9]. They are relatively immobile and, therefore, are representative for the area of study [11]. The banded dye-murex are predators and resistant to hypoxia and anoxia, as they close their shells under hypoxic conditions [12]. Today, they are mainly used because of fishing demands, whereas, in the past, they were mainly used because of their purple color [9]. The color is the consequence of the pigments indirubin ($\text{C}_{16}\text{H}_{10}\text{O}_2\text{N}_2$), indigotin, and other brominated derivatives, which are formed in the glands of *H. trunculus* [13]. Many previous studies were focused on the effect of TBT (tributyltin) and the development of imposex, whereas, unfortunately, only a few studies on isotope fingerprints of *H. trunculus* have been published to make a comparison.

The aim of this research was to compare individual sampling sites considering their different exposures to anthropogenic pollution and to assess if the Kornati National Park is an acceptable reference area. To determine this, the isotopic composition of organic carbon in the sediment as well as the isotopic composition of organic carbon and nitrogen in the muscle tissues of the banded dye murex were measured.

2. Materials and Methods

2.1. Environmental Setting

The Kornati National Park is a unit of several islands, cliffs, and sea reefs situated in the eastern part of the Adriatic Sea, Croatia (Figure 1). KNP ranges between the Zadar and Šibenik archipelagos, in the direction of the Dinaric Mountains [14,15]. The Kornati islands are composed of carbonate rocks, mostly Cretaceous limestones and dolomites [16,17]. Five different locations were included in this study: Klobučar (KLO), Lojena (LOJ), Mana (MAN), Piškera (PIŠ) and Vrulje (VRU). The geological outskirts of the locations mainly represent Cretaceous limestones with rudists and dolomites, with some Quaternary deposits of Terra Rossa [16]. In the area of the KNP no significantly important physical explorations have been made, and sedimentological charts of the sea bottom still need to be presented. The dynamics of the sea currents are comparable to those of the Žirje islands, where the long-term current in the NW (or N) direction occurs. The sea current strongly depends on the tidal oscillation and wind exposure, as these two factors increase or decrease the current and/or change its direction to the SE (or S). The oscillation mainly affects the bottom layer, in contrast to the wind which mainly affects the surface layers [18]. In the KNP, benthic algae (*Rhodophyta*, *Phaeophyta* and *Chlorophyta*) prevail, which are a source of food for many benthic organisms, such as *Pinna nobilis*, *Hexaplex trunculus*, *Posidonia oceanica*, and *Holothuroidea*. It is assumed that more than 3000 species of benthic and pelagic fauna are present in this area, but unfortunately only *Foramenifera* has been extensively explored [18].

