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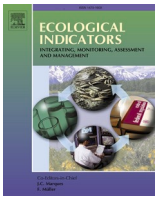

Estimating quality of archive urban stream macroinvertebrate samples for genomic, transcriptomic and proteomic assessment

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ABSTRACT

The existence of archival collections of macroinvertebrates opens the possibility of using these organisms as biological indicators and model organisms for developing indices of aquatic ecosystem health and stress, which provide a powerful basis for sustainable environmental monitoring and management of water resources. The environmental assessment of urbanization influence on urban stream biota over time can include molecular (proteomic, transcriptomic and genomic) analyses of aquatic insects isolated from archive samples. These analyses could include confirmation of detected species by barcoding, determination of the changes in the expression of specific genes and detection of changes in signaling pathways in cells of the studied organisms. Since the preservation of the organisms' macromolecules (DNA, RNA and proteins) is a prerequisite for the implementation of molecular methods, the aim of this study was to assess the quality of macromolecule preservation within archival samples of aquatic macroinvertebrates collected in urban streams during last 28 years. Results indicated that the optimal way for archive storage is 3.6% formaldehyde solution if they have a purpose of morphological assessment or protein analyses, and in absolute ethanol for nucleic acids evaluation. Furthermore, it is of great importance that samples are treated with fixative *in situ* and processed in a laboratory as soon as possible.

1. Introduction

Half of the world's population lives in urban areas, and it is predicted that the continuous population growth will result in an influx of around 2.5 billion people in cities by 2050, meaning that urban population could make up about 66% of the world's population (UN, 2014). The increase of urban population usually results in expansion of urban zones and landscape changes, which further leads to loss and fragmentation of natural habitats (Grimm et al., 2008; Seto et al., 2011). Sustainable coexistence of people and nature in urban areas is possible only if structure and functions of urban ecosystems are not lost. The urban ecosystem features can be detected, understood and forecasted via continuous (i.e., long-term) monitoring of the urban ecosystems' living components (flora and fauna) and their physical, biogeochemical, ecotoxicological and biological interactions and responses to the changes in urban environment (Naeem et al., 1999; Grimm et al., 2000; Filipović Marijić et al., 2016).

Urban streams provide habitats for diverse flora and fauna, they participate in circulation of matter and energy flow in urban ecosystems, and thus indirectly affect the quality of life of urban populations (Sweeney et al., 2004; Walsh et al., 2005). As a degree of urbanization increases, urban streams get degraded, i.e., threatened by channelization, in-stream habitat fragmentation, increase in impervious surface cover, nutrient and toxicant loads, modification of hydrological and biogeochemical cycles, leading to consequent biodiversity losses and ecotoxicological effects on aquatic organisms (Paul and Meyer, 2001; Walsh et al., 2005; Grimm et al., 2008; Seto et al., 2011).

Aquatic macroinvertebrates (i.e., macrozoobenthos including aquatic insect forms) are organisms that play an essential role in stream ecosystems. Being responsible for organic matter processing and serving as a trophic link between detrital and algal food base and fish in the streams, they are of vital importance for stream ecosystem functioning (Cummins, 1974; Anderson and Sedell, 1979). Macroinvertebrates have been widely used as biological indicators and model organisms for developing indices of aquatic ecosystem health and stress, providing a powerful basis for sustainable environmental monitoring and

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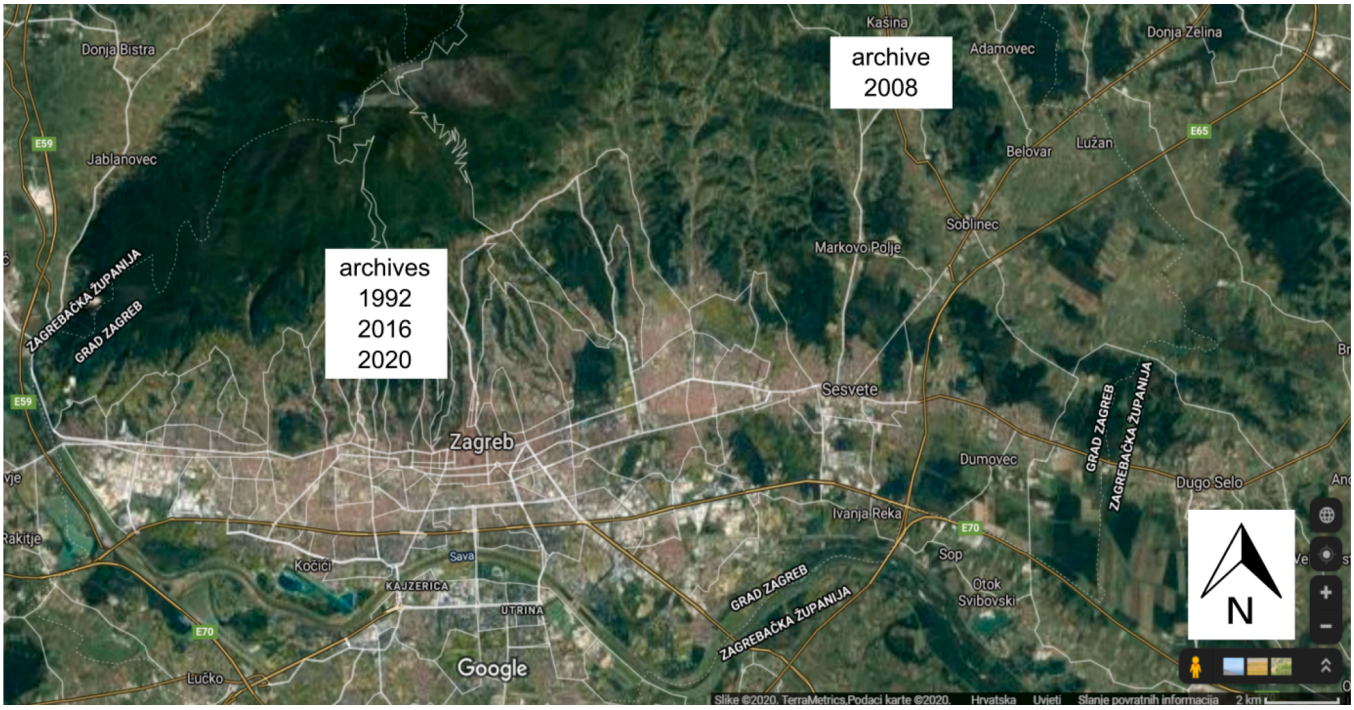

Fig. 1. City of Zagreb satellite map (Google maps application on 4th June 2020). Approximate locations of archive sampling points are marked with the respective year.

management of water resources (Adams, 2002). Some aquatic insect forms are real opportunists showing adaptability, resistance and resilience to environmental change, while others are highly sensitive to any changes in their surroundings (Giller and Malmqvist, 1998). Furthermore, because of their relatively small sizes and limited mobility (in comparison to fish, for example), and relatively long-lasting developmental stages within aquatic environment, aquatic insects can reflect both short and long term shifts in water quality (Giller and Malmqvist, 1998; Uherek and Gouveia, 2014). It all makes them ideal model organisms for capturing various subtle and profound effects of environmental changes on aquatic life (Woodward et al., 2010; Uherek and Gouveia, 2014).

