



Mediterranean Marine Science

Vol 24, No 2 (2023)

VOL 24, No 2 (2023)



First application of environmental eDNA for detecting the presence of the European eel [Anguilla anguilla, (Linnaeus, 1758)] in the Adriatic, as a basis for conservation remarks

MATEJ VUCIĆ, DUSAN JELIĆ, GORAN KLOBUČAR, ŽELJKO PAVLINEC, FEITOUMATT LEMATT GHRIB, MATEA JARAK, THOMAS BAUDRY, ANA GALOV

doi: 10.12681/mms.31829

To cite this article:

VUCIĆ, M., JELIĆ, D., KLOBUČAR, G., PAVLINEC, ŽELJKO, GHRIB, F. L., JARAK, M., BAUDRY, T., & GALOV, A. (2023). First application of environmental eDNA for detecting the presence of the European eel [Anguilla anguilla, (Linnaeus, 1758)] in the Adriatic, as a basis for conservation remarks. *Mediterranean Marine Science*, *24*(2), 364–376. https://doi.org/10.12681/mms.31829

First application of environmental eDNA for detecting the presence of the European eel [*Anguilla anguilla*, (Linnaeus, 1758)] in the Adriatic, as a basis for conservation remarks

Matej VUCIĆ¹, Dušan JELIĆ², Göran KLOBUČAR¹, Željko PAVLINEC³, Feitoumatt Lematt GHRIB², Matea JARAK⁴, Thomas BAUDRY⁵ and Ana GALOV¹

¹University of Zagreb, Faculty of Science, Department of Biology, Rooseveltov trg 6, HR-10000 Zagreb, Croatia ²Croatian Institute for Biodiversity, Maksimirska cesta 129/5, HR-10000 Zagreb, Croatia

³WWF Adria, Gundulićeva 63, HR-10000 Zagreb, Croatia

⁴Institute for Ornithology, Croatian Academy for Science and Arts, Gundulićeva 24, HR-10000 Zagreb, Croatia ⁵Laboratoire Écologie et Biologie des Interactions, 3 rue Jacques Fort, FR-86073 Poitiers, France

Corresponding author: Matej VUCIĆ; matej.vucic@biol.pmf.hr

Contributing Editor: Costas TSIGENOPOULOS

Received: 12 November 2022; Accepted: 26 May 2023; Published online: 29 June 2023

Abstract

Anguilla anguilla, the European eel, is an important species for aquaculture and fisheries. Its population has dropped dramatically in recent decades, reaching an all-time low. As a result, it has been listed as critically endangered on the IUCN Red List of Endangered Species since 2007. Therefore, constant population monitoring is essential to ensure the survival of this iconic species. Glass eel recruitment is declining worldwide, including the populations in the Mediterranean region. Despite the negative impact of man-made activities in Mediterranean coastal waters over the past few decades, data on spawning biomass escaping from the Mediterranean highlights the region's importance for the global eel supply. Eel research and monitoring is done using conventional techniques, which have certain drawbacks. Therefore, the use of molecular-based detection as a credible choice for monitoring species in aquatic ecosystems was recently shown to be an effective management plan alternative. We present the first use of environmental DNA for monitoring eel populations in the Adriatic Sea and in the complex Dinaric karst freshwater ecosystem. The method has been demonstrated to be accurate and useful for detecting the presence of *A. anguilla* eDNA and identifying conservation areas. This is also the first study investigating the range and presence of the European eel in the Adriatic and in the Mediterranean Sea, as well as in underground karst systems, springs, and in the karst poljes of the Eastern Adriatic coast.

Keywords: eels; Anguilla; environmental monitoring; Mediterranean region; environmental DNA; conservation genetics.

Introduction

The European eel, Anguilla anguilla, (Linnaeus, 1758), is a catadromous fish made up of a single mating population that spawns in the Sargasso Sea (Cresci, 2020). Eels migrate from the Atlantic Ocean as larvae and reach Europe's continental slope, where they transform into post-larval glass eels. The latter make it to the continent, where some enter fresh water, others stay in the marine environment, and still others move between the two (Cresci, 2020). In Europe, eels inhabit the area from Norway to the southern parts of the Mediterranean Sea (Dekker, 2000; 2003). Historically, the European eel was, and still is, an important species in aquaculture and fisheries (Cresci, 2020). This species is one of the most important commercial fish in the world (Violi et al., 2015), especially since the demand for glass eels from eel farms in Asia drives the glass eel trade. Due to the fact

Schlegel, 1846) were in short supply in the 1990s, European glass eels were frequently used as a substitute (Stein et al., 2016). Unfortunately, eel stocks have reached their all-time low and concerns regarding the state of the stock have been highlighted by a drop in captures of this species at all stages (Capoccioni et al., 2020). Over the last three decades, glass eel recruitment has plummeted to 10% of what it was in the 1960s and 1970s (Dekker, 2016). The main cause of decline of A. anguilla populations is the combination of natural and anthropogenic causes, such as uncontrolled exploitation, illegal trade, habitat alterations, and habitat loss due to human activities, contamination, and diseases. These impacts act together, affecting all developmental stages of the European eel, leading to decreased biomass of all stocks (Dekker & Beaulaton, 2016; Miller et al., 2016; Bevacqua et al., 2009; Stein et al., 2016; Jacoby et al., 2015). For that reason, the Euro-

that Japanese glass eels (Anguilla japonica, Temminck &

pean Union adopted an eel protection and recovery plan in 2007 (Anonymous, 2007). This regulation required the EU Member States to adopt national Eel Management Plans (EMPs) by 2009, with the goal of reducing anthropogenic mortality and restoring a spawner run. As a result, in nineteen EU countries, national management plans have been formed, preventive measures have been adopted, and additional information on the stock's status has been collated (Dekker, 2016; Dekker & Beaulaton, 2016). Furthermore, in September of 2007, A. anguilla was listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), imposing regulation on its international trade. Still, the European eel remains a critically endangered species according to the last IUCN assessment (Pike et al., 2020).

As evidenced by a concurrent decline in glass eel recruitment, including local stocks in the Mediterranean region (Aalto et al., 2016; Amilhat et al., 2014), the decline of the global eel stock affects the entire geographical range of the European eel, including the southern part of its distribution area. The eels are largely associated with coastal lagoons in the Mediterranean region (Nielsen & Prouzet, 2008), areas which encompass a total surface area of 5800 km² and account for a substantial fraction of the entire continental eel habitat (Cataudella et al., 2014; Capoccioni et al., 2020). A steep decrease of eel productivity in these coastal lagoons is indicative of significant ongoing negative changes in the quality of these habitats. Several man-made activities have impacted the Mediterranean coastal lagoons throughout the last several decades, resulting in increased eutrophication and pollution of these shallow water habitats with pesticides and pharmaceutical loads (Parolini et al., 2010; Pinto et al., 2016; Riascos-Flores et al., 2021). This may have influenced the reproductive potential of the Mediterranean eel stocks. Strong evidence supports chemical pollution being one of the main reasons behind the sharp decline in recruitment and abundance of the European eel (Belpaire et al., 2019). Pesticides and pharmaceuticals are well known to be a source of xenoestrogens. Xenoestrogens interfere with the natural functions of estrogens and induce reproductive issues, such as reduced sperm count in males and reduced fecundity and egg hatchability in females (Badamasi et al., 2020). Studies suggest that roughly 35% of the healthy spawning biomass is still escaping from Mediterranean lagoons (Aalto et al., 2016), which emphasizes their importance in contributing to the world eel supply (Capoccioni et al., 2020).