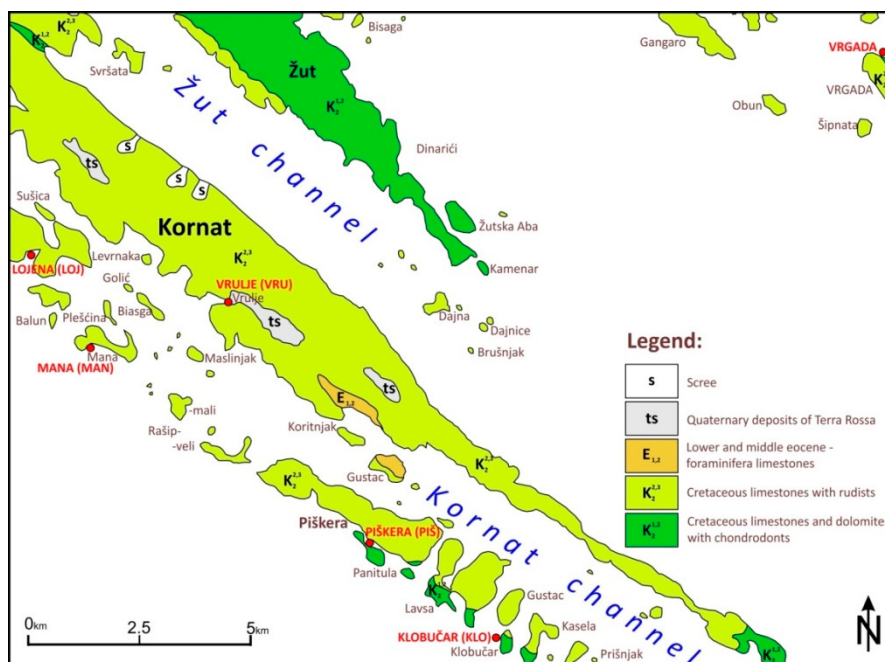


Figure 1. A map showing the sampling locations, exerted from a geological map of SFRY [16].

The locations were selected according to the estimated large, lower, and minimal possibility of anthropogenic pollution. The minimal possibility of anthropogenic influence on the sediment and organisms was estimated in Klobučar, which is located in the northern part of the island of Klobučar and is part of a strictly protected area of the KNP. Locations with a slightly higher possibility of anthropogenic pollution were identified in Lojena and Mana. These are located on the western part of the islands of Levernak and Mana and are highly popular among tourists during the summer. In the year 2016, some 165,200 tourists visited the park, according to the Ministry of Tourism of Croatia. [19] Among those tourists, there are also 2047 inhabitants predominately living in the Murter area [20]. Even higher tourists' demands had an effect on the islands Piškera and Vruļje, which are also locations this paper is focused on, which presented the highest possibility of anthropogenic pollution. The sampling site of Piškera is located in the strait between the Piškera and the Velika Panitula islands, where Piškera marina is located. The sampling site Vruļje is in the bay to the west of the village of Vruļje, which is uninhabited and without an adequate communal infrastructure. On both islands, overnight stays are permitted, and also the Vruļje visitor center is based there. The center will improve the visitor system management and provide sufficient data for further statistical analysis. A total of 53,276 tickets were sold for individual and tourist boats between 2011 and 2014. These also contributed to the increased amount of waste, consisting of plastic and glass bottles, cans and batteries, which represent 24.35% of the whole pollution in this area [20,21].

2.2. Sample Preparations and Methodology

For the purpose of the isotopic analysis, 23 samples of sediment and 11 samples of muscle tissues were collected (Figure 1). The samples were collected from five different locations, according to the estimated possibility of anthropogenic pollution: large possibility (Piškera, Vruļje), lower possibility (Mana, Lojena), and minimal possibility (Klobučar). The samples of *H. trunculus* were collected from four locations, because of the lack of sample material. All samples were gathered manually and with a help of plastic cores in June 2016, with permission from the Ministry of Environmental Protection and Nature of the Republic of Croatia and the Kornati National Park, and were collected at depths between 0 and 10 m. At Klobučar, three sediment samples and two samples of *H. trunculus* were obtained. At other locations (LOJ, MAN, PIŠ, VRU) five samples of sediment and three samples of *H. trunculus*

were collected. The sea bottoms at KLO, LOJ, and MAN were composed of sand, whereas the bottoms of PIŠ and VRU were composed of silt. The isotopic composition of organic carbon ($\delta^{13}\text{C}_{\text{org}}$) was determined for all samples, while the isotopic composition of nitrogen ($\delta^{15}\text{N}$) was determined in the soft tissues of *H. trunculus*.

The sediment samples were dry-sieved at the University of Ljubljana, Faculty of Natural Science, through two ASTM standard stainless-steel sieves, with aperture sizes of 2 mm and 0.315 mm. In this way, we eliminated all the larger particles of organic residues, as well as the remains of shells and skeletons. The fractions smaller than 0.315 mm were divided by a Johnson's riffle and were grinded into a homogeneous powder. For the isotope analysis of sedimentary organic carbon, the sediment was acidified with 1.5 M HCl to remove the carbonate fraction and kept at approximately 50 °C in a sand bath overnight. Afterwards, the isotopic composition of carbon in the dried residue was measured.