The effects of urban stressors on aquatic biota can be indirectly inferred from the proteomics indicators of early molecular responses of organisms to environmental stressors, as suggested by the relatively novel ecotoxicoproteomics approach (Dowling and Sheehan, 2006; Lemos et al., 2010; Gouveia et al., 2019). This approach can identify protein composition, structure, and activity as well as toxicity pathways and novel biomarkers, not only in the existing model bioindicator organisms (for which critical molecular biomarkers of environmental stress have already been defined), but also in non-model bioindicator organisms with highly divergent phylogenetic backgrounds (Lemos et al., 2010; Gouveia et al., 2019). However, to disclose molecular information, protein features and critical toxicity pathways in the non-model species, it is beneficial to couple the proteomics methods with comparative genomics, which could detect the functional similarities between the non-model and model (most sensitive) species (Gouveia et al., 2019).

We suggest that the assessment of urbanization influence on urban stream biota could greatly benefit from the inclusion of temporally spaced sampling, long-term monitoring strategies and/or proteomic, transcriptomic and genomic analyses of archive samples of aquatic insects. Sequencing DNA from temporally spaced samples can provide information on stressor influences in the past, as time series data allow direct quantification of population genetic parameters collected before, during, and after environmental changes driven by the increased urbanization (cf. Taus et al., 2017; He et al., 2019). This could advance our understanding of the temporal pace and chronology of genomic change driven by increasing urban influence. Archive samples can provide quality evidence of aquatic taxa responses to anthropogenic influences, and if coupled with contemporary sampling, they can provide high-resolution time series of the species' responses to environmental change (cf. Burrell et al., 2015; Lang et al., 2020).

Identifying genetic diversity is straightforward for model species with small genomes and existing reference sequence, while for non-model species - with small or large genomes - restriction-enzyme based sequencing is used (Lang et al., 2020). However, there are certain limitations of using this approach with archive samples due to potential postmortem DNA damage, fragmentation and decay often caused by various damaging and/or fixation agents (e.g., light, oxygen, formaldehyde, non-absolute ethanol) on free nucleotides (Lindahl, 1993; Zimmermann et al., 2008; Allentoft et al., 2012; Dehasque et al., 2020). Traditionally in biomonitoring studies, aquatic macroinvertebrates have been preserved by formaldehyde (37–40% aqueous formaldehyde gas solution). Formaldehyde forms crosslinks between DNA and/or proteins, thereby preserving the morphological structure of the organism tissues, but denaturing their DNA structure (Stein et al., 2013; Hoffman et al., 2015). Due to its tendency to induce toxic, allergenic and carcinogenic effects on the exposed humans (i.e., researchers) (Elshaer and Mahmoud, 2017), in most biomonitoring routines formaldehyde was replaced by ethanol (Hauer and Lamberti, 1996). Aquatic macroinvertebrates are usually preserved in $\leq$70%-ethanol solutions, but if the organisms are aimed to be used for molecular analyses, higher ethanol concentrations (e.g., $\geq$95%) are used, to ensure denaturation of proteins that might degrade DNA, and to enable the respective DNA preservation (Nagy, 2010; Stein et al., 2013). However, the effects of

Table 1
Species selected from different archives.

Species	Archive			
	1992	2008	2016	2020
Antocha vitripennis	+	+	+	
Bezzia flavicornis			+	+
Ecdyonurus starmachi				+
Gammarus fossarum			+	
Habrophlebia lauta				+
Ibisia marginata	+	+		
Simulium sp.	+	+	+	+
Wiedemannia ouedorum	+	+	+	

prolonged exposure of DNA (e.g., storage of samples) to ethanol are not yet known. Thus, some studies suggest to emerge organisms in 4–6% formaldehyde solution for a short time (up to one week) after the sampling, and then transfer the specimens to ethanol, which is a good medium for removing formaldehyde from specimens and avoiding DNA degradation (Schander and Halanych, 2003; Vivien et al., 2016).

Because of the potential time-dependent DNA damage, the targeted DNA can be impossible to amplify, so obtaining genetic information from archive samples can be difficult (Jackson et al., 2012). Since preservation of DNA, RNA and protein molecules is a prerequisite for the implementation of transcriptomic, genomic and proteomic analysis, the aim of this study was to assess the quality of macromolecule preservation within archive samples of several macroinvertebrate taxa fixed in formaldehyde solution and/or ethanol. We combined DNA sequencing and assessments of RNA quality and protein preservation, with tissue preservation for morphometric measurements on contemporary and archive specimens of several non-model aquatic insect taxa commonly found in urban streams. The same analyses also were run on the species of well-known reference genome/proteome as positive controls for specific methods. This study provides important information on the influence of commonly used fixatives on DNA, RNA and protein preservation on contemporary and archive, model and non-model insect specimens. Our findings might help to develop a reliable strategies for processing macroinvertebrate samples within future assessments of anthropogenic influences on aquatic life. Such strategies should incorporate time series of the macroinvertebrate responses to environmental change, i.e., genome-wide genetic trends of aquatic taxa over time, independent of genome size and presence of a reference genome.

2. Materials and methods

2.1. Archive samples

In order to evaluate quality of freshwater macroinvertebrate samples for analysis of anthropogenic influence through years, we used available archive samples collected within City of Zagreb area in 1992, 2008, 2016 and January 2020 (Fig. 1).

Archive 1992 consisted of samples collected at Veliki potok stream (Fig. 1) and stored at room temperature in 3.6% formaldehyde solution immediately upon the sampling (as suggested by APHA, Greenberg et al., 1992). Archive 2008 was collected at Kašina stream (Fig. 1) and stored at room temperature in 3.6% formaldehyde solution >2 h after the sampling. Samples were stored for one year and after this time period the specimens were transferred to 70% ethanol (as suggested by Rosenberg et al., 1997; Greenberg et al., 1992). Archive 2016 consisted of samples collected at Veliki potok (Fig. 1), stored in 70% ethanol immediately upon the sampling. Archive 2020 consisted of two series, as all samples were taken in duplicates, so one sample series was stored in 36% formaldehyde solution and the other in absolute ethanol, both treated with fixative solutions immediately upon the sampling.