Currently, research and monitoring of eels relies on traditional methods like electrofishing and trapping, which have certain limitations. These methods are increasingly controversial because they are recognized to be non-selective (with certain non-target species captured) and very disruptive for the ecosystem, sometimes resulting in the death of certain specimens (Robinson *et al.*, 2018; Wang *et al.*, 2021). Though some research requires capturing individual specimens using traditional methods (i.e., monitoring the population density or population genetics studies), problems still exist, particularly in the context of monitoring protected species such as the European eel and aiming to determine only species presence/absence. As a result, these traditional methods tend to be replaced by molecular methods, which have been widely developed over the past ten years (Thomsen & Willerslev, 2015). This method is commonly called environmental DNA (eDNA) and allows the detection of an organism's DNA which comes from various tissues, e.g., skin, eggs, and mucus, shed in its surroundings, without the requirement to see the target species at any stage of its life (Ficetola et al., 2008). The effectiveness of eDNA-based detection techniques has been proven for the early detection of rare, endangered, and endemic organisms (Piggott, 2016), as well as for non-native and invasive species (Baudry et al., 2021, Dubreuil et al., 2022). This strategy has also been demonstrated as a sustainable practice for anguillid species in their native habitats, making it a valuable technique for research and conservation (Hänfling et al., 2016; Weldon et al., 2020; Burgoa Cardás et al., 2020).

In the Croatian part of the Adriatic Sea drainage system, the European eel occurs in all rivers of the basin (Milošević *et al.*, 2021). Based on data provided by the Croatian Institute for Biodiversity, records indicate a wide distribution of *A. anguilla* in the Croatian coast of the Adriatic Sea during the last 120 years (Fig. 1). It is interesting and important to notice that the European eel is not only present in marine or freshwater habitats in the Adriatic region, it is also frequently detected in lakes and rivers more than 100 kilometers inland. Moreover, the eel was also detected in different karst fields (poljes) like in Gacko, Ličko, Imotsko, Vrgoračko, and Konavosko polje.

Large populations of this species have been recorded in the lower parts and estuaries of large rivers (Neretva, Zrmanja, Krka, Cetina, Jadro, Žrnovnica, etc.), and in lakes (Vrana Lake near Biograd, Baćina Lakes near Neretva River, Vrana Lake on Cres, etc.) (Piria et al., 2014; Dulčić & Glamuzina, 2006). Zrmanja and Neretva rivers are large freshwater systems that drain into the Adriatic Sea (Bonacci, 1999; Riđanović et al., 2010). Estuaries of both rivers are areas of high biodiversity, especially for the freshwater, brackish, and marine ichthyofauna (Mrakovčić et al., 2006; Glamuzina & Dobroslavić, 2020). Large populations of A. anguilla can be found in both rivers, making them important habitats for this species in the Adriatic Basin. Moreover, the European eel is one of the most dominant species in the Neretva River, with 3.75% of the biomass share (Glamuzina & Dobroslavić, 2020). The existence of eel populations on Pag, Ugljan, and Pašman Islands is historically known, especially in the larger bodies of water (e.g., lakes Kolansko Blato, Velo, and Malo Blato on Pag Island). Interestingly, even though A. anguilla is not considered a stygophilic species, it has also been detected in the underground habitats of the Dinaric karst. Underground eel migrations were already mentioned in the early 20th century with the detection of eels in karst poljes (Curčić, 1916). Research conducted on the Timavo River (Slovenia, Italy) confirmed the underground migrations of eels. A. anguilla was also detected in other karst poljes – Mostarsko blato



Fig. 1: Literature data on A. anguilla presence in the last 120 years in the Croatian Adriatic.

(230 m.a.s.l.), Imotsko blato (150 m.a.s.l), Popovo polje (300 m.a.s.l.) (Ćurčić, 1916; Dojmi, 1939).

Materials and Methods

At present, despite its critically endangered species status, the efforts for its protection, as well as its commercial importance, surprisingly little effort was put into studying the ecology and distribution of *A. anguilla* in Croatia and in the Adriatic region in general. Most of the published research has been focused on the length-weight relationship (e.g., Dulčić & Glamuzina, 2006; Piria *et al.*, 2014; Castadelli *et al.*, 2014), otoliths (Kanjuh *et al.*, 2018; Milošević *et al.*, 2021), parasites (Di Cave *et al.*, 2007). The majority of data on the presence of *A. anguilla* in the Croatian Adriatic is collected from the studies which aim at other freshwater or marine species, where the eel was detected as a bycatch.

The study we present here is the first eDNA study conducted in the broader Adriatic region, with the aim of monitoring eel populations in this area of major ecological importance. Our field sampling was carried out with consideration of the historical data on the presence of the European eel, and our aim was to be as exhaustive as possible. The laboratory protocol was optimized, following the scale of Thalinger *et al.* (2021), resulting in robust, specific, and highly sensitive results for our target species. In this study, we report the first data on the presence of European eel DNA, thanks to an effective, non-disruptive, and reproducible method, in the context of future monitoring studies of these endangered eel populations.

Sampling sites

Sampling was conducted during ten days in September of 2021 on islands and one week in December of 2021 in the Neretva River delta. It included a total of 24 locations - fifteen on the four islands of the Zadar County (Pag, Ugljan, Pašman, Dugi otok), and nine localities in the Neretva River Delta (Table 1, Fig. 2). The sampling localities were selected considering the available literature data on *A. anguilla* in Croatia, the existing data on wet habitats on the chosen islands, unpublished data, and the habitats that correspond to the ecology of the target species.

Sample collection

To avoid potential field cross-contamination, filter housing and tubing were totally disinfected in a 10% bleach (sodium hypochlorite) solution for 20 minutes after each filtration step, then transferred and thoroughly rinsed using tap water to eliminate the bleach. Though tap water is not sterile, it can be assumed that it is completely free from eel DNA, single-targeted species in this research, thus not affecting possible cross-site contamination.

After collecting water from the water bodies, filtration took place on-site using an electric vacuum pump

| No. | Locality | Coordi- Locality nates Habitat type Detec | | Detection | Proportion of qPCR positive replicates | Mean Ct Values ± SD | |
|-----|----------------------------------|--|-----------|--------------------------|---|---------------------------|------------------|
| | | Longitude | Latitude | | | | |
| 1 | Dugi otok, Velo jezero | 15.10932 | 43.941757 | Freshwater | - | 0 | - |
| 2 | Dugi otok, pond | 15.10992 | 43.962694 | Freshwater | - | 0 | - |
| 3 | Pašman, Jelenići | 15.237878 | 44.015086 | Marine | - | 0 | - |
| 4 | Pašman, Barotul | 15.362688 | 43.964398 | Marine | + | 1 | 31.94 ± 0.49 |
| 5 | Ugljan, M. Lukoran | 15.165024 | 44.096911 | Brackish | + | 1 | 30.54 ± 0.45 |
| 6 | Ugljan, pond in the Vela Lamjana | 15.197886 | 44.051487 | Brackish | - | 0 | - |
| 7 | Ugljan, Vela Lamjana | 15.198543 | 44.05038 | Marine | + | 0.67 | 35.32 ± 0.76 |
| 8 | Pag, Solana Pag (saltern) | 15.07988 | 44.416991 | Marine, hyper- saline | + | 0.67 | 35.3 ± 0.66 |
| 9 | Dugi otok, pond Dugo polje | 15.117474 | 43.93644 | Freshwater | - | 0 | - |
| 10 | Dugi otok, lake Mir | 15.162003 | 43.889349 | Marine, hyper- saline | - | 0 | - |
| 11 | Dugi otok, Malo jezero | 15.101682 | 43.948398 | Freshwater | - | 0 | - |
| 12 | Pag, Velo Blato | 15.151155 | 44.351151 | Slightly brackish | - | 0 | - |
| 13 | Pag, Sega lagoon | 15.096664 | 44.355539 | Marine | + | 1 | 28.9 ± 0.54 |
| 14 | Pag, Malo blato mouth | 15.114092 | 44.369691 | Marine | - | 0 | - |
| 15 | Pag, Kolansko blato | 14.918247 | 44.514459 | Brackish | + | 1 | 33.26 ± 0.49 |
| 16 | Mliništa | 17.615854 | 42.992206 | Freshwater | + | 1 | 31.21 ± 0.8 |
| 17 | Podolac | 17.658710 | 43.046335 | Freshwater | + | 1 | 33.95 ± 1.09 |
| 18 | Bađula, karst spring | 17.610419 | 42.962383 | Freshwater, spring | + | 0.89 | 34.34 ± 0.71 |
| 19 | Bijeli Vir | 17.653863 | 43.012689 | Freshwater | - | 0 | - |
| 20 | Sv. Mihovil | 17.629369 | 43.000556 | Freshwater | + | 1 | 33.47 ± 0.67 |
| 21 | Bijeli Vir, karst spring | 17.654834 | 43.010184 | Freshwater, spring | + | 0.67 | 34.68 ± 1.84 |
| 22 | Glušci, karst spring | 17.679228 | 43.016968 | Freshwater | - | 0 | - |
| 23 | Čekrk, karst spring | 17.673341 | 43.019067 | Freshwater | + | 0.89 | 34.69 ± 1.15 |
| 24 | Bijeli Vir, main | 17.656233 | 43.009189 | Freshwater | + | 0.44 | 34.58 ± 0.82 |