The frozen samples of *H. trunculus* were cut with a diamond saw and freeze-dried at −110 °C. The muscle tissues were separated from the intestines and manually grinded into powder. The isotopic composition of nitrogen and organic carbon in *H. trunculus* muscle tissue was determined. For carbon isotope analysis, the samples were pre-treated with 1 M HCl to remove any remains of carbonate shells, while the untreated samples were analyzed for $\delta^{15}\text{N}$. The measurements of the isotopic composition of carbon and nitrogen were carried out at the Jozef Stefan Institute with an isotope ratio mass spectrometer (Europa 20–20) with a preparatory module ANCA-SL. Results are given as relative δ (delta) values per mille (‰), according to:

$$\delta[\text{‰}] = \frac{R(\text{sample}) - R(\text{standard})}{R(\text{standard})} \times 1000 \quad (1)$$

where R is the ratio of heavy to light isotopes ($^{15}\text{N}/^{14}\text{N}$, $^{13}\text{C}/^{12}\text{C}$) of the sample and standard, i.e., Vienna Pee Dee Belemnite (VPDB) for C and atmospheric nitrogen (air) for N.

The isotope measurements were calibrated to the VPDB scale using USGS-40 and USGS-41 (L-glutamic acid) reference materials, and the accuracy was monitored with the commercially available isotope standards (Sercon) OAS Protein Cat. No. B2155, Batch No. 114859 and Sorghum flour Cat. No. B2159, Batch No. 114855. All measurements were carried out in triplicate, and average values were used for further analysis. The N isotope measurements were calibrated against USGS 25 and USGS 26, and the controls were the same as for C. The measurement uncertainty was equal to or less than 0.25‰ for both elements, determined as the long-term reproducibility (1 σ) of the working standard (protein).

3. Results

The values obtained in this research were compared by the t-test, with an assumption of equal variances and the differences between individual locations distinguishable in box-plot diagrams and in the distribution maps. The distribution maps presented below were created with a Surfer 8.0 (Golden Software, LLC, Golden, CO, USA), using an inverse distance to a power gridding method. The method is a weighted average interpolator, with which the influence of one data point to another decreases with the distance. One of the important characteristics is a formation of “bull's eyes” [22,23].

3.1. Isotopic Analysis of Organic Carbon in the Sediment

The $\delta^{13}\text{C}_{\text{org}}$ values for the sediment in the different samples, presented in the Table 1, varied from −20.35‰ to −17.84‰, with an overall mean of −19.34‰ (n = 23). The statistically important differences in the $\delta^{13}\text{C}$ values between individual locations are presented in the Table 2.

Table 1. Average values of $\delta^{13}\text{C}_{\text{org}}$ in the sediment, (n = number of samples collected).

	$\delta^{13}\text{C}_{\text{org}}$ [‰]
KLO (n = 3)	−19.96
LOJ (n = 5)	−20.35
MAN (n = 5)	−20.05
PIŠ (n = 5)	−17.84
VRU (n = 5)	−18.50

Table 2. Results of the t-test for the $\delta^{13}\text{C}_{\text{org}}$ values of the sediments (NSD = no statistical significance, * = statistical significance at a confidence level of $p \leq 0.05$).

	KLO	LOJ	MAN	PIŠ	VRU
KLO	-	NSD	NSD	*	NSD
LOJ	NSD	-	NSD	*	*
MAN	NSD	NSD	-	*	*
PIŠ	*	*	*	-	NSD
VRU	NSD	*	*	NSD	-

The results in Figures 2 and 3 show that the measured $\delta^{13}\text{C}_{\text{org}}$ values in the location of Piškera and Vrulje were increased compared to those of other locations.