From each archive, four insect species were selected and five specimens belonging to each species were collected for further assessment (Table 1).

The species were identified based primarily on morphological characteristics, following the respective determination keys for individual insect orders (Bertrand, 1954; Elson-Harris, 1990; Mey, 1997). Species selected for further assessment were chosen based on their abundance in the respective archive and all were considered non-model organisms. Afterwards, the morphological species from our dataset were associated to a unique gene sequence as described in the Barcoding section and Table 3 below. Additionally, from Archive 2016, the amphipod *Gammarus fossarum* was chosen because of its abundance.

For all experiments, model organism *Drosophila melanogaster* samples (5 adult and 5 larvae specimens) were used as controls. *Drosophila* samples were collected in laboratory in 2020, and stored in both 3.6% formaldehyde solution and absolute ethanol.

2.2. Preparation of formalin-fixed paraffin embedded (FFPE) tissue samples

Formalin-fixed paraffin embedded (FFPE) samples were prepared for all selected archived species as well as for *Drosophila melanogaster* with the purpose of long-term tissue and macromolecules preservation following the concept of FFPE-archives for human tissues (Blow, 2007). For this study segment, 5 specimens representing selected species from all archives were treated with 70% ethanol incubation 2 × 1 h, 95% ethanol 2 × 1 h, absolute ethanol 2 × 1 h, and xylene substitute 2 × 1 h at 37 °C, prior to incubation in liquid paraffin 3 × 1 h at 60 °C. Paraffin tissue blocks were cut into 2 μm thick sections using a standard sliding manual microtome (Microm), and sections were mounted onto glass slides (HistoBond adhesive microscope slides, Paul Marienfeld) for hemalum and eosin (H&E) staining and immunofluorescent labeling. Additional 10 μm thick sections were used for isolation of nucleic acids.

2.3. Hemalum and eosin (H&E) staining

Hemalum and eosin (H&E) staining was done with an aim of tissue quality assessment for morphometry-based analyses, e.g. shape variation of organs and organisms assessment. FFPE sections were deparaffinized using xylene substitution, rehydrated through decreasing ethanol series (absolute, 95% and 70% ethanol, 2 min each) and washed with distilled water. Modified hematoxylin according to Harris was applied for 5 min. Slides were rinsed with tap water for 2 min, and eosin Y aqueous solution was applied for 2 min. Slides were then dehydrated with increasing ethanol series (70%, 95% and absolute ethanol, each 2 min), cleared in xylene and mounted with xylene-based mounting media for glass coverslipping. Tissue quality was evaluated using Olympus BX51 microscope in order to asses sample quality for morphometrical measurements.

2.4. Nucleic acids isolation

In order to evaluate the best method for nucleic acid isolation, two species from the oldest archives were used. DNA was isolated from FFPE samples (*Antocha vitripennis* 1992 and 2008, *Ibisia marginata* 1992 and 2008) using commercially available kits (Quick-DNA/RNA FFPE Miniprep Kit, FFPE RNA/DNA Purification Plus Kit) and in-house protocol consisting of the following steps - the sections were: (i) deparaffinized with xylen substitution, (ii) washed with 100% ethanol, (iv) resuspended in digestion buffer (50 mM Tris-HCl pH 8.5, 1 mM, EDTA, 0.5% Tween 20) and (v) digested with proteinase K (Roche Diagnostics, Mannheim, Germany) at final concentration of 200 mg mL^{-1} overnight at 37 °C. The following day, the digestion samples were boiled for 8 min

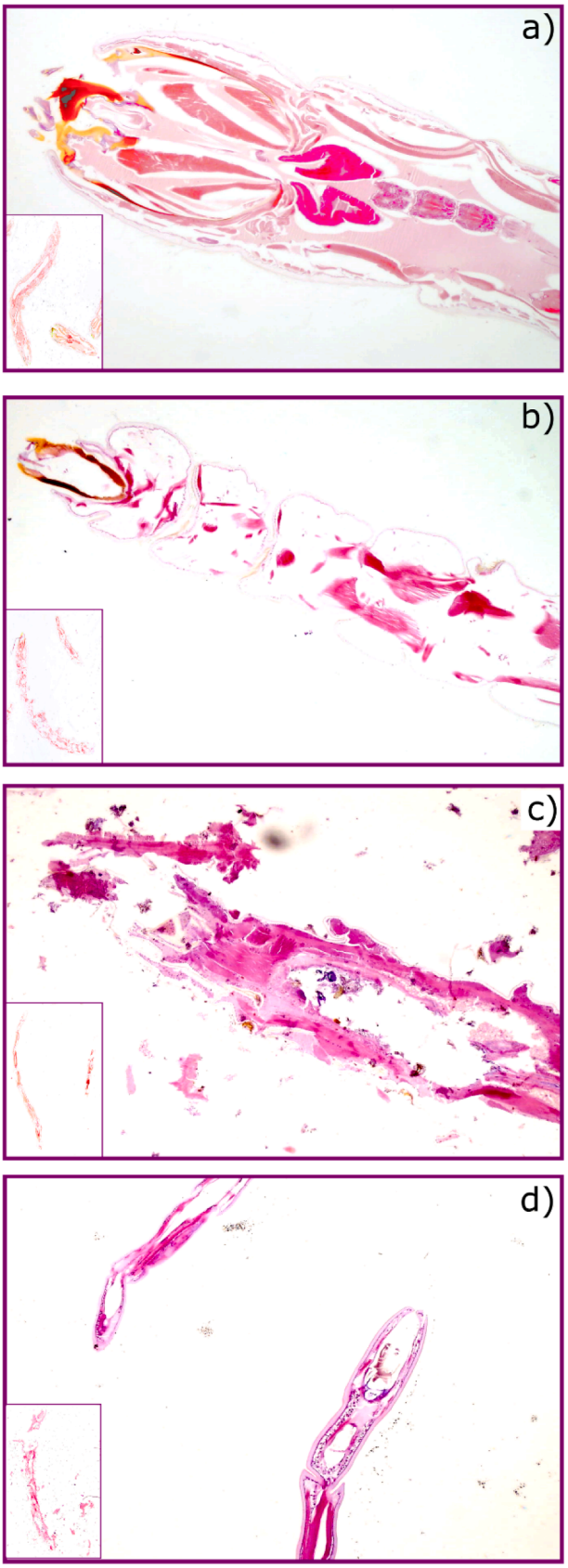

Fig. 2. H&E-stained sections (magnifications 20x and 100x, Olympus BX51) of larval: a) *Antocha vitripennis* 1992, b) *Antocha vitripennis* 2008, c) *Antocha vitripennis* 2016, d) *Bezzia flavicornis* 2020. The bottom left corner shows the entire organism, whereas the large central picture shows the frontal body part of the organism.