Table 1. Eel sampling locations, positive or negative detection, and habitat type. Island locations are marked with grey cells (1-15); and the rest are locations from the Neretva River Delta (16-24).



Fig. 2: Localities included in this study. Red triangles represent localities with no detection of *A. anguilla*, green triangles represent localities where *A. anguilla* was detected.

(Rocker Lafil300 OilFree Pump), as well as a 1L filtering unit (NalgeneTM) and nitrocellulose filters (Sartorius® 47 mm diameter and 0.45 µm size pore) (Lawson Handley et al., 2019). Surface water samples were sampled in three different ways, depending on the size of the water body: i) for small water bodies sampling was performed from the bank, ii) for medium water bodies it was done by entering the water body using plastic waders, and iii) for large water bodies it was done by using a small rubber boat. Between every sampling site, all equipment was disinfected using 10% bleach and 96% ethanol. Samples were collected using a decontaminated plastic bottle, thoroughly rinsed by submerging them into the water just before the effective sampling, and wearing non-powdered gloves. Following the filtration process, the filter was removed and folded in quarters into a 1.5 mL tube using sterile forceps. The filters were stored in 1 mL of 100% molecular-grade ethanol, in a cooler box, until it was returned to the laboratory. Three independent natural replicates, each consisting of 1L of filtered water, were collected at each sampling location.

DNA extraction

DNA extraction from tissue

Muscle tissue from a live specimen caught in the Neretva River, Croatia, was used for genomic *A. anguilla* DNA extraction, which was done following the manu-

facturer's guidelines in the Qiagen DNeasy® Blood & Tissue Kit.

Environmental DNA extraction

To avoid contamination, extractions were performed in a separate sterile laboratory, different from the one in which the preparation of the qPCR mixture took place. Half of each filter was cut with sterilized tweezers and scissors, and dried for thirty minutes permitting the ethanol to evaporate from the filter. After this procedure, one half of the filter was further cut into small pieces and placed in a new 2 mL Eppendorf tube. The Qiagen DNeasy® Blood & Tissue Kit for DNA extraction was used, following the manufacturer's guidelines with slight modifications as shown in Baudry et al. (2021). The modifications were as follows, 450 µL of ATL buffer and 50 µL of Proteinase K were added to the fragment filter tube, vortexed for 15 seconds, and incubated at 56°C overnight. Then, 500 µL of AL buffer and 500 µL of 100% ethanol were added. The remaining steps were performed following the manufacturer's protocol. The extracted DNA was stored at -20°C until further analysis.

qPCR primers and probe specificity

Species-specific primers and probes used in this study were those developed by Weldon *et al.* (2020), tar-

geting a cytochrome *b* region of *A. anguilla* (Forward: Aangcytb1F 5'- TTGCCCTATTCTACCCGAACC-3', Reverse: Aangcytb1R 5'- ACAAGGCTAATACCCCG-CC-3' and specific-fluorescent labelled probe: Aangcytb1P 5'- TTGGAGACCCAGACAACTTCACCCCG-GCA-3'). The specificity of the primers was determined *in silico* using the primer-BLAST tool.

In vitro tests have already been carried out by Weldon *et al.* (2020) on 17 species, and they indicate the absence of amplification in these taxa (Table 2), attesting to the specificity of the primers for *A. anguilla*. Due to different biotic contexts in the Croatian Adriatic, especially in terms of faunal composition, we performed *in vitro* tests with 5 new species found in Croatian freshwaters, to test the specificity of the primers (Table 2). The qPCR parameters (primer and probe concentrations, and annealing temperature) used are those described and optimized in Weldon *et al.* (2020), providing good amplification yields.

qPCR treatments

For the detection of A. anguila DNA, we performed real-time PCR using the primers Aangevtb1F (5'-TTGCCCTATTCTACCCGAACC-3') and Aangcytb1R (5'- ACAAGGCTAATACCCCGCC-3'), and a fluorescently labeled probe Aangcytb1P (5'-FAM-TTGGAGACCCAGACAACTTCACCCCGGCA-BHQ1-3'), designed by Weldon et al. (2020). All oligonucleotides were manufactured by Macrogen Europe. Each natural replicate (i.e., water sample) was analyzed in three technical/qPCR replicates, providing nine replicates per sampling station. Each qPCR reaction contained 10 µl of GoTaq® Probe qPCR Master Mix (Promega, USA), 0.2 µM of each primer, a 0.1 µM probe, 5 µl of DNA, and water up to 20 µl. Thermocycling and detection was performed on a qTower³ (Analytik Jena, Germany), with the following protocol: denaturation at 95°C

Table 2. List of fish species used to test primer specificity.

for 2 min, followed by 40 cycles of 95°C for 15 s and 59 °C for 1 min. Each assay included three replicates of the positive control – *A. anguilla* genomic DNA (extracted from *A. anguilla* muscle tissue as described above) and three replicates of 10x diluted positive control. Three replicates of the negative control (H_2O) were also included in each assay. Field positive controls weren't performed, since the sampling referred to past known presence data. Field negative controls were not performed as well, since the sampling protocol was based on published methodologies (e.g., Baudry *et al.*, 2021; Dubreuil *et al.*, 2022), showing the effectiveness of the disinfection protocol used in this study.

Positive signals were considered when a C_t value (cycle threshold; the value defining positive and negative amplifications) below 36 at a site was defined as "harboring *A. anguilla*", as well as if at least one replicate of the nine (per station) was positive (following Weldon *et al.*, 2020). This threshold for positive results is validated and used in many published studies (Bedwell & Goldberd, 2020). It can be assumed that this amount of eDNA is low, however, we have taken a more cautious reading of our results. Most studies consider a positive qPCR result for a Ct value of <45 (e.g., Bedwell & Goldberg, 2020) or <42 (Agersnap *et al.*, 2017), but we have chosen to lower the limit to Ct <36 in this study, thus attesting to the robustness of our results.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.*, 2009). For that purpose, standard dilutions (of known concentrations) were done from an *A. anguilla* DNA extract (53,58 ng μ L⁻¹, measured using Nanodrop Spectrophotometer), which were then treated in 10 replicates by

| Species | Source | qPCR results | |
|-----------------------------|-----------------------------|--------------|--|
| A. anguilla | this study | + | |
| A. anguilla | Weldon et al. (2020) | + | |
| Alburnus alburnus | this study | - | |
| Rutilus rutilus | this study | - | |
| Phoxinus lumaireul | this study | - | |
| Squalius illyricus | this study | - | |
| Salmo faroides | this study | - | |
| Scardinius erythrophthalmus | Weldon et al. (2020) | - | |
| Petromyzon marinus | Weldon <i>et al.</i> (2020) | - | |
| Perca fluviatilis | Weldon et al. (2020) | - | |
| A. rostrata | Weldon et al. (2020) | + (weak) | |

qPCR. LOD corresponds to the lowest concentration at which organismal DNA can be detected by qPCR and LOQ corresponds to the lowest concentration at which targeted DNA can be quantified. Our qPCR results were modeled following Klymus *et al.* (2019), with slight modifications, as our concentrations were measured in ng μ L⁻¹. The model was performed with the "*Best*" parameter for *LOD.FCT* and *LOQ.FCT* functions, and 0.7 for the *LOQ.threshold* function.

