

Ingression of the hydromedusa *Neotima lucullana* (delle chiaje, 1822) into the ecosystem of the Neretva river estuary (south-eastern Adriatic, Croatia)

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Hydromedusa Neotima lucullana is an endemic species of the Mediterranean Sea. In this study we document the first numerous and permanent occurrence of N. lucullana in the estuary of the Neretva River, an unusual habitat for this typical marine species. We provide COI barcode sequence of this species, which is also first genetic sequence ever published of N. lucullana. Weekly data on the occurrence and abundance of N. lucullana were obtained from reports and records of autonomous divers and local fishermen in the area from Opuzen to Metković from mid-June to the end of October 2021. The individuals were always located in the layer above the bottom with salinity between 35.2 and 38.2. The diameters of the jellyfish bells ranged from 15 mm to 72 mm, most of them between 40 mm and 58 mm. Differential development of the gonads was observed in all specimens larger than 25 mm. In mid-June, the specimens were common but solitary. A week later, the jellyfish were more frequent and in smaller aggregations. From late June to mid-September, the jellyfish were constantly observed in larger aggregations. Toward the autumn, the number of individuals gradually decreased, and by the last week of October, only single individuals were observed. Our results indicate significant changes in the ecosystem of the Neretva estuary and confirm the observed composition of zooplankton communities dominated by marine species in summer and autumn.

Key words: jellyfish; Eirenidae; zooplankton; karstic estuary ecosystem; Mediterranean Sea

INTRODUCTION

Historical observations and knowledge of jellyfish in the Mediterranean Sea contribute significantly to our current knowledge of the biology and ecology of cnidarians in planktonic communities (BOERO & BOUILLON, 1993; GRAVILI *et al.*, 2013). Medusa *Neotima lucullana* (Hydrozoa, Leptothecata) is a Mediterranean endemic species known for the western Mediterranean and Adriatic Sea (BOUILLON *et al.*, 2004). The first description of the species was based on finds in the Gulf of Naples (DELLE CHIAJE, 1882 as *Dianaea lucullana*) and then in the Gulf of Trieste (NEPPI & STIASNY, 1913 as *Tima flavilabris*). In later finds the species was described as *Tima lucullana*, again referring to the Naples area (PETERSEN, 1962; VANNUCCI, 1966; BRINCKMANN-VOSS, 1987) and to the area around Catalonia and the Tyrrhenian Sea (GILI, 1986; MADIN, 1991; MEDEL & LÓPEZ-GONZÁLEZ, 1996; GRAVILI *et al.*, 2008). On the eastern Adriatic coast, *N. lucullana* has been found only in the marine lakes of the island of Mljet in southern Croatia (BENOVIĆ *et al.*, 2000 determined as *Tima* sp). From an underwater photo taken in September 2014 near Pula (northern Adriatic), it could be concluded that the recorded jellyfish might be *N. lucullana* (Fig. 1).

Thus, although the species has been described for a long time, very little is known about its distribution. All observations have revealed the presence of a small number of individuals, usu-



Figure 1. *Neotima lucullana*, Pula, northern Adriatic (photo: D. Petricioli, September 2014)

ally solitary. Only PETERSEN (1962) mentions the finding of 26 adults from July to October near Naples, at a depth of 40-50 m above the bottom.

The aim of this article is to report the unusual occurrence of *Neotima lucullana*, a typically marine and rarely observed hydromedusa, in a completely new environment of the Neretva River estuary. For this purpose, hydrographic parameters of the water column were described, biometric measurements of medusae were performed and abundance of individuals was estimated. Molecular analysis was performed to provide COI genetic barcode of the species.

MATERIAL AND METHODS

Study area

The valley of the Neretva River is located in the southern part of the Republic of Croatia. It is surrounded by the Adriatic Sea in the west and by karst hills in the north, east and south (Figure 2). The Neretva River is the largest watercourse on the east coast of the Adriatic Sea, and represents a typical river that flows into the Adriatic Sea. The length of the river is 215 km, of which 25 km are in the territory of the Republic of Croatia. The mean year-round discharge of the Neretva River at Metković is estimated at 355 m³/s. It was found that saltwater in Metković occurs at a freshwater flow of less than 180 m³/s, while saltwater is completely displaced from the riverbed at a water flow of more than 500 m³/s. (LJUBENKOV & VRANJEŠ, 2012). Many fish species spawn and grow up in this area, which makes it a very important fishing area (GLAMUZINA, 2010). There are also several ornithological and ichthyological protected areas in this area, and it is considered one of the most important wetlands in Europe as a resting and wintering area for migratory birds (STUMBERGER & SCHNEIDERJACOBY, 2010).