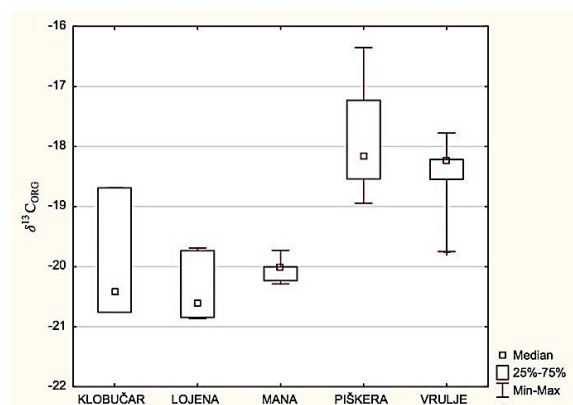


Figure 2. Box-plot diagrams of the $\delta^{13}\text{C}_{\text{org}}$ values of the sediment from different locations.

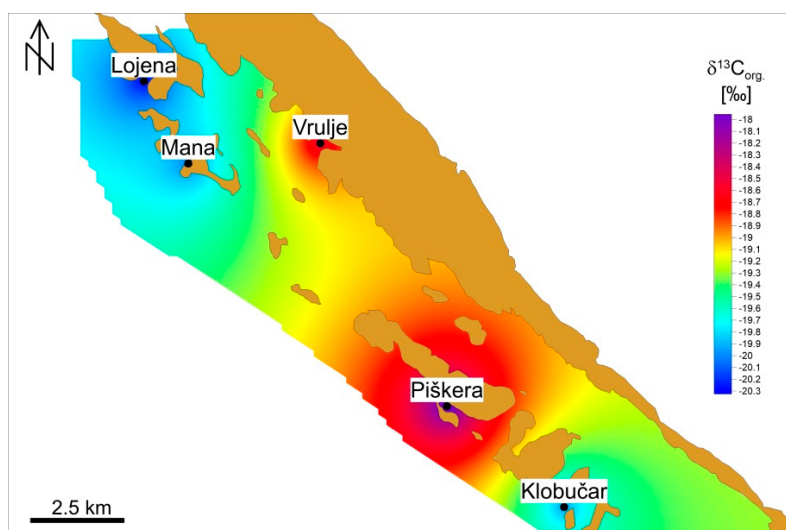


Figure 3. Distribution map of the $\delta^{13}\text{C}_{\text{org}}$ values of the sediment.

3.2. Isotopic Analysis of Nitrogen and Organic Carbon in the Muscle Tissues of the Banded Dye-Murex *H. trunculus*

The $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ data obtained from the muscle tissues ranged between +10.4‰ and +5.65‰, and between −19.15‰ and −14.47‰, respectively, with an overall mean of +7.76‰ (n = 11) for $\delta^{15}\text{N}$ and −16.45‰ for $\delta^{13}\text{C}$ (n = 11). The measured values for $\delta^{15}\text{N}$ are presented in Table 3, and the $\delta^{13}\text{C}$ values in Table 4.

Table 3. Average $\delta^{15}\text{N}$ values for *H. trunculus* (n = number of samples collected).

	$\delta^{15}\text{N}$ [‰]
KLO (n = 2)	+6.13
MAN (n = 3)	+5.65
PIŠ (n = 3)	+8.87
VRU (n = 3)	+10.4

Table 4. Average $\delta^{13}\text{C}$ values for *H. trunculus* (n = number of samples collected).

	$\delta^{13}\text{C}$ [‰]
KLO (n = 2)	−19.15
MAN (n = 3)	−16.55
PIŠ (n = 3)	−14.47
VRU (n = 3)	−15.66

Data in Table 5 support the statistically important differences in the $\delta^{15}\text{N}$ values between Klobučar and Vrulje and between Mana and the sampling sites of Piškera and Vrulje.

Table 5. Results of the t-test of the $\delta^{15}\text{N}$ values of the muscle tissues (NSD = no statistical significance, * = statistical significance at a confidence level of $p \leq 0.05$).