Data analysis

In this paper, all older literature data available was analyzed, together with recent data gathered by the author and colleagues, in the period from 2010 to 2021. All field data (sites, coordinates, and habitat) and lab results (qPCR positive replicates and mean Ct values \pm SD) were compiled in Table 2. All data was further analyzed in QGIS v. 3.26 software (QGIS Development Team, 2022) to plot maps, both for the historic records of *A. anguilla* in Croatia and for detection based on the eDNA method from the current study.

Rstudio V1.1.463 (Core Team R Development, 2019) was used to perform statistical analyses and modelling. The Shapiro-Wilk normality test and the Bartlett homogeneity test were used to verify the normal and homogeneous distribution of the data (p > 0.05). Lastly, the effect of habitat type on detection efficiency and sensitivity was investigated with a one-way ANOVA.

Results

qPCR assays

In silico tests performed using the alignment primer-BLAST tool showed no risk of cross-amplification with non-target species and other closely related Anguillid species. Primers and the probe experiment set showed 100% specificity for *A. anguilla* and the best hit for another species is 96% specificity for *Crenicichla lepidota*, a cichlid species native to South America and absent from the Adriatic and the Mediterranean Sea.

In vitro testing confirmed this high specificity, with no DNA amplification of co-occurring species in Croatia (Table 1). Only a weak amplification for the American eel (A. rostrata) (Weldon et al., 2020) occurred, but it does not pose a problem for the specificity of the tested primers and the probe, since this species, like the aforementioned C. lepidota, is a priori absent in Europe.

The qPCR assays show a high sensitivity, with LOD and LOQ corresponding respectively to concentrations of $5.1 \times 10^{-4} \text{ ng.} \mu \text{L}^{-1}$ and $8.1 \times 10^{-4} \text{ ng.} \mu \text{L}^{-1}$ (p-value < 0.05).

In-situ detection and habitat effect

The European eel was detected by the environmental DNA-based method in 13 out of the 24 studied localities,

with an amplification rate ranging from four to nine, out of the nine technical replicates (Table 2, Fig. 2). qPCR sensitivity (mean Ct values) for these positive stations ranged from 28.9 ± 0.54 , with a proportion of qPCR positive replicates reaching 100% (for Pag, Sega lagoon; Marine habitat), to 35.32 ± 0.76 , with a proportion of qPCR positive replicates reaching 67% (for Ugljan, Vela Lamjana; Marine habitat). Interestingly, *A. anguilla* was detected in six localities at three Adriatic islands: Pag, Pašman, and Ugljan, but not at Dugi otok Island. Eel DNA was detected in seven out of the nine sampled localities in the Neretva River Delta (Table 2, Fig. 2).

Interestingly, the qPCR results show that *A. anguilla* can be detected in all prospected habitats, i.e., fresh water, marine water, and brackish water (Table 2). Eel eDNA was detected at two out of the four brackish sampling sites, seven out of the 13 freshwater sampling sites, and four out of the seven marine sampling sites.

Ratios of positive qPCR replicates reach 0.5 ± 0.58 , 0.45 ± 0.46 , and 0.48 ± 0.47 for brackish, freshwater, and marine habitats, respectively. No significant difference was found in this detection probability, but the sensitivity difference was significant (mean Ct values). The eel eDNA seemed to be significantly more frequently detected in marine and brackish habitats (32.11 ± 2.67 Ct and 31.9 ± 1.47 Ct) than in freshwater (33.6 ± 1.57 Ct) (F = 7.67; p-value < 0.001) (Fig. 3).

Discussion

Environmental DNA application

The use of eDNA as a tool for monitoring A. anguilla presence in Croatia was found to be accurate and reliable. We used the eDNA identification setup (primers and probe) developed by Weldon et al. (2020) to detect A. anguilla in Ireland. In the latter study, Weldon et al. (2020) have proven the specificity of the primers and the probe for the A. anguilla species through in silico and in vitro testing on co-occurring species, without any match possibility with them. In our study, we performed additional tests to prove the specificity of the primers and the probe for applications in Croatia, where other species inhabit naturally. In silico and in vitro tests showed that the qPCR assay developed by Weldon et al. (2020) for the detection of A. anguilla is species-specific enough to be also applied in the Croatian Adriatic, and LOD calculations show a high sensitivity of the method.

At all sites, sampling was conducted on surface waters. Although Burgoa Cardás *et al.* (2020) detected higher proportion of positive DNA amplifications in the bottom than in the surface water samples, other studies showed no difference between surface and subsurface water, even for benthic species (Hinlo *et al.*, 2017; Forsström & Vasemägi, 2016). Furthermore, Weldon *et al.* (2020) also sampled surface water for the detection of *A. anguilla* and confirmed this method to be effective. The results of this study show that *A. anguilla* eDNA can be successfully recovered and amplified from freshwater, brackish, and



Fig. 3: Influence of habitat types, brackish, freshwater, and marine, on detection sensitivity (Ct values). The annotations (***) highlight a significant difference between the groups.

marine habitats. The qPCR results seem to highlight an effect of the habitat on detection by eDNA, especially on detection sensitivity (Mean Ct value). It seems that detection is less effective in freshwater than in brackish and marine habitats. However, this can be a consequence of the eel's ecological preferences, the sampling sites, as well as the characteristics of environmental DNA. First, the eel is a catadromous species, adapted to both freshwater and marine water, and therefore, its presence can be detected in the entire basin, from upstream to downstream reaches of rivers and their estuaries. This makes the sampling area very large and, depending on seasonal variations, the species may be absent from certain localities within the sampling systems (e.g., upstream/freshwater). Second, the sampling conducted here represents nearly twice as many sites sampled in fresh water as those in brackish or marine water. This inevitably impacts the detection efficiency of the species, due to prospecting areas where the eel has never been detected, and therefore the probability of detection will be reduced in freshwater areas. Finally, eDNA is well-known to persist in the environment and diffuse downstream, sometimes up to several tens of kilometers (Deiner & Altermatt, 2014). In this case, eDNA will eventually end up in the downstream zones (brackish or estuarine/marine), well known to be the final recipient of the entire hydrographic flow. Nevertheless, the sampling was carried out in a thoughtful way, with points distributed from the upstream (freshwater) to the transition zones (brackish), then marine, thus giving precise and robust location data for A. anguilla. Thus, our study represents the only application of eDNA for research and monitoring purposes in Croatia, after the study on the olm, Proteus anguinus (Vörös et al., 2017). It is the first eDNA research of any fish species in Croatia,

Medit. Mar. Sci., 24/2 2023, 364-376

the first eDNA monitoring of *A. anguilla* in the Adriatic Sea and, to our knowledge, the first one in the Mediterranean region. Burgoa Cardás *et al.* (2020) applied eDNA analysis for monitoring the European eel in Spain, however, monitoring was performed in the rivers of the Atlantic drainage basin. Additionally, our study is the first one investigating the presence of the European eel in the Adriatic basin, a species of major ecological importance for which many conservation plans have been deployed around the world.