for enzyme inactivation. RNA was isolated from the same samples using commercially available sets of chemicals according to manufacturers' protocols (Quick-DNA/RNA FFPE Miniprep Kit, FFPE RNA/DNA Purification Plus Kit, High Pure FFPE RNA Micro Kit). Based on the preliminary results on nucleic acids concentration and quality, optimal protocols for further isolations from all selected species and archives were selected. Specimens of selected species from all archives were used for nucleic acids isolation in two forms: directly from the original fixative they were stored in (non-embedded samples), and as FFPE-sections cut from FFPE-blocks prepared for histological staining. Non-embedded samples were taken from the archives, dried and further processed according to the specific isolation protocol.

2.5. PCR amplification and barcoding

Isolated DNA molecules (both from FFPE-sections and non-embedded samples) were used as templates for PCR amplification of *COI* barcode region using previously described protocol with BF1-BR2 primers specifically designed for freshwater macrozoobenthos species (Elbrecht et al., 2017). PCR-amplicons were sent to service institution (Macrogen) for Sanger-based sequencing. Gained sequences were assessed using BLAST. Detection of 95% barcode sequence alignment was used for genus, and for species identification alignment of 98% or more.

2.6. Immunofluorescent labeling

In order to assess protein preservation, immunofluorescent labeling was done. FFPE-sections from all species were deparaffinized using xylene substitution, rehydrated through decreasing ethanol series (absolute, 85% and 70% ethanol, each 2 min) and washed with distilled water. After heat-induced epitope retrieval (HIER), blocking in 4% BSA (Sigma-Aldrich) and 0.5% Triton X-100 in PBS for 30 min at room temperature was carried out. Slides were then incubated with anti-tubulin antibody (1:200, Ab6046, Abcam) overnight and with FITC-labeled secondary antibody (1:100, Ab6717, Abcam) for 1 h. Counter-staining was done with DAPI (1 μg mL^{-1}, Sigma-Aldrich). Tissues were mounted with Fluorescence Mounting Medium (Dako) and analyzed using an Olympus BX51 microscope.

In order to optimize the HIER protocol we undertook the following procedure: As selected primary antibody was, according to the manufacturer, produced to have following species reactivity: Mouse, Rat, Chicken, Human, Pig, *Xenopus laevis*, Zebrafish, Chinese hamster, firstly we assessed its reactivity for Diptera model organism – *Drosophila melanogaster* using human tonsil as a control. Optimal HIER conditions were evaluated using human tonsil FFPE sections and adult *Drosophila melanogaster* FFPE sections. Three sets of conditions were used: EDTA treatment at 97 °C for 20 min, EDTA treatment at 125 °C during 2 min, and citrate buffer treatment at 125 °C during 2 min. Optimal conditions were further used for protocols applied on all samples representing selected species from all archives.

2.7. Statistical analyses

Kruskal–Wallis (K–W test) and Mann–Whitney U Test (M−W test) were used to test the differences between groups due to violations of the normality assumption. Chi-square was used to determine association between the variables. Statistical analyses were performed with the STATISTICA software, version 13.0 (StatSoft Inc. Tulsa, OK, USA). The level of significance was set at $p < 0.05$.

3. Results and discussion

Present study was conducted in order to assess the macromolecule (DNA, RNA, protein) quality in archived macroinvertebrate samples for morphometric and genomic/transcriptomic/proteomic analyses.

Table 2
Comparison of different methods for DNA and RNA isolation from various archive macroinvertebrate samples. ($A_{260/280}$ and $A_{260/230}$ ratios show nucleic acid purity based on absorbance at 230, 260 and 280 nm, *c* denotes the concentration of the isolated molecule.)

samples	DNA								
	Quick-DNA/RNA FFPE Miniprep Kit			FFPE RNA/DNA Purification Plus Kit			in-house method		
	c (ng μL^{-1})	$A_{260/280}$*	$A_{260/230}$*	*c* (ng μL^{-1})	$A_{260/280}$	$A_{260/230}$	*c* (ng μL^{-1})	$A_{260/280}$	$A_{260/230}$
*Ibisia*_1992	21	1.17	0.58	20	1.07	0.65	193.5	0.97	0.45
*Antocha*_1992	17.5	1.04	0.89	17.5	1.11	0.69	86.4	0.93	0.35
*Ibisia*_2008	19.5	1.12	0.87	16.6	1.15	0.85	42.9	1.03	0.28
*Antocha*_2008	23.5	1.16	0.82	19.5	1.3	0.79	39.9	0.87	0.21
	RNA								
	Quick-DNA/RNA FFPE Miniprep Kit			FFPE RNA/DNA Purification Plus Kit			High Pure FFPE RNA Micro Kit		
	c (ng μL^{-1})	$A_{260/280}$	$A_{260/230}$	*c* (ng μL^{-1})	$A_{260/280}$	$A_{260/230}$	*c* (ng μL^{-1})	$A_{260/280}$	$A_{260/230}$
*Ibisia*_1992	8.6	3.5	0.083	11.4	1.42	0.52	2.35	0.83	0.39
*Antocha*_1992	7.9	1	0.06	9.4	1.27	1.7	3.85	0.91	0.7
*Ibisia*_2008	22.4	1.3	0.052	16.8	1.15	0.81	2.4	0.72	1.02
*Antocha*_2008	8.1	0.87	−0.028	10.1	7.4	0.73	10.05	1.15	0.91

Existing archives were, at the time, collected for different environmental assessment purposes, mostly with an aim of investigating macrozoobenthos distribution patterns and species composition based on morphological species identification. However, storage and preservation of the existing archives could be beneficial for present studies aiming to assess long-term environmental changes based on DNA-dependent identification methods and data analysis (cf. Sweeney et al., 2011; Baird and Hajibabaei, 2012).

3.1. Morphology assessment

For the purpose of tissue evaluation and morphometric measurements, selected organisms were taken from the fixative they were stored in and processed to FFPE blocks. Tissue sections cut from those blocks were than used for hemalaum and eosin staining, which revealed preserved tissue morphology for all archives stored in formaldehyde solution. Samples stored in formaldehyde solution for 28 years or few weeks, as well as samples initially stored in formaldehyde solution and after the first years transferred to 70% ethanol for another 11 years, showed adequate quality for morphological analyses, i.e., the aquatic macroinvertebrate larvae showed the common appearances with well-developed and distinguished body regions of the larvae (Fig. 2 a, b, d).