	KLO	MAN	PIŠ	VRU
KLO	-	NSD	NSD	*
MAN	NSD	-	*	*
PIŠ	NSD	*	-	NSD
VRU	*	*	NSD	-

The results from Figures 4 and 5 demonstrate that the $\delta^{15}\text{N}$ values obtained from Piškera and Vrulje are increased as well.

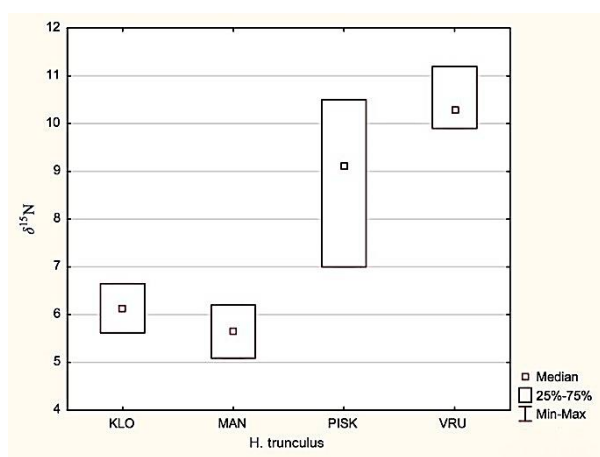


Figure 4. Box-plot diagram of the $\delta^{15}\text{N}$ values of *H. trunculus*.

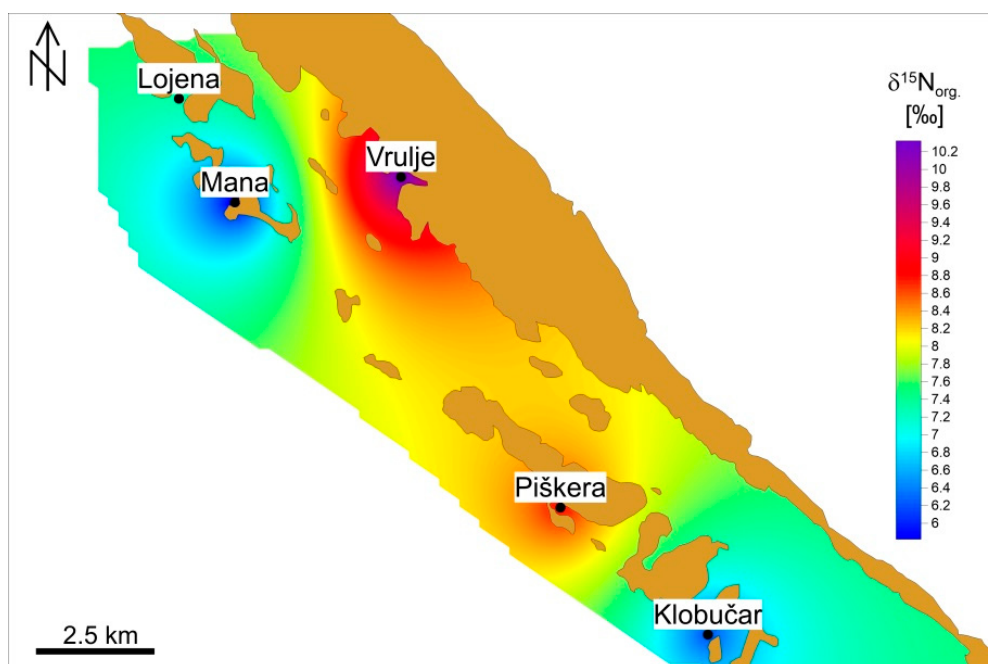


Figure 5. Distribution map of the $\delta^{15}\text{N}$ values of *H. trunculus*.

The results of the t-test presented in Table 6 show that there were statistically important differences between the $\delta^{13}\text{C}$ values obtained at Klobučar and those of all the other locations.

Table 6. Results of the t-test of the $\delta^{13}\text{C}$ values of the muscle tissues (NSD = no statistical difference, * = statistical significance at a confidence level of $p \leq 0.05$).