Detected eel populations

Conventional research methods using electrofishing did not prove to be very effective for detecting eels in the coastal lagoon habitats of the Adriatic Islands, since these are unique habitats with very high salinity, which makes electrofishing either inefficient or impossible. On the other hand, even in freshwater systems, electrofishing has certain limitations that affect its efficiency due to electrical conductivity, turbidity, habitat complexity, fish size, and species (Lieschke et al., 2019). Additionally, efficiency of electrofishing decreases with the increase in the width and depth of the stream, causing considerable variation in effectiveness, especially in large lowland systems (Pottier et al., 2020; Lyon et al., 2014). Using eDNA as an alternative to electrofishing sampling, even for freshwater fish communities; it is already established to be more effective than the traditional methods (Mc-Coll-Gausden et al., 2021). In some localities in this research (e.g., 12 and 15 in Table 1), the surface layer of the water body is freshwater (approx. 50 cm), but below that is salt water, where electrofishing is inefficient. Eels

are usually hidden in the bottom layer, under rocks and vegetation, or buried in the mud (Froese & Pauly, 2021). Hand nets are ineffective during the day when the eels are hiding, and even if spotted they are very difficult to catch. Light traps could also be used, but some problems still remain, similar to hand nets and pull nets. De Graaf et al. (2010) discuss that the optimal positioning of light traps is still unknown, and their biggest problem is zero catches, demonstrating that light traps are not a suitable alternative. In addition to all this, it is necessary to consider that eel populations in these locations are expected to be smaller, since these are not their most optimal habitats, like river estuaries and lakes (Moriarty, 2003). However, ideal habitats appear to be strongly linked to areas with extensive freshwater-saltwater mixing. Some modest differences in eel detection among sample sites might be explained by the migratory behavior of eels and their environmental preferences. When glass eels arrive from the Sargasso Sea, they spend more time near a river's mouth than in the estuary (Harrison et al., 2014). Due to the mixing of saltwater and freshwater (brackish waters) in some Adriatic islands (especially Pag, Pašman, and Ugljan), the presence of the eel might be explained by this environmental condition. Furthermore, the detection of the European eel on these islands and its lack of detection on the Dugi Otok Island can be explained by the geography of these locations. The islands Pag, Pašman, and Ugljan are all positioned closer to the mainland and close to the Zrmanja River estuary, making them the most convenient locations for eels to migrate. The eel development period in continental waters ends with the silvering process, after which A. anguilla begins migrating to marine waters. Migrating silver-stage A. anguilla, like other diadromous fish, pass through fertile estuarine ecosystems with vast populations of birds, mammals, and other fish predators. Predation pressure on migratory fish in such areas may be considerable and it is known that cormorants (Phalacrocorax sp.), a frequent bird species in estuary habitats, prey substantially on smaller A. anguilla specimens (Keller, 1995; Barry et al., 2016). Sites 6 and 7 on Ugljan Island, although geographically close, are physically separated from one another. Site 6 is a pond divided from the sea, and it is connected to it only during high sea water (storm tides, low pressure atmospheric systems, etc.). So, this result indicates that there is no underground connection either, since the eels were not detected. On the other hand, Site 7 is a lagoon directly connected to the open sea waters, which makes it an easily accessible and a suitable locality for the eels. Therefore, smaller island habitats, such as aforementioned coastal lagoons and lakes, are suitable for eels escaping predatory pressure. Furthermore, it seems that A. anguilla is not present in habitats which are under heavy anthropogenic pressure. Site 3 on Pašman Island is positioned in the area of frequent ferry and catamaran lines (Zadar-Zaglav, Zadar-Sali, Zadar-Bršanj-Mala Rava, Zadar-Rava, etc.) with beaches attractive to tourists, and the eels were not detected there, indicating that heavy anthropogenic pressure makes this habitat not suitable for the eels.

On the other hand, A. anguilla is a common species

in the Neretva River Delta and eel DNA detection is not surprising. The detection of eel DNA in the upper parts of this area, and outside of the main course of the river, is unsurprising as well, since it is known that eels migrate far upstream in the Neretva River, up to 100 kilometers inland, e.g., to Hutovo Blato, Bosnia and Herzegovina (Has-Schön et al., 2008; Đeđibegović et al., 2012). Burgoa Cardás et al. (2020) detected eels in the upstream parts of rivers during different sampling seasons (November, February, April, July). However, in the lower parts of rivers, eels were not detected in the November sampling. According to this, we detected A. anguilla at almost all sampling sites in the Neretva River (7 out of 9), positioned upstream from the river mouth. Furthermore, sampling for this research was performed during early December, which is the period of the early entry season of the glass eel (Burgoa Cardás et al., 2020).

Additionally, eel DNA detection in the karst springs (localities 18 and 21 in Table 1) was also expected, since A. anguilla uses underground corridors in its upstream migration (Dojmi, 1939). This is the reason why it can be found in karst poljes that have no direct surface water connection to the Adriatic Sea, such as Imotsko, Mostarsko, Popovo, and Dabarsko polje, positioned 150, 230, 300, and 550 meters above sea level (m.a.s.l.), respectively. (Ćurčić, 1916; Dojmi, 1939). It is also known that the eels migrate up to 100 kilometers upstream in the Neretva River, with maximum gained elevation of 640 m.a.s.l. (Curčić, 1916). Interestingly, sites 22 and 23 are geographically close karstic springs, but the eel has been detected in only one of them. The main problem with underground karstic water is that we usually cannot determine where the water comes from. Even though springs, sinkholes, or estavelles can be geographically really close, often they do not share the same water, due to different underground connections from those on the ground itself (Bonacci, 1999; Bonacci, 2015; Bonacci et al., 2013; Bonacci & Andrić, 2008; Palandačić et al., 2012).

Remarks on conservation

The European eel is an important species worldwide, both in fisheries and in aquaculture (Violi et al., 2015). This critically endangered species faces imminent and drastic population decline, with its current population at its all-time low (Pike et al., 2020). Management plans for the eels are being developed in order to protect and restore their populations through the reduction in anthropogenic mortalities and by enabling a high probability of escapement to the sea. Even though the implementation of management measures has shown certain improvement, the impact of those measures is still not adequate and the European eel remains a critically endangered species included in the last IUCN assessment (Pike et al., 2020). Another main problem is the limited understanding of the complex relationship between recruitment, the growth phase, and the escapement of eels, due to the lack of data on ecology and distribution of the species, especially in its southern range of distribution. Filling these

gaps will make management plans more efficient and allow a more comprehensive assessment of this species (Pike et al., 2020). Adriatic Sea harbors a large part of the A. anguilla stock, as it can be found in all Adriatic rivers. Furthermore, the presence of eels in bodies of water across the Croatian Adriatic islands highlights the importance of these habitats for its migrating silver-stage. These habitats can play an important role for the migration paths of the species and as a refuge from predators. In order to implement the Eel Regulation Act and design efficient Ecological Management Plans (EMPs), considerably more data is needed, especially on the distribution of this species. Once more, this study has demonstrated the efficiency of eDNA as a powerful tool in detecting rare and elusive species like A. anguilla. Similar to previous studies (Hänfling et al., 2016; Weldon et al., 2020; Burgoa Cardás et al., 2020), our study also suggests that this strategy might be a long-term solution for the detection and monitoring of anguillid species in their natural habitats. The application of eDNA provides the opportunity to asses populations and habitats which are important for European eels in the Adriatic Sea, to act fast in order to protect them, and to enhance eel stock recruitment in the long run.

Finally, it is necessary to emphasize the importance of this method due to its application in karstic waters. Eels use underground pathways regularly to enter and travel far inland (Ćurčić, 1916; Dojmi, 1939). This is also confirmed by the findings of eel DNA in Gacko polje (Croatia) (Fig. 1), where the only connection with the Adriatic Sea is through several sinkholes and underground waterways below the Velebit mountains. Similar situations can be observed in other karst poljes in Croatia, such as Ličko, Vrgoračko, Imotsko, and Sinjsko. As a result, the eDNA approach is also critical for detecting this species in karstic waters and for determining significant inland habitats and migration pathways.