Among those samples, species from 2008 archive showed the lowest quality (i.e., the most pronouncedly damaged morphology) (Fig. 2b). The low 2008 archive quality could be due to the increased time interval (>2h) between sampling of the organisms and storing the samples in fixative, as suggested by the available information on the archive (extracted from the respective field and lab diaries). The time interval between collection and processing of biological samples as well as temperature during the sample storage may greatly affect the sample stability (Winikoff et al., 2005). It is likely associated to cell viability, which decreases with time and increasing temperature (Winikoff et al., 2005). The available historical information suggest that for the 1992 and 2020 samples formaldehyde solution was used as a fixative, which was applied directly in the field (the organisms were immersed into the fixative immediately after the sampling). On the other hand, 2008 samples were collected, taken to the lab and then immersed into the fixative a few hours after the sampling. Until applying the fixative, organisms were kept in the stream water at the room temperature. As it was a summer period, it is likely that the high ambient temperature affected the organisms' soft tissue decay (Rivers and Dahlem, 2014). Additionally, FFPE-sections of species stored in 70% ethanol directly in the field and kept for 4 years in the fixative (archive 2016: *Antocha vitripennis, Bezzia flavicornis, Gammarus fossarum, Simulium* sp., *Wiedemannia ouedorum*) showed poor quality (i.e., pronouncedly damaged morphology) for microscopic analysis and morphometry (Fig. 2c).

Generally, our results indicate that the best fixative for macrozoobenthos organisms collected for morphology assessment is formaldehyde solution. Moreover, time interval between sampling of the organisms and storing the samples in fixative should be the shortest possible. However, although formaldehyde solution has shown to be the best fixative for morphological determination of macrozoobenthos, it is important to consider its toxic, allergenic and carcinogenic effects on researchers (Elshaer and Mahmoud, 2017). It would therefore be desirable to use alternative (substitute) fixatives to preserve the organism tissues. Numerous studies are aiming to target a fixative that would have the same or similar chemical properties for tissue/protein preservation as formaldehyde solution, and ensure a high level of environmental and human health protection by its usage (e.g., Brenner, 2014). For example, high-molecular-weight aldehydes such as glyoxal and some product-type PT 22 "embalming and taxidermist fluids" appear to be less toxic than formaldehyde, but the information on their human and environmental toxicity is still limited and their usage should be further investigated (National Research Council (US) Committee on Aldehydes, 1981; Brenner, 2014).

3.2. Nucleic acids quality

For the purpose of macromolecular analyses, DNA and RNA isolation protocols were firstly evaluated on the samples of species belonging to the oldest archives. Statistical analyses showed significant difference between *in-house* method for DNA isolation and both DNA isolation methods conducted using commercially available kits. *In-house* method resulted with significantly higher DNA concentration (K-W test and M−W tests, $p < 0.05$), while both kits provided DNA isolates with higher purity (M−W test, $p < 0.05$). There was no statistical difference in RNA concentration between three isolation protocols, but RNA isolate purity significantly varied between different methods (M−W test $p < 0.05$). These results together with time and cost of the methods indicated that the most optimal protocol for further analysis is Quick-DNA/RNA FFPE Miniprep Kit (Table 2).

Nucleic acids were further isolated from organisms stored directly in the fixative of choice (non-embedded samples) as well as organisms taken from the same fixative and processed to FFPE blocks. There was no statistically significant difference in DNA and RNA purity or concentration between two analyzed types of samples (M−W test, $p > 0.05$). DNA was further used for PCR amplification of the barcode gene and Sanger sequencing of the barcode in both types of samples. Statistical analyses showed significantly different outcome - only non-embedded samples stored in ethanol showed interpretable results (Chi-square, *p*