The sea penetrates like a salty wedge into the bed of the Neretva. In between there is a relatively narrow transition layer between salt and fresh water. The intensity of injection depends on the tides, wind action and, most importantly, on the current in the river, which is mainly gen-



Figure 2. Study area showing locations of permanent monitoring stations (yellow squares).

erated by the operation of upstream hydroelectric power plants in the dry season and by floods in the rainy season (LJUBENKOV & VRANJEŠ, 2012; KRVAVICA & RUŽIĆ, 2020; LOVRINOVIĆ *et al.*, 2021). As a rule, the winter period is markedly rainy and summers are characterised by drought. In the area of the lower Neretva, there are two natural directions for the intrusion of the salt sea: the first through the Neretva riverbed and the second through the deep underground layers. The sea usually begins to penetrate the Neretva riverbed in spring, and the influence of the salt water decreases with increasing precipitation in autumn. It was found that saline water in Metković occurs at a freshwater flow of less than $180 \text{ m}^3/\text{s}$, while saline water is completely displaced from the riverbed at a water flow of more than $500 \text{ m}^3/\text{s}$ (LJUBENKOV & VRANJEŠ, 2012). As a result of the strong influence of saltwater in the Neretva, migrations of some marine cephalopods and fish have therefore been recorded (GLAMUZINA & DOBROSLAVIĆ, 2020; TUTMAN *et al.*, 2021).

Hydrographic features

Monthly temperature and salinity measurements from June to October 2021 were made using a mobile probe SEBA Hydrometrie KLL-Q-2. Measurements were taken at the Opuzen station (Fig. 1), about 11 km from the river mouth, at intervals of one meter from the surface to the bottom (7,5 m depth).

Zooplankton monitoring and sampling

Data on the occurrence and abundance of *Neotima lucullana* were obtained from reports and records of autonomous divers and local fishermen. Observations were conducted weekly in the area from Opuzen to Metković. Abundance estimates were based on divers observations of whether single jellyfish or jellyfish in groups of several individuals were observed. Based on these estimates, the following categories were formed: a) sporadic occurrence of single organisms; b) frequent occurrence of jellyfish and small aggregations (3-6 individuals per 10 m^3); c) large aggregations (> 6 individuals per 10 m^3).

The jellyfish collected by hand by autonomous divers were stored in plastic bags and transferred to a bucket of sea water on the shore. The diameter of the bell was measured on a 1 mm scale on freshly collected jellyfish flattened on a glass plate with the oral side facing up. A total of 45 individuals were measured. Morphological characteristics of jellyfish of different bell diameter sizes were performed in the laboratory on the basis of preserved specimens with 4.5% neutralized formaldehyde. Details of the individuals were observed using an Olympus SZX 9 stereomicroscope.

Zooplankton samples were collected at Opuzen station on August 23 with two separate vertical hauls from the bottom to the surface using a modified Nansen net with $53 \mu\text{m}$ and

125 µm mesh size and 40 cm mouth diameter. Taxonomic identification and abundance of zooplankton were performed using an Olympus SZX16 stereomicroscope at a magnification of 80×.

Morphological and Molecular Analysis

The detailed descriptions of the species published by KRAMP (1959), PETERSEN (1962) and BOUILLON *et al.* (2004) were used for the morphological analysis.

DNA extraction and PCR

Approximately 1 mm³ of tissue was taken for DNA extraction. DNA was extracted using the ammonium acetate precipitation method. The DNA extraction method and PCR conditions for COI amplification using primer pairs LCO1490 and HCO2198 (FOLMER *et al.*, 1994) are described in detail in GARIĆ & BATISTIĆ (2022). The PCR product was sequenced by Macrogen. The COI sequence obtained was deposited with GenBank under accession number ON615603.