Acknowledgements

This study was performed through the research project of BIOTA Ltd. and was partly financed by the WWF Adria and the Public Institution for the Management of Protected Areas in the Dubrovnik-Neretva County, Croatia. The authors declare that they have no known conflicts of interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Archiving Statement: The data supporting the findings of this study will be available on the Zenodo open digital repository. **Author contribution**: MV: Conceptualization, Methodology, Formal analysis, Writing - original draft. DJ: Methodology, review & editing, Conceptualization, Project administration, Funding acquisition. GK: Methodology, review & editing. ŽP: Methodology, Formal analysis, review & editing. LMH: Methodology, review & editing. MJ: review & editing, Project administration, Funding acquisition. GK: Methodology, review & editing. LMH: Methodology, review & editing. MJ: review & editing, Project administration, Funding acquisition. TB: Review & editing, Modelling. AG: Methodology, review & editing.

References

- Aalto, E., Capoccioni, F., Terradez Mas, J., Schiavina, M., et al., 2016. Quantifying 60 years of declining European eel (Anguilla anguilla L., 1758) fishery yields in Mediterranean coastal lagoons. ICES Journal of Marine Science, 73, 101-110.
- Agersnap, S., Larsen, W.B., Knudsen, S.W., Strand, D., Thomsen, P.F. et al., 2017. Monitoring of noble, signal and narrow-clawed crayfish using environmental DNA from freshwater samples. *PloS One*, 12(6), e0179261.
- Amilhat, E., Fazio, G., Simon, G., Manetti, M., Paris, S., et al., 2014. Silver European eels health in Mediterranean habitats. Ecology of Freshwater Fish, 23, 49-64.
- Anonymous, 2007. Council Regulation (EC) No 1100/2007 of 18 September 2007 establishing measures for the recovery of the stock of European eel. Official Journal of the European Union L 248/17. https://eur-lex.europa.eu/legal-content/ EN/TXT/?uri=celex%3A32007R1100 (Accessed 8 December 2021).
- Badamasi, I., Odong, R., Masembe, C., 2020. Threats posed by xenoestrogenic chemicals to the aquatic ecosystem, fish reproduction and humans: a review. *African Journal of Aquatic Science*, 45 (3), 243-258.
- Barry, J., Newton, M., Dodd, J. A., Lucas, M.C., Boylan, P. et al., 2016. Freshwater and coastal migration patterns in the silver-stage eel Anguilla anguilla. Journal of Fish Biology, 88, 676-689.
- Baudry, T., Mauvisseau, Q., Goût, J.P., Arqué, A., Delaunay, C. et al., 2021. Mapping a super-invader in a biodiversity hotspot, an eDNA-based success story. *Ecological Indicators*, 126, 107637.
- Bedwell, M.E., Goldberg, C.S., 2020. Spatial and temporal patterns of environmental DNA detection to inform sampling protocols in lentic and lotic systems. *Ecology and Evolution*, 10, 1602-1612.
- Belpaire, C., Hodson, P., Pierron, F., Freese, M., 2019. Impact of chemical pollution on Atlantic eels: Facts, research needs, and implications for management. *Current Opinion in Environmental Science & Health*, 11, 26-36.
- Bevacqua, D., Melià, P., Crivelli, A. J., Gatto, M., De Leo, G.A., 2009. Assessing management plans for the recovery of the European eel: a need for multi-objective analyses. *American Fisheries Society Symposium*, 69, 637-647.
- Bonacci, O., 1999. Water circulation in karst and determination of catchment areas: example of the River Zrmanja. *Hydrological Sciences Journal*, 44, 373-386.
- Bonacci, O., 2015. Surface waters and groundwater in karst. p. 149-169. In: *Karst aquifers—characterization and engineering*. Stevanović, Z. (Ed.). Springer International Publishing, Switzerland.
- Bonacci, O., Andrić, I., 2008. Karst rivers hydrology: case of the Lika and Gacka (Croatia). Acta carsologica, 37 (2-3), 185-196.
- Bonacci, O., Željković, I., Galić, A., 2013. Karst rivers' particularity: an example from Dinaric karst (Croatia/Bosnia and Herzegovina). *Environmental earth sciences*, 70, 963-974.
- Bustin, S.A., Vandesompele, J., Pfaffl, M., 2009. Standardization of qPCR and RT-qPCR. *Gen Eng Biotechnol News* 2009b, 29.

- Burgoa Cardás, J.B., Deconinck, D., Márquez, I., Torre, P.P., Garcia-Vazquez, E. *et al.*, 2020. New eDNA based tool applied to the specific detection and monitoring of the endangered European eel. *Biological Conservation*, 250, 108750.
- Castadelli, G., Aschonitis, V., Lanzoni, M., Gelli, F., Rossi, R. et al., 2014. An update of the length–weight and length–age relationships of the European eel (Anguilla anguilla, Linnaeus 1758) in the Comacchio Lagoon, northeast Adriatic Sea, Italy. Journal of Applied Ichthyology, 30 (3), 558-559.
- Cataudella, S., Crosetti, D., Ciccotti, E., Massa, F., 2014. Sustainable management in Mediterranean coastal lagoons: Interactions among capture fisheries, aquaculture and environment. p. 7-49. In: *Mediterranean coastal lagoons: sustainable management and interactions among aquaculture, capture fisheries and environment.* S. Cataudella, D. Crosetti, F. Massa (Eds.). General Fisheries Commission for the Mediterranean. Studies and reviews, N. 95. FAO, Rome.
- Capoccioni, F., Leone, C., Belpaire, C., Malarvannan, G., Poma, G. *et al.*, 2020. Quality assessment of escaping silver eel (*Anguilla anguilla* L.) to support management and conservation strategies in Mediterranean coastal lagoons. *Environmental Monitoring and Assessment*, 192, 1-22.
- Core Team R Development, 2019. R: a language and environment for statistical computing.
- Cresci, A., 2020. A comprehensive hypothesis on the migration of European glass eels (*Anguilla anguilla*). *Biological Reviews*, 95, 1273-1286.
- Ćurčić, V., 1916. Narodno ribarstvo u Bosni i Hercegovini: II. Hercegovina. (in Croatian) [Folk fishing in Bosnia and Herzegovina: II. Herzegovina]. Zemaljska Štamparija, Sarajevo, Bosnia and Hercegovina, 208 pp.
- de Graaf, M., van der Heul, J.W., van Willigen, J. A., Leijzer, T.B., 2010. *The use of light traps in monitoring abundance of glass eel.* No. C167/10, IMARES, 16 pp.
- Deiner K., Altermatt F., 2014. Transport Distance of Invertebrate Environmental DNA in a Natural River. *PLoS ONE*, 9, 2, e88786.
- Dekker, W., 2000. The fractal geometry of the European eel stock. *ICES Journal of Marine Science*, 57, 109-121.
- Dekker, W., 2003. On the distribution of the European eel and its fisheries. *Canadian Journal of Fisheries and Aquatic Sciences*, 60, 787-799.
- Dekker, W., 2016. Management of the eel is slipping through our hands! Distribute control and orchestrate national protection. *ICES Journal of Marine Science*, 73, 2442-2452.
- Dekker, W., Beaulaton, L., 2016. Climbing back up what slippery slope? Dynamics of the European eel stock ad its management in historical perspective. *ICES Journal of Marine Science*, 73, 5-13.
- Dezfuli, B.S., Giari, L., Castaldelli, G., Lanzoni, M., Rossi, R. et al., 2014. Temporal and spatial changes in the composition and structure of helminth component communities in European eels Anguilla anguilla in an Adriatic coastal lagoon and some freshwaters in Italy. Parasitology Research, 113, 113-120.
- Di Cave, D., Berrilli, F., De Liberato, C., Orecchia, P., Kennedy, C.R., 2001. Helminth communities in eels Anguilla anguilla from Adriatic coastal lagoons in Italy. Journal of Helminthology, 75, 7-13.