	KLO	MAN	PIŠ	VRU
KLO	-	*	*	*
MAN	*	-	NSD	NSD
PIŠ	*	NSD	-	NSD
VRU	*	NSD	NSD	-

The results in Figures 6 and 7 show that the measured values in the location of Piškera were elevated compared to those of other locations.

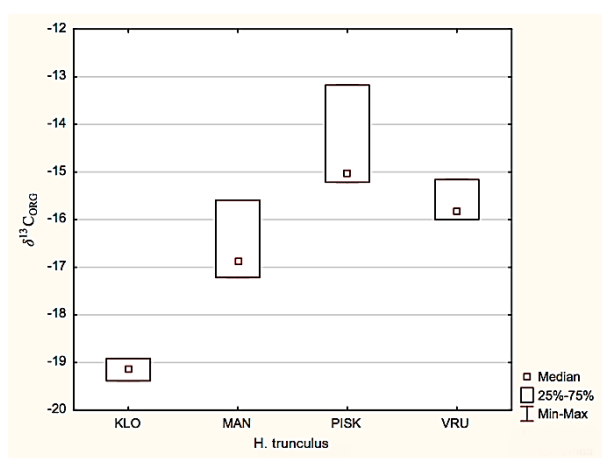


Figure 6. Box-plot diagram of the $\delta^{13}\text{C}$ values of *H. trunculus*.

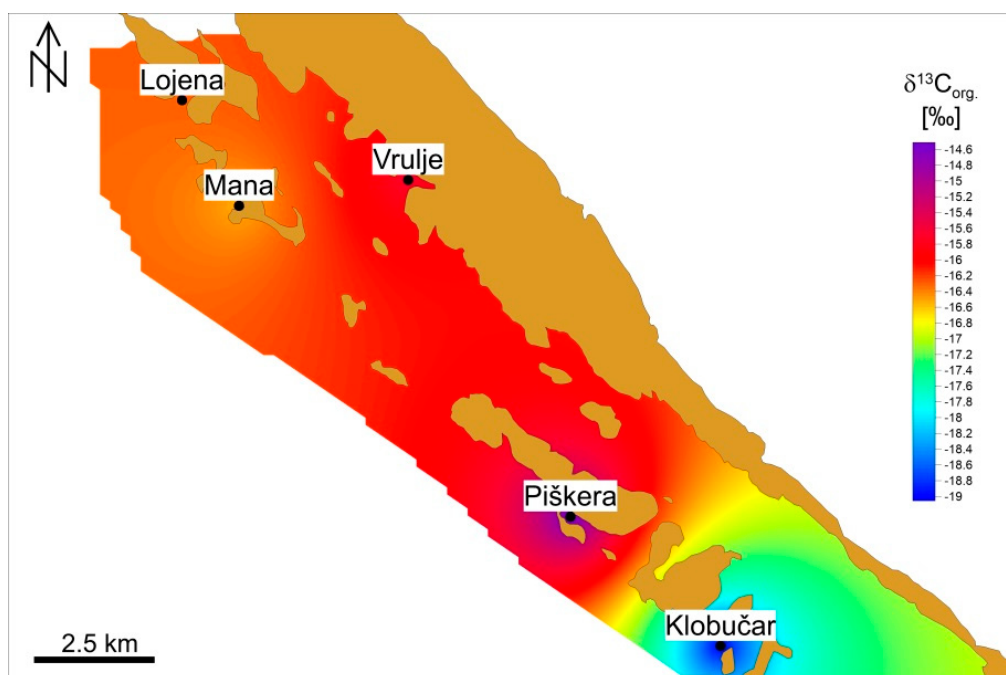


Figure 7. Distribution map of the $\delta^{13}\text{C}$ values of *H. trunculus*.

