

# Environmental DNA captured on the fish skin mucus – a potential bias to molecular diet analyses

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**Abstract** – Molecular diet analyses from faeces appear to be an ideal alternative to traditional feeding ecology studies. Nevertheless, this method can carry a risk of contamination from the environment or from body surface of the fish itself. To tackle the contamination problem, an experiment was performed with the main aim to identify whether foreign DNA is present or absent on the fish skin mucus, and if so, the second aim was to find out if this environmental DNA (eDNA) can be removed by repeated wiping of the skin mucus. Specimens of fish were exposed to eDNA and then their fish skin mucus was wiped with two consecutive smears (using a forensic swab) that were subjected to molecular analysis. The results demonstrate that eDNA from other organisms can be captured and persist on the fish skin mucus, posing a potential risk of contamination of faeces samples. Repeated wiping of mucus reduces or eliminates foreign DNA. This study provides new insights that can contribute to the development of the molecular methods, reducing the bias and increasing the accuracy of the diet spectrum analyses.

**Keywords:** eDNA contamination / experiment / feeding ecology / fish / molecular approach

## 1 Introduction

Trophic interactions represent a very important topic, as well as a methodological challenge in ecological studies. In particular, the accurate identification of prey is a fundamental requirement in elucidating the food-web structure (Jo *et al.*, 2014). In fishes, traditional approaches of diet analysis (dissected gut/stomach content analysis) suffer one serious weakness: difficulties to identify items in the gut content to species level, because the soft-bodied organisms are often digested beyond the possibility of visual recognition. The traditional approaches thus often fail to identify many diet items, which inevitably leads to loss of important information (Jo *et al.*, 2016; Legler *et al.*, 2010). Moreover, obtaining a sufficient number of fish samples for trophic studies is a problem, especially when considering endangered/protected species or small populations. As the abundance of native fish populations decreases, killing large numbers of fish for scientific purposes should be avoided whenever possible (Bammer *et al.*, 2015).

Molecular methods of diet analysis appear more precise than traditional visual techniques, and they have become an important tool for studying feeding interactions (Carreon-Martinez *et al.*, 2011; Sint *et al.*, 2011). However, DNA degradation rates due to digestion, assay sensitivity and the ability to quantify the amount of individual components of the diet still pose major limitations to this approach (Deagle *et al.*, 2013). On the other hand, these methods often provide a greater taxonomic resolution (more species identified) than the visual analyses of gut/stomach content and can also identify highly digested prey that have lost all physical characteristics (Carreon-Martinez *et al.*, 2011; Guilleraut *et al.*, 2017; Smith *et al.*, 2005). The dietary samples like regurgitates or faeces, which may be a very good source of information for molecular prey analyses, can be obtained non-lethally (Briem *et al.*, 2018; Kamenova *et al.*, 2018; Oehm *et al.*, 2016; Thalinger *et al.*, 2016). For example, in fishes, gently squeezing the abdomen, *i.e.* lightly pressing the peritoneal cavity towards the rectum allows to obtain faeces samples with no harm to the examined specimen (Corse *et al.*, 2010; Guilleraut *et al.*, 2017).

Despite all the advantages, potential contamination from the environment and from the body surface of the organism itself is a major challenge in molecular approaches of diet analysis. Field collected specimens may be externally

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contaminated by DNA from the environment they live in, therefore potentially contaminating and biasing the results of diet analysis (Briem *et al.*, 2018; Greenstone *et al.*, 2012). In fishes, the question is whether foreign DNA (contamination from the environment) can be captured on the surface of their bodies, which might lead to contamination of faeces samples directly from the fish's own skin mucus. In other organisms (*e.g.* insects), the external contamination can be removed using proper bleaching protocol, that is lethal to the sampled specimen (Briem *et al.*, 2018). The problem comes when the examined organisms are not allowed to be sacrificed during the research. Although the fish skin mucus produces substances that can cause environmental DNA (eDNA) degradation, and it is constantly secreted and replaced (Alexander and Ingram, 1992; Nagashima *et al.*, 2003; Guardiola *et al.*, 2015; Kumari *et al.*, 2019; Patel *et al.*, 2020; Wang *et al.*, 2019), the question whether eDNA can be captured on the fish skin mucus has never been answered satisfactorily. Thus, to reduce the risk of contamination when collecting faeces from fishes, it might be helpful to wipe out the mucus around their anus area.

In this study, we tested the hypothesis that eDNA (*i.e.* foreign DNA) is present on the fish skin mucus. For doing so we performed a laboratory experiment in which fish individuals were exposed to the target contaminant (eDNA). Molecular analyses were applied to find out whether the foreign DNA is detectable on mucus. Subsequently we tested whether the potential foreign DNA detection could be removed by repeated wiping of the fish mucus.