Dojmi, L., 1939. Podzemne migracije jegulja. (in Croatian)

[Underground migrations of eel]. *Priroda: popularni časopis Hrvatskog prirodoslovnog društva*, 9, 263-269.

- Dubreuil, T., Baudry, T., Mauvisseau, Q., Arqué, A.. Courty, C. et al., 2022. The development of early monitoring tools to detect aquatic invasive species: eDNA assay development and the case of the armored catfish *Hypostomus robinii*. Environmental DNA, 1-14.
- Dulčić, J., Glamuzina, B., 2006. Length–weight relationships for selected fish species from three eastern Adriatic estuarine systems (Croatia). *Journal of Applied Ichthyology*, 22 (4), 254-256.
- Dedibegović, J., Larssen, T., Skrbo, A., Marjanović, A., Sober, M., 2012. Contents of cadmium, copper, mercury and lead in fish from the Neretva river (Bosnia and Herzegovina) determined by inductively coupled plasma mass spectrometry (ICP-MS). *Food Chemistry*, 131, 469-476.
- Ficetola, G. F., Miaud, C., Pompanon, F., Taberlet, P., 2008. Species detection using environmental DNA from water samples. *Biology letters*, 4 (4), 423-425.
- Forsström, T., Vasemägi, A., 2016. Can environmental DNA (eDNA) be used for detection and monitoring of introduced crab species in the Baltic Sea? *Marine Pollution Bulletin*, 109, 350-355.
- Froese, R., Pauly D., 2021. *FishBase*. World Wide Web electronic publication. www.fishbase.org. (Accessed 25 January 2022).
- Glamuzina, L., Dobroslavić, T., 2020. Summer fish migrations in the River Neretva (South-Eastern Adriatic coast, Croatia) as a consequence of salinization. *NAŠE MORE: znanstveni* časopis za more i pomorstvo, 67, 103-116.
- Has-Schön, E., Bogut, I., Rajković, V., Bogut, S., Čačić, M. et al., 2008. Heavy metal distribution in tissues of six fish species included in human diet, inhabiting freshwaters of the Nature Park "Hutovo Blato" (Bosnia and Herzegovina). Archives of Environmental Contamination and Toxicology, 54, 75-83.
- Hänfling, B., Handley, L.L., Read, D.S., Hahn, C., Li, J. *et al.*, 2016. Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. *Molecular Ecology* 25, 3101-3119.
- Harrison, A.J., Walker, A.M., Pinder, A.C., Briand, C., Aprahamian, M.W., 2014. A review of glass eel migratory behaviour, sampling techniques and abundance estimates in estuaries: implications for assessing recruitment, local production and exploitation. *Reviews in Fish Biology and Fisheries*, 24, 967-983.
- Hinlo, R., Furlan, E., Suitor, L., Gleeson, D., 2017. Environmental DNA monitoring and management of invasive fish: Comparison of eDNA and fyke netting. *Management of Biological Invasions*, 8, 89-100.
- Jacoby, D.M.P., Casselman, J.M., Crook, V., DeLucia, M.-B., Ahn, H. et al., 2015. Synergistic patterns of threat and the challenges facing global anguillid eel conservation. *Global Ecology and Conservation*, 4, 321-333.
- Kanjuh, T., Mrdak, D., Piria, M., Tomljanović, T., Joksimović, A. *et al.*, 2018. Relationships of otolith dimension with body length of European eel *Anguilla anguilla* (Linnaeus, 1758) from Adriatic catchment of Montenegro. *Acta Adriatica: International journal of Marine Sciences*, 59, 91-96.
- Keller, T., 1995. Food of cormorants Phalacrocorax carbo

sinensis wintering in Bavaria, southern Germany. Ardea, 83, 185-192.

- Klymus, K.E., Merkes, C.M., Allison, M.J., Goldberg, C.S., Helbing, C.C. *et al.*, 2019. Reporting the limits of detection and quantification for environmental DNA assays. *Environmental DNA*, 1-12.
- Lawson Handley, L., Read, D.S., Winfield, I.J., Kimbell, H., Johnson, H. *et al.*, 2019. Temporal and spatial variation in distribution of fish environmental DNA in England's largest lake. *Environmental DNA*, 1, 26-39.
- Lieschke, J.A., Dean, J.C., Pickworth, A., 2019. Extending the effectiveness of electrofishing to estuarine habitats: Laboratory and field assessments. *Transactions of the American Fisheries Society*, 148, 584-591.
- Lyon, J.P., Bird, T., Nicol, S., Kearns, J., O'Mahony, J. et al., 2014. Efficiency of electrofishing in turbid lowland rivers: implications for measuring temporal change in fish populations. *Canadian Journal of Fisheries and Aquatic Sciences*, 71, 878-886.
- McColl-Gausden, E. F., Weeks, A. R., Coleman, R. A., Robinson, K.L., Song, S. *et al.*, 2021. Multispecies models reveal that eDNA metabarcoding is more sensitive than backpack electrofishing for conducting fish surveys in freshwater streams. *Molecular Ecology*, 30, 3111-3126.
- Miller, M.J., Feunteun, E., Tsukamoto, K., 2016. Did a "perfect storm" of oceanic changes and continental anthropogenic impacts cause northern hemisphere anguillid recruitment reductions? *ICES Journal of Marine Science*, 73, 43-56.
- Milošević, D., Bigović, M., Mrdak, D., Milašević, I., Piria, M., 2021. Otolith morphology and microchemistry fingerprints of European eel, *Anguilla anguilla* (Linnaeus, 1758) stocks from the Adriatic Basin in Croatia and Montenegro. *Science* of The Total Environment, 786, 147478.
- Moriarty, C., 2003. The yellow eel. p. 89-105. In: *Eel biology*. Aida, K., Tsukamoto, K., Yamauchi, K. (Eds). Springer, Tokyo, Japan.
- Mrakovčić, M., Brigić, A., Buj, I., Ćaleta, M., Mustafić, P. *et al.*, 2006. *Red book of freshwater fishes of Croatia*. (in Croatian with English abstract). Ministarstvo kulture, Državni zavod za zaštitu prirode, Zagreb, 253 pp.
- Nielsen, T., Prouzet, P., 2008. Capture-based aquaculture of the wild European eel (*Anguilla anguilla*). *FAO Fisheries Technical Paper*, 508, 141-149.
- Palandačić, A., Bonacci, O., Snoj, A., 2012. Molecular data as a possible tool for tracing groundwater flow in karst environment: example of *Delminichthys adspersus* in Dinaric karst system. *Ecohydrology*, 5(6), 791-797.
- Parolini, M., Binelli, A., Matozzo, V., Marin, M.G., 2010. Persistent organic pollutants in sediments from the Lagoon of Venice - a possible hazard for sediment-dwelling organisms. *Journal of Soils and Sediments*, 10, 1362-1379.
- Piggott, M.P., 2016. Evaluating the effects of laboratory protocols on eDNA detection probability for an endangered freshwater fish. *Ecology and Evolution*, 6, 2739-2750.
- Pinto, M.I., Burrows, H.D., Sontag, G., Vale, C., Noronha, J.P., 2016. Priority pesticides in sediments of European coastal lagoons: a review. *Marine Pollution Bulletin*, 112, 6-16.
- Pike, C., Crook, V. Gollock, M., 2020. Anguilla anguilla. The IUCN Red List of Threatened Species 2020, e.

T60344A152845178.