Sequence and phylogenetic analysis

The obtained COI sequence of *Neotima lucullana* was aligned to 23 Eirenidae sequences from GenBank using ClustalW software. The COI sequence of *N. lucullana* was translated from nucleotide to amino acid sequence with the ExPASy translate tool using the genetic code of coelenterates (<https://web.expasy.org/translate/>). Phylogenetic analysis was performed using the

nucleotide alignment with MegaX software (KUMAR *et al.*, 2018), the maximum likelihood method, and the GTR+G+I evolutionary model with 4 discrete gamma categories, which was determined to be the best model by the model selection test in MegaX software. The resulting phylogeny was tested with 1000 bootstrap replicates.

RESULTS AND DISCUSSION

Hydrography features

Based on the hydrographic parameters, the water column of the estuary was divided into three layers (Table 1): surface layer (0-2 m) with colder temperature values and low salinity; middle transition layer (3-4 m), where the seasonal halocline formed; bottom layer (5-7 m) with higher temperatures and high salinity.

In early June, higher temperature values (19-20 °C) were measured below 5 m depth, while they did not reach 14 °C in the upper layer (Table 1). The temperature increased from the end of June to August and September. A sharp decrease was observed in October. Monthly variations in values within the deep layers were negligible, except in the lowest layer in October (Table 1). In general, the highest monthly temperatures were recorded in the bottom layer and the lowest at the surface.

Salinity values in the surface layer were consistently low, ranging from 1.5 (early June) to 5.2 (September). Large variations were observed in the middle layer (Figure 3). Low values (1.6-2.5) were measured in early June and October,

Table 1. Distribution of temperature values (°C) at Opuzen station in three vertical layers of the water column (June-October 2021).

Date	Surface Layer (0-2 m)		Middle Layer (3-4 m)		Bottom Layer (5-7 m)	
	range	average	range	average	range	average
2-Jun	13.7-13.9	13.82±0.09	13.7-13.9	13.79±0.11	18.7-19.2	19.03±0.28
29-Jun	18.5-18.7	18.57±0.11	20.9-21.8	21.31±0.62	21.2-22.1	21.67±0.47
19-Jul	18.3-18.4	18.32±0.04	23.2-23.9	23.56±0.51	22.6-24.5	23.58±0.95
24-Aug	21.5-21.6	21.51±0.01	21.5-21.6	21.51±0.01	24.8-24.9	24.86±0.01
20-Sep	18.4-18.7	18.58±0.13	23.3-24.1	23.68±0.59	24.2-24.2	24.20±0.00
27-Oct	11.4-11.8	11.64±0.22	11.9-11.9	11.93±0.01	13.9-19.6	17.67±3.25

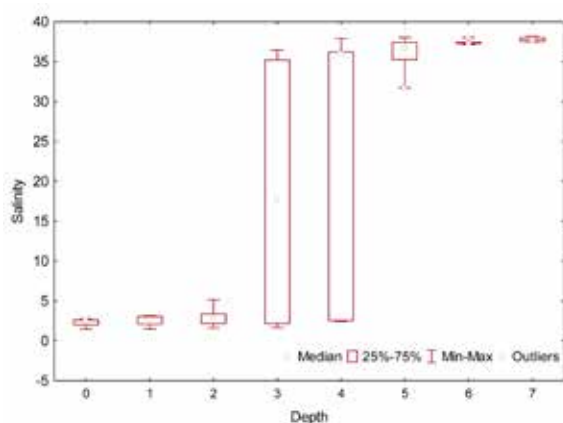


Figure 3. Box plot of salinity variations at Opuzen stations from June to October 2021.

while in the other months they ranged from 32.3 (late June) to 37.9 (September). In the bottom layer, the value ranged from 35.2 (early June at 5 m depth) to 38.2 (October at 7 m depth). In the entire part of the bottom layer, a salinity of 38 was measured in September.

Our hydrographic measurements revealed particularly high salinity values in summer and early autumn, which is consistent with previous studies from the last two decades (LJUBENKOV & VRANJEŠ, 2012; GLAMUZINA & DOBROSLAVIĆ, 2020). Continuous monitoring of values using the same method from June to November for the period from 2009 to 2021 showed that the highest values were obtained in the layer below 5 m depth in 2017 (VRANJEŠ, unpublished data), as well as in our measurements in 2021.