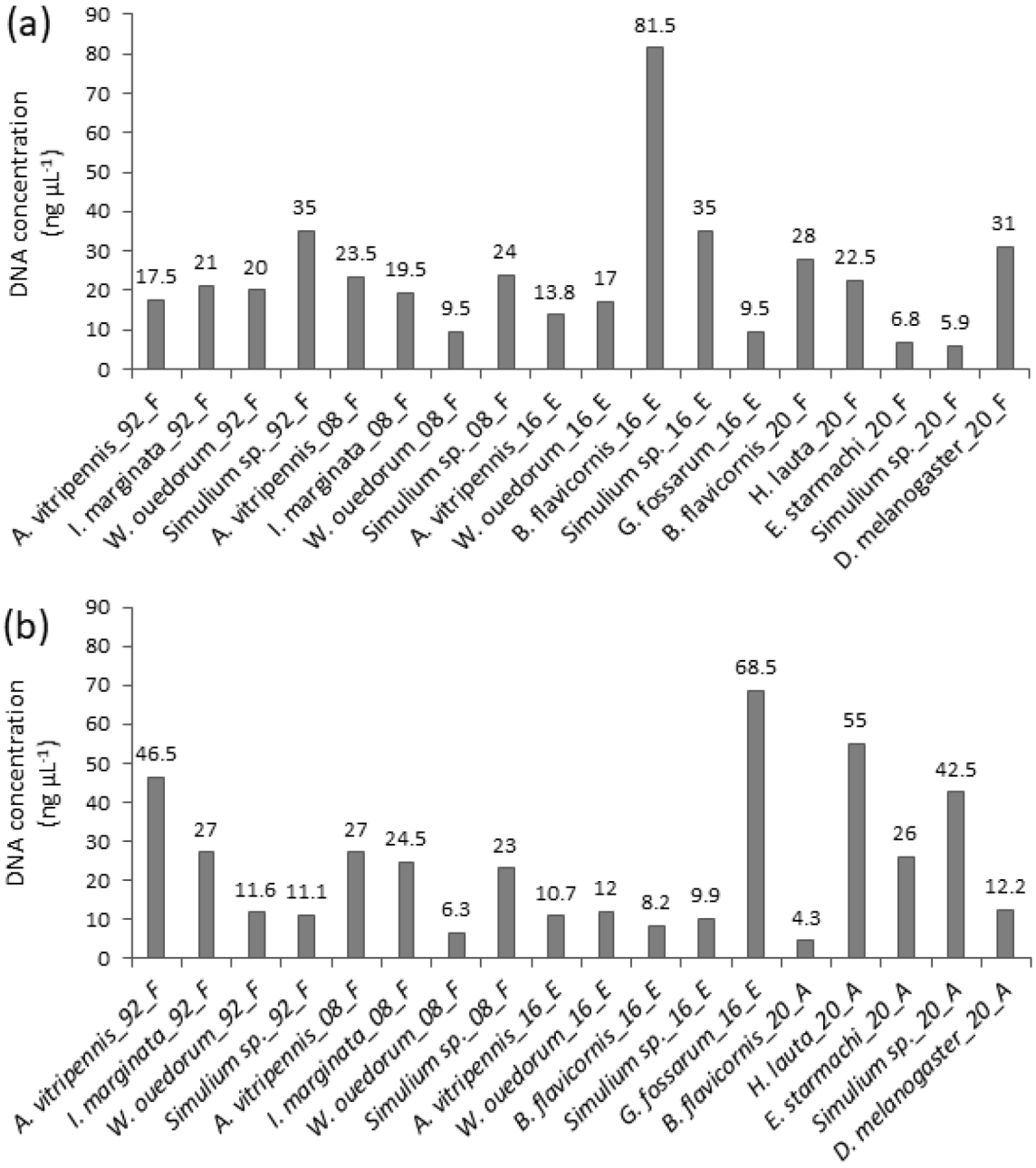

Fig. 3. DNA concentration from a) FFPE-embedded samples, and b) non-embedded samples. The numbers in the continuation of the macrozoobenthos species name indicates the year of the archive (i.e., sampling year); the letters F, A and E denote the fixative used when collecting samples (F – formalin, A – absolute ethanol, E – 70% ethanol).

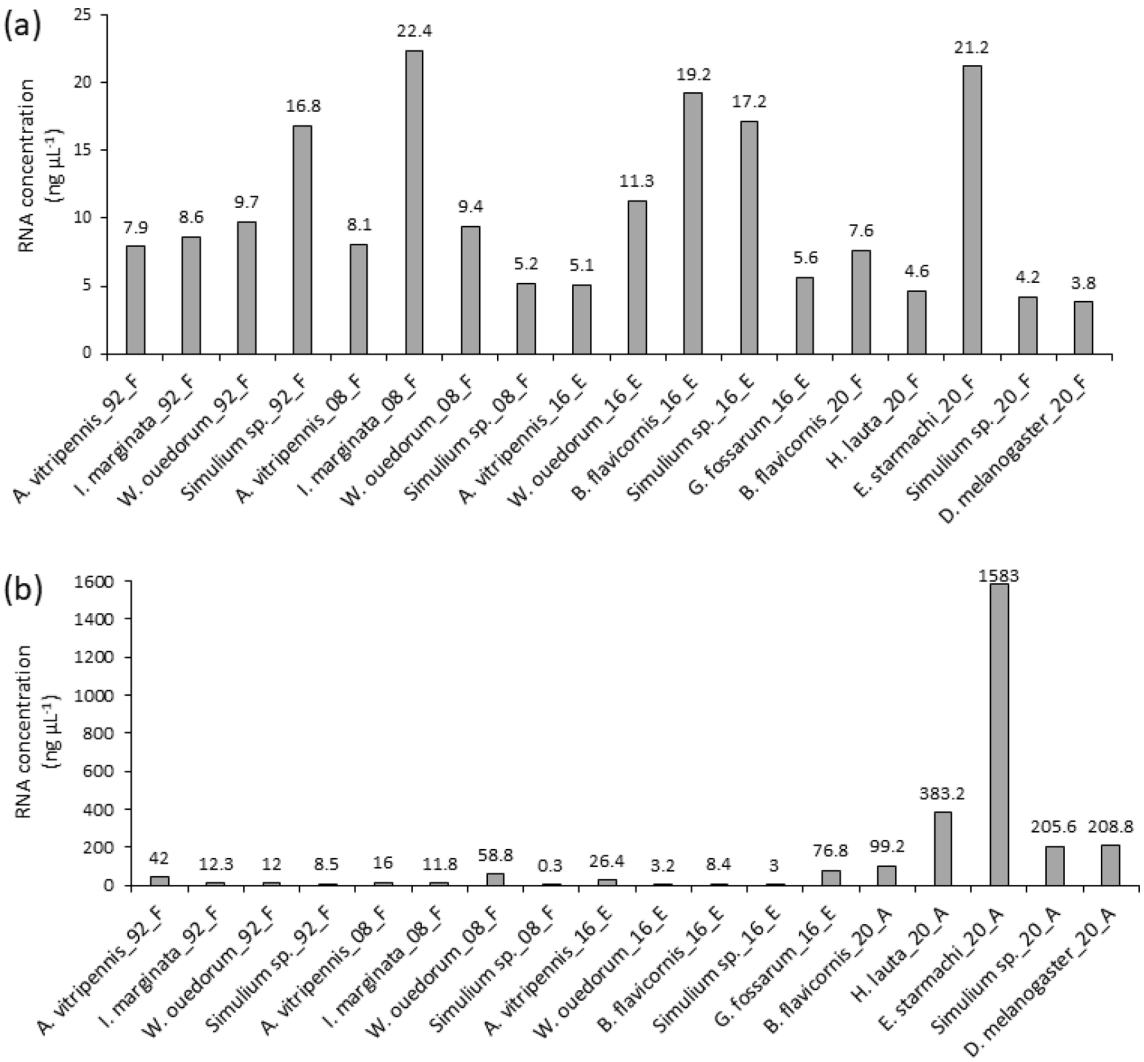

Fig. 4. RNA concentration from a) FFPE-embedded samples, and b) non-embedded samples. The numbers in the continuation of the macrozoobenthos species name indicates the year of the archive (i.e., sampling year); the letters F, A and E denote the fixative used when collecting samples (F – formalin, A – absolute ethanol, E – 70% ethanol). Note different scaling of the y-axes.

= 0.01), (Figs. 3 and 4; supplementary material Tables 1–3).

Formaldehyde solution used as fixative degrades nucleic acids and although there is sufficient DNA and/or RNA amount for the downstream analyses after PCR amplification, fragmentation is the main concern for molecular analyses (Guyard et al., 2017; Watanabe et al., 2017; Groelz et al., 2018). PCR and other downstream methods limit the usage of samples depending on the fragment length required for those methods, mainly based on primer design e. g. minimal fragment that can be amplified using specific pair of primers. In our study, according to previously described optimal primers for *COI* barcode amplification, only samples that had DNA fragments longer than 300 bp could be used (Elbrecht et al., 2017). Our results showed that following this criteria, DNA isolates from macroinvertebrate forms stored in ethanol (both 70% and absolute) were adequate for barcoding, while the ones that were stored in formaldehyde fixative or processed to FFPE-blocks were degraded beyond the required fragment length.