The statistical evaluations of $\delta^{13}\text{C}_{\text{org}}$ and $\delta^{15}\text{N}$ in the sediments and in the muscle tissues from all the sampling sites indicated the highest values at Piškera and Vrulje in comparison with the other examined locations of the Kornati National Park.

4. Discussion

4.1. Sediment

In the area of the KNP, carbonate minerals, such as calcite, aragonite, and dolomite prevail. In the marine sediment, silicate minerals (quartz, plagioclase, potassium feldspar, and muscovite) were also determined. The highest levels of quartz and feldspars were measured in Vrulje, where Terra rossa is present [24]. Organic matter produced in the marine environments is degraded (re-mineralized) and only partially stored in the marine sediment as sedimentary organic matter. In the coastal areas, the biggest contributor to the sedimentary organic C (C_{org}) is phytoplankton along with terrestrial organic debris, as a consequence of human interference [6].

The small variability of the measured $\delta^{13}\text{C}_{\text{org}}$ values in this study (-20.35‰ to -17.84‰) is attributed to the low number of sampling sites. The measured values varied in the same range as those of particulate organic matter (POM) around the Istria Peninsula, which generally ranged from -26.5‰ to -18.8‰ , and were also comparable to those obtained at other Mediterranean sites [5].

The increased $\delta^{13}\text{C}_{\text{org}}$ values measured at Piškera and Vrulje are related to the presence of the seagrass *Posidonia oceanica*, which is one of the most common and widespread types of seagrass in the Adriatic Sea [25]. Some previously reported $\delta^{13}\text{C}_{\text{org}}$ values for the *P. oceanica* were measured in the research of Lepoint et al. [26] and ranged from -10.8‰ to -19.7‰ for the leaves, from -11.3‰ to -17.1‰ for the roots, and from -13.3‰ to -17.5‰ for the rhizomes. Kennedy et al. [21] published a mean value for the seagrass tissue of -12.2‰ , and the report of Gacia et al. [27] indicated values in the same range of the spectrum (-12.24‰).

The measured mean $\delta^{15}\text{N}$ value of *P. oceanica* in Revellata Bay (Calvi, NW Corsica, France) [26] was $+2.2\text{‰}$, while the $\delta^{15}\text{N}$ values of the same species in the Adriatic varied in a large range of values between $+2.4$ and $+6.7\text{‰}$ [28], depending upon the presence of ^{15}N -enriched anthropogenic wastes. It is therefore reasonable to expect that the presence of ^{15}N -enriched seagrass will also be reflected in

the increased $\delta^{15}\text{N}$ of sedimentary nitrogen at or in the vicinity of seagrass meadows and in organisms, depending on nutrients released from decomposing seagrass detritus in the sediment.

The measurements of potentially toxic elements (PTE) in the sediment showed slightly elevated concentrations in the location of Vrulje, which can be partly attributed to the mineralogical composition of the sediment (higher fraction of silicates at this site compared to others). However, the high levels of some elements (Zn, As, Cu, Cr) can be ascribed to the use of fertilizers and phytopharmaceuticals in viticulture in the coastal area close to this sampling site, which also delivers nutrients to the sea [24].

4.2. *Hexaplex trunculus*

H. trunculus is an opportunistic and generalist predator, which includes invertebrates into its diet but excludes plant material [29,30]. Even though *H. trunculus* is one of the most common organisms in the Mediterranean Sea, not many studies on isotope fingerprints related to it have been published.

The positive $\delta^{15}\text{N}$ values of *H. trunculus* are typical of its predatory feeding behavior, as is the presence of the pigments indirubin ($\text{C}_{16}\text{H}_{10}\text{O}_2\text{N}_2$), indigotin ($\text{C}_{16}\text{H}_8\text{N}_2\text{Na}_2\text{O}_8\text{S}_2$), and other mono- and dibrominated derivatives, which are formed in the glands of this organism [13,29]. Surowiec et al. [13] have proved the presence of the pigment indirubin ($\text{C}_{16}\text{H}_{10}\text{O}_2\text{N}_2$) in the glands of *H. trunculus*, where compounds substituted one CO group with a CNH group [13].