Net zooplankton assemblages

The total abundance of zooplankton collected with the 53 μm net was high, 43 735 ind. m^{-3} . The most numerous were: copepods nauplii (15 019 ind. m^{-3}), Calanoida copepodites (10 240 ind. m^{-3}), *Oithona nana* (5803 ind. m^{-3}), Cyclopoida copepodites (3755 ind. m^{-3}), Bivalvia larvae (3413 ind. m^{-3}) and Harpacticoida copepodites (2048 ind. m^{-3}). Among the other adult specimens, the cladocerans *Evadne spinifera* and *Pseudevadne tergestina*, the copepods *Paracalanus parvus parvus*, *Microsetella norvegica* and *Euterpina acutifrons* were numerous. Two protists were detected in the fraction of the

53- μm net: *Tintinnopsis cylindrica* (2048 ind. m^{-3}) and *Codonellopsis schabi* (85 ind. m^{-3}). Both tintinnids are common and numerous in summer in the coastal areas of the Adriatic Sea (KRŠINIĆ, 2010).

The total zooplankton abundance collected with the 125- μm net was lower, 3203 ind. m^{-3} . Seventeen taxa of typical coastal marine zooplankton species were detected. Copepodites (1109 ind. m^{-3}), *Oithona nana* (896 ind. m^{-3}) and meroplanktonic larvae (600 ind. m^{-3}) were the most numerous. Among adult calanoids, the highest abundance was recorded in copepods *Acartia (Acartiura) clausi* (213 ind. m^{-3}) and *Centropages kröyeri* (85 ind. m^{-3}). Three individuals (two males and one female) of the invasive non-indigenous copepod *Pseudodiaptomus marinus* were also recorded. Previously, this species was found at the mouth of the river Neretva in the area of the port of Ploče (UTTIERI *et al.*, 2000). Our values of total zooplankton numbers and the ratio of abundance between samples collected with 125- μm and 53- μm nets are consistent with the results of the zooplankton study of the Krka estuary (eastern Adriatic coast) (VIDJAK *et al.*, 2009).

In general, the composition of zooplankton consisted exclusively of coastal marine species characteristic of the summer period of the nearby Neretva Channel (VIDJAK *et al.*, 2007) and Mali Ston Bay (KRŠINIĆ & LUČIĆ, 1998). We can assume that high zooplankton abundances could be the reason for the invasion and residence of *N. lucullana* jellyfish in the Neretva estuary, an area with less food competition and without potential predators.

Neotima lucullana – distribution, taxonomic features and abundance

According to the World Register of Marine Species (WoRMS), only two other species besides *Neotima lucullana* are known for this genus: *N. peterseni*, found in the Bismarck Sea and originally described by BOUILLON (1984), and *N. galei* from the Persian Gulf, described by SCHUCHERT (2017). No polyps are known for species of the genus *Neotima* (BOUILLON *et al.*, 2004; SCHUCHERT, 2017).

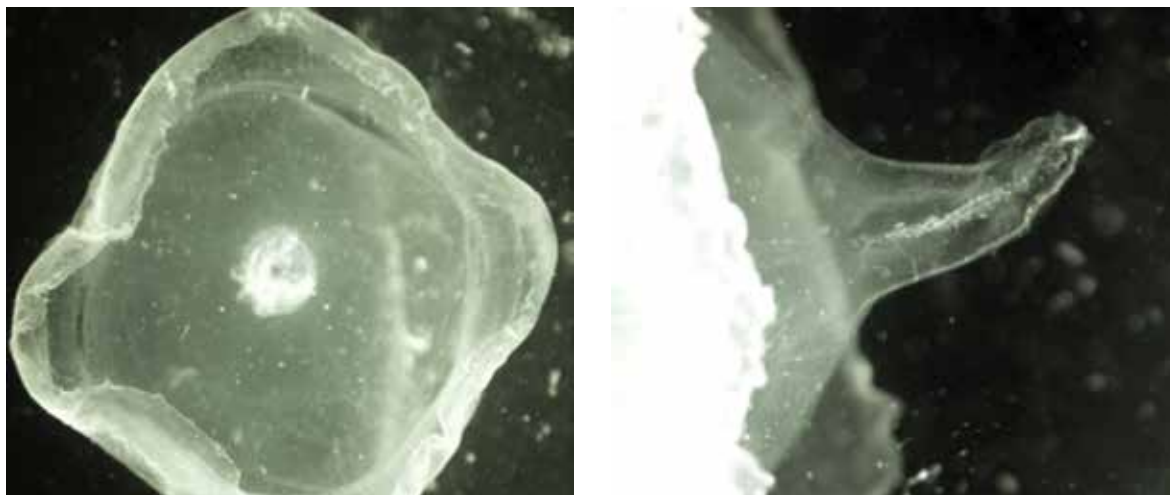


Figure 4. The bell shape (20 mm) and manubrium of juveniles of *Neotima lucullana* from the Neretva estuary.