- Piria, M., Šprem, N., Tomljanović, T., Slišković, M., Jelić Mrčelić, G. *et al.*, 2014. Length weight relationships of the European eel *Anguilla anguilla* (Linnaeus, 1758) from six karst catchments of the Adriatic basin, Croatia. *Croatian Journal of Fisheries: Ribarstvo*, 72, 32-35.
- Pottier, G., Beaumont, W.R., Marchand, F., Le Bail, P.Y., Azam, D. et al., 2020. Electrofishing in streams of low water conductivity but high biodiversity value: Challenges, limits and perspectives. Fisheries Management and Ecology, 27, 52-63.
- QGIS Development Team, 2022. QGIS Geographic Information System. Open Source Geospatial Foundation Project. http://qgis.osgeo.org (Accessed 8 December 2021).
- Riascos-Flores, L., Bruneel, S., Van der Heyden, C., Deknock, A., Van Echelpoel, W. *et al.*, 2021. Polluted paradise: Occurrence of pesticide residues within the urban coastal zones of Santa Cruz and Isabela (Galapagos, Ecuador). *Science of the Total Environment*, 763, 142956.
- Ridanović, L., Ridanović, S., Jurica, D., Spasojević, P., Bijedić, D., 2010. Evaluation of water temperature and dissolved oxygen regimes in River Neretva. *BALWOIS Ohrid*, 2010, 1-8.
- Robinson, C.V., Uren Webster, T.M., Cable, J., James, J., Consuegra, S., 2018. Simultaneous detection of invasive signal crayfish, endangered white-clawed crayfish and the crayfish plague pathogen using environmental DNA. *Biological Conservation*, 222, 241-252.
- Stein, F.M., Wong, J.C., Sheng, V., Law, C.S., Schröder, B. et al., 2016. First genetic evidence of illegal trade in endangered European eel (Anguilla anguilla) from Europe to Asia. Conservation Genetics Resources, 8, 533-537.
- Storelli, M.M., Barone, G., Garofalo, R., Marcotrigiano, G.O., 2007. Metals and organochlorine compounds in eel (*An-guilla anguilla*) from the Lesina lagoon, Adriatic Sea (Italy). *Food Chemistry*, 100, 1337-1341.
- Thalinger, B., Deiner, K., Harper, L.R., Rees, H.C., Blackman, R.C. et al. 2021. A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. *Environmental DNA*, 3, 823-836.
- Thomsen, P.F., Willerslev, E., 2015. Environmental DNA An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, 183, 4-18.
- Vörös, J., Márton, O., Schmidt, B.R., Gál, J.T., Jelić, D., 2017. Surveying Europe's only cave-dwelling chordate species (*Proteus anguinus*) using environmental DNA. *PloS* one, 12, e0170945.
- Violi, L., Falcone, G., De Luca, A.I., Chies, L., 2015. Sustainability European Eel Population: A Statistical Survey on Production, Conservation Status and Market Trends. *Calitatea*, 16 (148), 83.
- Wang, S., Yan, Z., Hänfling, B., Zheng, X., Wang, P. et al., 2021. Methodology of fish eDNA and its applications in ecology and environment. Science of the Total Environment, 755.
- Weldon, L., O'Leary, C., Steer, M., Newton, L. Macdonald, H. et al., 2020. A comparison of European eel Anguilla anguilla eDNA concentrations to fyke net catches in five Irish lakes. Environmental DNA, 2, 587-600.

Appendix A. Historic data on A. anguilla presence in Croatia in the 10x10 km EEA grid.

| 0 | 10kmE460N247 | 4600000 | 2470000 | 33 | 10kmE479N231 | 4790000 | 2310000 |
|----|--------------|---------|---------|----|--------------|---------|---------|
| 1 | 10kmE460N248 | 4600000 | 2480000 | 34 | 10kmE479N232 | 4790000 | 2320000 |
| 2 | 10kmE461N247 | 4610000 | 2470000 | 35 | 10kmE479N235 | 4790000 | 2350000 |
| 3 | 10kmE462N246 | 4620000 | 2460000 | 36 | 10kmE479N236 | 4790000 | 2360000 |
| 4 | 10kmE462N247 | 4620000 | 2470000 | 37 | 10kmE480N230 | 4800000 | 2300000 |
| 5 | 10kmE462N248 | 4620000 | 2480000 | 38 | 10kmE480N231 | 4800000 | 2310000 |
| 6 | 10kmE463N245 | 4630000 | 2450000 | 39 | 10kmE480N232 | 4800000 | 2320000 |
| 7 | 10kmE463N246 | 4630000 | 2460000 | 40 | 10kmE480N233 | 4800000 | 2330000 |
| 8 | 10kmE464N246 | 4640000 | 2460000 | 41 | 10kmE482N228 | 4820000 | 2280000 |
| 9 | 10kmE466N242 | 4660000 | 2420000 | 42 | 10kmE482N229 | 4820000 | 2290000 |
| 10 | 10kmE466N248 | 4660000 | 2480000 | 43 | 10kmE483N229 | 4830000 | 2290000 |
| 11 | 10kmE467N247 | 4670000 | 2470000 | 44 | 10kmE484N229 | 4840000 | 2290000 |
| 12 | 10kmE468N245 | 4680000 | 2450000 | 45 | 10kmE484N232 | 4840000 | 2320000 |
| 13 | 10kmE468N246 | 4680000 | 2460000 | 46 | 10kmE485N229 | 4850000 | 2290000 |
| 14 | 10kmE469N245 | 4690000 | 2450000 | 47 | 10kmE486N228 | 4860000 | 2280000 |
| 15 | 10kmE469N246 | 4690000 | 2460000 | 48 | 10kmE486N229 | 4860000 | 2290000 |
| 16 | 10kmE472N237 | 4720000 | 2370000 | 49 | 10kmE486N230 | 4860000 | 2300000 |
| 17 | 10kmE472N243 | 4720000 | 2430000 | 50 | 10kmE487N228 | 4870000 | 2280000 |
| 18 | 10kmE473N237 | 4730000 | 2370000 | 51 | 10kmE489N228 | 4890000 | 2280000 |
| 19 | 10kmE473N241 | 4730000 | 2410000 | 52 | 10kmE490N228 | 4900000 | 2280000 |
| 20 | 10kmE473N242 | 4730000 | 2420000 | 53 | 10kmE492N224 | 4920000 | 2240000 |
| 21 | 10kmE473N243 | 4730000 | 2430000 | 54 | 10kmE492N225 | 4920000 | 2250000 |
| 22 | 10kmE474N234 | 4740000 | 2340000 | 55 | 10kmE493N221 | 4930000 | 2210000 |
| 23 | 10kmE474N235 | 4740000 | 2350000 | 56 | 10kmE493N224 | 4930000 | 2240000 |
| 24 | 10kmE474N240 | 4740000 | 2400000 | 57 | 10kmE494N223 | 4940000 | 2230000 |
| 25 | 10kmE476N232 | 4760000 | 2320000 | 58 | 10kmE494N224 | 4940000 | 2240000 |
| 26 | 10kmE476N233 | 4760000 | 2330000 | 59 | 10kmE494N225 | 4940000 | 2250000 |
| 27 | 10kmE476N235 | 4760000 | 2350000 | 60 | 10kmE498N220 | 4980000 | 2200000 |
| 28 | 10kmE476N236 | 4760000 | 2360000 | 61 | 10kmE498N221 | 4980000 | 2210000 |
| 29 | 10kmE477N232 | 4770000 | 2320000 | 62 | 10kmE499N220 | 4990000 | 2200000 |
| 30 | 10kmE477N235 | 4770000 | 2350000 | 63 | 10kmE500N219 | 5000000 | 2190000 |
| 31 | 10kmE477N236 | 4770000 | 2360000 | 64 | 10kmE501N219 | 5010000 | 2190000 |
| 32 | 10kmE478N236 | 4780000 | 2360000 | | | | |