The measured $\delta^{15}\text{N}$ values of *H. trunculus* in the present study (+8.87‰ to +10.4‰) are in the same range of values as those determined by Cresson et al. [2], Mancinelli et al. [29], and Erdelez [31], reporting average values of +8.69‰, approximately +12‰, and +9.89‰, respectively. Marić [32] reported slightly lower values (+6.8‰ to +7.0‰).

^{15}N enrichment by several per mille of organisms thriving in anthropogenically impacted sites in the area around Kornati islands was also previously reported for filtrators such as *Balanus perforatus*, *Anemonia sulcata*, and *Aplysina aerophoba* [33], as well as for the mussel *Mytilus galloprovincialis* [8,34]. Namely, ^{15}N enriched the nitrate from human or animal waste (ranging usually between +10‰ and +20‰ [8,28]), was assimilated by primary producers, and processed by consumers, and therefore affects, and usually elevates, their $\delta^{15}\text{N}$ values [8]. The increased $\delta^{15}\text{N}$ values of *H. trunculus* at Piškera and Vrulje can thus be attributed to the discharge and recycling of ^{15}N -enriched sewage from the nearby marina of Piškera and the village of Vrulje. This implies that the installation of adequate waste water treatment plants along the Adriatic coast and, in particular, in protected areas such as KNP is desperately needed [31].

The $\delta^{13}\text{C}$ values of organisms' tissues usually indicate the source of C rather than specifying the trophic levels [6,8]. The $\delta^{13}\text{C}$ values measured in the *H. trunculus* varied significantly between different locations, from −19.15‰ to −14.47‰, and are comparable with those reported in some other studies. The average $\delta^{13}\text{C}$ value of *H. trunculus* was −17.94‰ in a study by Cresson et al. [2], −17.32‰ in a study by Erdelez [31], and between −17.3‰ and −14.6‰ in a report by Marić [32]. These $\delta^{13}\text{C}$ values are slightly higher than those measured in *H. trunculus* in the study of Mancinelli et al. [29], where the average $\delta^{13}\text{C}$ value was around −15.0‰. Apart from the presence of carbon derived from the decomposing macroalgae or seagrasses, the increased $\delta^{13}\text{C}$ value of dissolved or particulate organic carbon can also be related to the presence of anthropogenic septic wastes. Spies [35] reported $\delta^{13}\text{C}$ values around −16.5‰ for composite sewage.

Thus, it is reasonable to expect that organisms growing at the anthropogenically affected areas would be enriched in both ^{13}C and ^{15}N .

5. Conclusions

The main purpose of this research was to assess the adequacy of the Kornati National Park as an acceptable reference area for environmental studies. For this purpose, we compared different locations and evaluated the possible anthropogenic influence at the locations of Klobučar, Mana, Lojena, Piškera, and Vrulje.

On the basis of the isotopic analysis, the analyzed sites were divided into two subgroups: (a) Klobučar, Lojena, and Mana, where the anthropogenic influence on the environment is negligible, and (b) Piškera and Vrulje, where the anthropogenic influence on the environment is slightly elevated.

Even though some parts of the Kornati National Park are still preserved and intact, there are some areas where human activity has already made an evident impact and has consequently contributed to changes of the environment. Our pilot study shows that the areas closer to marinas and villages are more likely to be under the influence of anthropogenic pollution, yet, more distant areas remain anthropogenically unaltered. To extrapolate these results to the entire Kornati National Park, the analysis of more sampling sites is necessary, and the entire food web, including the dissolved and particulate load of the seawater, should be taken into consideration, so as to acquire a complete set of environmental data.

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Conflicts of Interest: The authors declare no conflict of interest.

Correction Statement: This article has been republished with a minor change. The change does not affect the scientific content of the article and further details are available within the backmatter of the website version of this article.

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