Fifty-five specimens of *N. lucullana* found in the Neretva estuary had bell diameters ranging from 15 mm to 72 mm. The appearance of tentacles and manubria was noted in the smallest specimens (Figure 4). Juveniles were not recorded in any of the previous findings and have not been described. Differential development of gonads was observed in all specimens larger than 25 mm. Most specimens had a diameter between 40 mm and 58 mm (73%). Tentacles and manubrium were well developed in individuals with bell diameters greater than 40 mm (Fig. 5). The morphological details were in complete agreement with the description of the species *Neotima lucullana* (PETERSEN, 1962; BOUILLON *et al.*, 2004; SCHUCHERT, 2017). In individuals larger than 50 mm in diameter,

the tentacles, velum and manubrium were fully developed and longer than the bell diameter (Fig. 6). Our results regarding the maximum bell diameter of *N. lucullana* were similar to most previous results for the Mediterranean region. According to BOUILLON *et al.* (2004), the bell diameter of this medusa was up to 74 mm. In the Mljet lakes, all individuals had a diameter of 60-84 mm (BENOVIĆ *et al.*, 2000). PETERSEN (1962) found specimens up to 87 mm in diameter, while the estimate of the individual found near Pula was about 80 mm (D. PETRICIOLI, pers. comm.).

The obtained COI sequence of *Neotima lucullana* was 419 bp long. The nucleotide sequence database was searched for the COI sequence of *N. lucullana* using the Blastn tool (<https://blast.ncbi.nlm.nih.gov/>) and yielded no

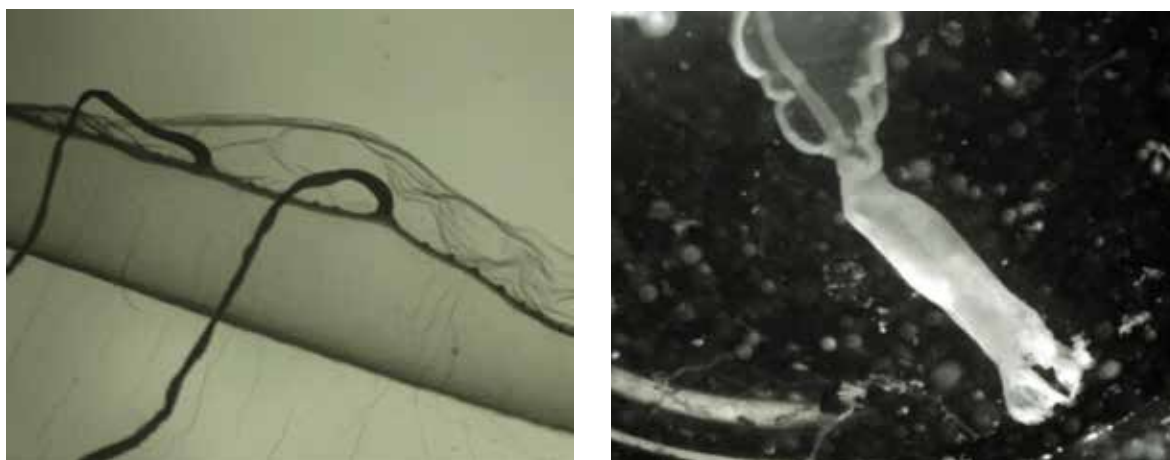


Figure 5. Tentacles and manubrium of *Neotima lucullana* from the Neretva estuary with a bell diameter of 40 mm.



Figure 6. Fully developed *Neotima lucullana* (70 mm) in the Neretva estuary (photo: N. Kaleb, August 2021).

close hits. The translated amino acid sequence did not contain stop codons in the correct reading frame, indicating that it was not a pseudogene sequence. Obtained COI sequence represents the first *N. lucullana* sequence in GenBank. The amino acid sequence of the obtained COI fragment of *N. lucullana* was 100% identical to 8 species of Eirenidae: *Eutima krampi* (accession number: FJ418664), *E. gegenbauri* (MF000515), *E. curva* (MF000511), *E. gracilis* (KC440059), *E. viridula* (KC440019), *Eutoniina indicans* (JF884213), *Helgicirrha brevistyla* (FJ418667), *H. cari* (MF000519). Phylogenetic analysis produced a tree with unsupported branches (most branches had bootstrap support of less than 50), indicating that the COI fragment was not informative enough to generate a meaningful phylogeny (data not shown).