The results of our morphological and barcode-based species determination (Table 3) mostly matched.

However, morphologically determined Ceratopogonidae species *Bezzia flavicornis* was by barcoding confirmed as *Palpomyia flavipes*, whereas the Heptageniidae species initially determined as *Ecdyonurus starmachi* was proven to be *Electrogena ujhelyii*, which belongs to the same Heptageniidae family. Although Ceratopogonidae are common in many aquatic and semi-aquatic habitats, many species are small and members of some genera can be very difficult to identify solely by morphological identification (Stur and Borkent, 2014). Thus, for the precise identification of the ceratopogonid species, either barcoding or morphological identification of the late larval instars are required. The mismatch between *Ecdyonurus* and *Electrogena* was likely due to the highly variable anterior margin of the head capsule between the two

Table 3
Morphological and barcode-based determination of species.

archive	morphologically determined species	barcoding results
2016.	*Antocha vitripennis*	"no significant similarity found"
	Wiedemannia ouedorum	"no significant similarity found"
	Bezzia flavicornis	"no significant similarity found"
	Simulium sp.	"no significant similarity found"
	Bezzia flavicornis	*Palpomyia flavipes* (95.89%)
	Habrophlebia lauta	*Habrophlebia lauta*(97.88%)
2020.	*Ecdyonurus starmachi*	*Electrogena ujhelyii* (97.65%)
	Simulium sp.	*Simulium velutinum*(98.98%)
	Drosophila melanogaster	*Drosophila melanogaster* (98.15%)

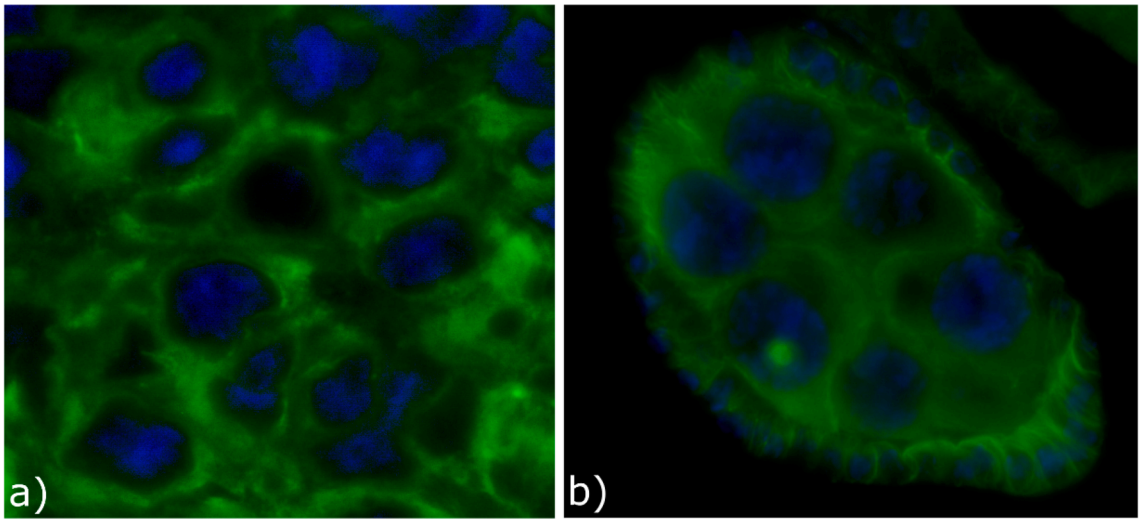

Fig. 5. FFPE sections of a) human tonsil and b) adult *Drosophila melanogaster* showed tubulin-specific FITC signals (green) after immunofluorescent staining using EDTA at 97 °C for 20 min as HIER step of the immunostaining protocol (magnification 1000×, Olympus BX51). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

heptagenid species. This could have caused morphological misidentification of the two species, but it was corrected by barcoding (Webb and McCafferty, 2008).

Our results show the importance of nucleic acid preservation for species determination using barcoding. For this purpose, organisms should be immersed and stored in ethanol immediately after the field sampling until nucleic acid isolation.

3.3. Protein preservation

When assessing macromolecule preservation, especially protein preservation of macroinvertebrates stored in different archives, we encountered another obstacle – most species are non-model species, their genomes were in most cases unknown (not yet sequenced) and there were no available antibodies specific for those species. For this reason, we decided to use antibody which is specific for well-known conserved protein (i. e., β-tubulin) and to test its reactivity for Diptera model organism - *Drosophila melanogaster*, using human tonsil as a control as it was clarified in the "Material and Methods" section within optimization of the immunostaining protocol. Retrieval protocols tested on human tonsil FFPE section and *Drosophila melanogaster* FFPE sample showed that the optimal HIER protocol is treatment with EDTA at 97 °C for 20 min (Fig. 5).

Our results (Fig. 6) showed that protein preservation is dependent not only on the fixative of choice, but also on the time gap between sampling and protein analysis.

Tubulin-specific signal was detected within samples initially fixed with formaldehyde solution and after 1 year stored in 70% ethanol, as well as within samples fixed with formaldehyde solution for few weeks, but not within the samples stored in formaldehyde solution for 28 years. Moreover, samples stored in ethanol and subsequently processed to FFPE block showed no immunofluorescent signal.

Immunofluorescent staining also confirmed our results about DNA degradation – samples from archives 1992 and 2008 showed no DAPI-staining, suggesting that in those samples DNA was fully degraded.

In general, protein preservation is best achieved if organisms are stored in 3.6% formaldehyde solution immediately after the sampling. Protein evaluation should be performed as soon as possible because of protein degradation over longer periods (e.g., Alber and Suter, 2019).

3.4. Overall archive quality evaluation

The main goal of this research was to assess the quality of macromolecule preservation in archival samples of aquatic macroinvertebrates collected in urban streams during few decades. The preservation of the organisms' DNA, RNA and proteins is of vast importance for studies of urbanization influence on aquatic life, as the environmental information recorded in these molecules during time (i. e., in archival vs. contemporary samples) could advance our understanding of the genomic change driven by increasing urban influence (Burrell et al., 2015; Lang et al., 2020). The organisms' DNA preservation is also a prerequisite for the successful confirmation of detected (archived) species by the DNA barcoding, which is presently often used to increase bioassessment metrics, i.e. to assess aquatic habitat environmental conditions based on increased taxonomic resolution of aquatic macroinvertebrate taxa (Stein et al., 2014). Thus, the results of the present study might improve the routinely used sample preservation methods to protect DNA from degradation that may pose a potential impediment to application of DNA barcoding and metagenomics for biomonitoring using benthic macroinvertebrates (Stein et al., 2013), especially if planning the long-term bioassessment studies.

Our results suggest that there is a great variety in the archive storage approaches, which consequently affect molecular analysis quality. This should be taken into account if planning temporally spaced macrozoobenthos sampling for proteomic, transcriptomic and genomic analyses of the organism. Present study was based on routinely used, widespread methods for DNA/RNA/protein quality evaluation. Based on our results on DNA and RNA quality gained through using the downstream molecular methods (e.g., PCR and Sanger sequencing), it is likely that other PCR-based methods such as Q-RT PCR or other more sensitive sequencing methods, such as next generation sequencing, would show similar efficiency for genomic studies on these type of samples. Protein preservation was evaluated using immunofluorescent labeling technique, which is one of the routinely used methods for antigen detection in histological and/or cytological samples. More sensitive methods (e.g., ELISA, Western blot), could likely confirm our results, but they are too based on antibody specificity and selectivity, and are thus dependent of available reagents for non-model organisms (Pillai-Kastoori et al., 2020).

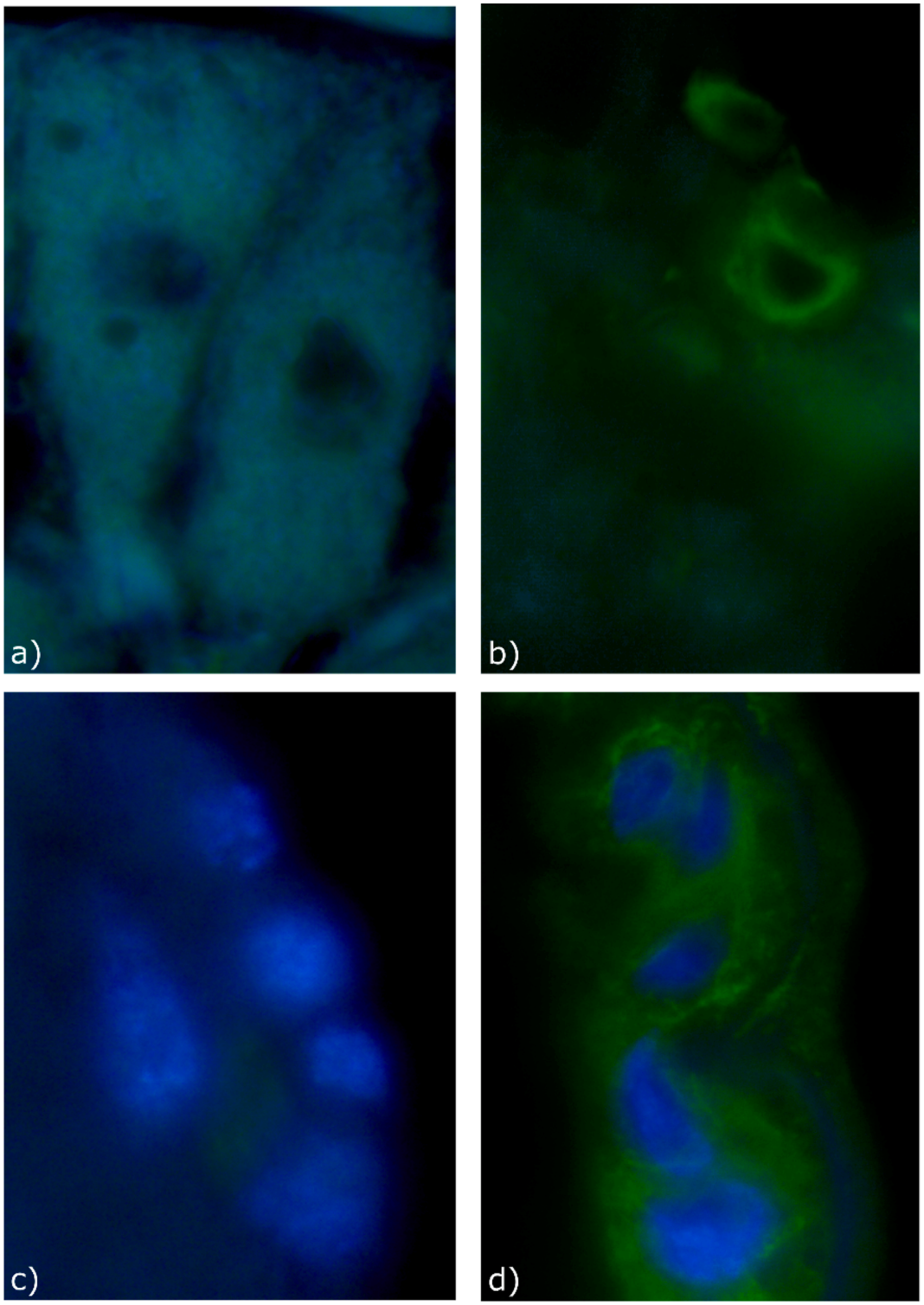

Fig. 6. Immunofluorescent staining of a) *Antocha vitripennis* 1992, b) *Antocha vitripennis* 2008, c) *Antocha vitripennis* 2016, and d) *Bezzia flavicornis* 2020. Magnification: 1000×; FITC (green) signal represents tubulin, while DAPI (blue) signal represents nucleus/DNA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In line with other studies including DNA/RNA/protein analyses (Nagy, 2010; Stein et al., 2013), our results suggest that organisms sampled for nucleic acids analyses should preferably be fixed and stored in absolute ethanol. However, if collected organisms are aimed for morphological determination or protein detection, we suggest to store them in 3.6% formaldehyde solution (whereby precautions should be taken due to the toxic effects of this compound), whereas for long-time storage and investigations, FFPE-blocks could be produced.

Our results further suggest that macrozoobenthos samples should be fixed *in situ* (directly in the field at the time of sampling) and processed in a lab as soon as possible at least to the level of macromolecule isolates; and the samples should be collected in duplicates to form dual archives (ethanol- and formaldehyde- or its substitute-based) and that would enable further studies on the ecosystem changes even several decades

after the sampling.

CRediT authorship contribution statement

Valerija Begić: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Mirela Sertić Perić:** Methodology, Writing - original draft, Writing - review & editing, Visualization. **Suzana Hančić:** Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Mihaela Štargl:** Formal analysis, Writing - review & editing. **Matea Svoboda:** Formal analysis, Writing - review & editing. **Petra Korać:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Validation, Visualization, Writing - original draft, Writing - review & editing. **Ines Radanović:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2021.107509.

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