The first reports of medusae in the Neretva estuary date from mid-June 2021, after fishermen reported the presence of unusual gelatinous substances in their fish traps. According to the divers' observations, the individuals were frequent but individually present. By the first week, the animals were more abundant and in smaller aggregations. From late June to mid-September, the jellyfish were constantly observed in larger aggregations. At the permanent monitoring sta-

tions Opuzen and Metković the estimated density was 6 to 12 individuals per 10 m³. During this period, fishermen reported dense accumulations of the gelatinous substance in their fish traps, which negatively affected expected catches. The number of individuals gradually decreased toward the fall, and single specimens were observed during the last week of October. No specimens of *N. lucullana* were observed in the surrounding arms of the river, in the mouth of the river, or in the surrounding sea. Our findings of this jellyfish can only be compared with the results of BENOVIĆ *et al.* (2000) for the Small Lake of Mljet Island: an aggregation of five adult specimens was observed near the bottom between 17 and 20 m depth, from August to September (V. ONOFRI, unpublished data). In the Big Lake of Mljet, the specimens were sporadic. BENOVIĆ *et al.* (2000) consider that the reason for the rare finds of this jellyfish is its very fragile body structure and its limited distribution near the bottom, which is why it cannot be caught with the standard vertical tows of plankton nets that miss the first meters above the bottom.

CONCLUSIONS

We document the first numerous and prolonged occurrence of *Neotima lucullana* in the Mediterranean Sea, in a habitat unusual for this typical marine species. Our results confirm the widespread ecological changes in the Neretva estuary ecosystem and also confirm the increasing seawater intrusion into the upper reaches of the river.

In addition, the results indicate changes in the composition of biological communities dominated by marine zooplankton species, as

well as the presence of alien invasive species in the upper estuaries, which is another threat to this fragile ecosystem.

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Ingresija hidromeduse *Neotima lucullana* (della chiaje, 1822) u ekosustavu estuarija rijeke Neretve (jugoistočni Jadran, Hrvatska)

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SAŽETAK

Hidromedusa *Neotima lucullana* je endemska vrsta Sredozemnog mora. Ovim istraživanjem dokumentiramo prve zapise o brojnoj i dugotrajnoj pojavi meduze *N. lucullana* u estuariju rijeke Neretve, neobičnom staništu za ovu tipično morsku vrstu. U sklopu ovog istraživanja dobivena je sekvenca podjedinice i citokrom oksidaze (COI), što je ujedno i prva genetička sekvenca ikad objavljena vrste *N. lucullana*. Tjedni podaci o pojavi i brojnosti *N. lucullana* dobiveni su na osnovi izvještaja i zapisa autonomnih ronilaca i lokalnih ribara na području od Opuzena do Metkovića od sredine lipnja do kraja listopada 2021. Jedinke meduza su uvijek bile u sloju iznad dna unutar vrijednosti saliniteta između 35,2 i 38,2. Promjer zvona meduza kretao se od 15 mm do 72 mm, glavnina između 40 mm i 58 mm. Diferencijalni razvoj spolnih žlijezda uočen je kod svih primjeraka većih od 25 mm. Sredinom lipnja jedinke su bile uobičajene, ali prisutne pojedinačno. Tjedan dana kasnije, meduze su bile često videne i u manjim skupinama. Od kraja lipnja do sredine rujna, meduze su bile prisutne u većim agregacijama. Broj jedinki postupno se smanjivao prema jeseni i samo pojedinačni primjerci bili su uočeni tijekom posljednjeg tjedna listopada. Naši rezultati ukazuju na znatne promjene ekosustava donjeg toka rijeke Neretve što potvrđuje zabilježen sastav zooplanktonskih zajednica u kojima dominiraju morske vrste tijekom ljeti i jeseni.

Ključne riječi: želatinozni plankton; Eirenidae; zooplankton; krški estuariji; Sredozemno more