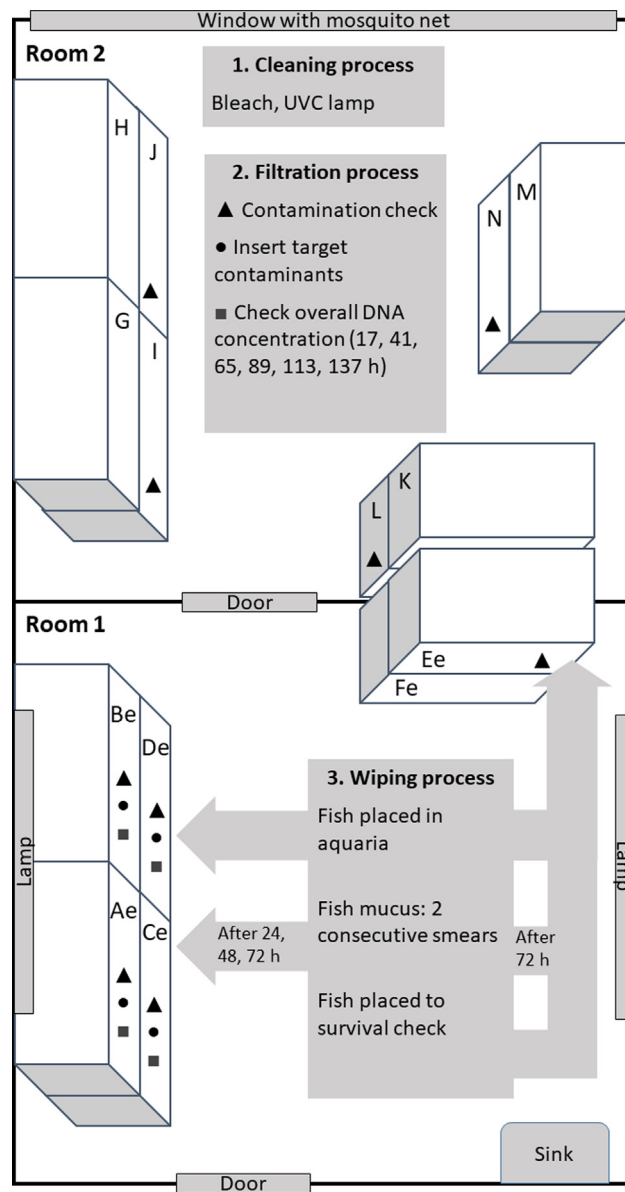
## 2 Material and methods

### 2.1 Experimental setup

The experiment was conducted in the fish laboratory with aquaria used for rearing fish only. The lab itself consists of two rooms, Room 1 was equipped with four experimental aquaria (214.5 l/aquarium, Ae, Be, Ce, De) and two additional aquaria (Ee, Fe). The Room 2 contained eight aquaria that served for rearing the experimental fish (Fig. 1). To remove possible DNA contamination, all aquaria in Room 1 were bleached (Sodium hypochlorite solution) and cleaned properly with tap water. Each aquarium was irradiated overnight using germicidal UVC lamp, and then filled with tap water, filtration was put on, and once again germicidal UVC radiation was used overnight in each aquarium. Similarly, every surface in the Room 1 was bleached and irradiated with germicidal UVC lamp for five nights before placing target contaminants in the experimental aquaria.

Common roach *Rutilus rutilus* (Linnaeus;  $n = 39$ ) was used for this experiment. This species is listed as Least Concern in the IUCN Red list and it is one of the most common and abundant species in the Danube water bodies. The fish were collected from the northern drainage channel of the Gabčíkovo Hydroelectric Scheme (the River Danube, 47.9385N, 17.4483E), and reared subsequently in aquaria for more than one year. Such acclimatized fish tolerated handling very well and did not show signs of stress.

Throughout the whole study, the standards set for fish rearing in laboratory conditions according to Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific



**Fig. 1.** Schema of the fish laboratory and experimental procedure. The fish laboratory consists of two separate rooms – Room 1 (experimental room) and Room 2 (rearing room). Aquaria in both rooms were placed on stands. Abbreviations: Ae, Be, Ce, De – experimental aquaria; Ee – negative control aquarium and the control of the fish survival; Fe – control of the fish survival; G, H, I, J, K, L, M, N – aquaria for rearing the fish (Room 2).

purposes were followed carefully. After the experiment, the fish were carefully released back to their natural environment.

### 2.2 Experimental procedure

To simulate the macrozoobenthos contamination, *i.e.* the presence of macrozoobenthos eDNA in aquaria, individuals of *Dugesia sp.* and chironomid larvae (*i.e.* target macrozoobenthos) were used. These two groups were selected due to easy availability in large quantities, whether by direct sampling in

the field (*Dugesia* sp.) or by purchase from external breeder (Chironomidae). To ensure a large amount of eDNA (from different types of organisms), uncontrolled algae contamination was also added at the beginning of the experiment (placed into the aquarium using a hose). Before the onset of the experiment, fishes in Room 2 were fed with dried granulated fish feed that contained no invertebrates to avoid bias contamination during experiment (SAK Mix, Exot Hobby s r.o.).

The experiment itself took place in four experimental aquaria (Ae, Be, Ce, De) and one negative control (Ee). Before the start of the experiment, 150 ml of water from each aquarium was filtered using a hand sampling vacuum pump (Schenchen pump SC600, flow rate: 0–2280 ml/min) and nitrate cellulose filters (Nalgene™ Single Use Analytical Filter Funnels, 0.2 µm pore size) to check for the contamination. The target macrozoobenthos was then placed into four experimental aquaria in the fish breeding boxes, to avoid predation from fish. In each of the four aquaria, the same amount of *Dugesia* sp. (29 g), as well as, chironomid larvae (59 g) was added. The water was filtered after 17, 41, 65, 89, 113 and 137 hours from each of the four experimental aquaria to check the overall DNA concentration (Fig. 1). To prepare suitable conditions in the experimental aquaria, approximately stable eDNA concentrations was necessary.

The negative control aquarium (Ee) was treated in the same way as the experimental aquaria, except for the insertion of target macrozoobenthos. Three fish individuals were placed in Ee aquarium for 3 days. Afterwards, the fish skin mucus was wiped with two consecutive smears. The smears were taken from the same body part in each fish, using a forensic swab (not performed around the anus to avoid contamination of samples by faeces). Before the onset of the experiment, the negative control aquarium (Ee) served to test, if the water samples and the skin mucus of fish from this aquarium did not contain target macrozoobenthos DNA. Afterwards, Ee and Fe aquaria served as the control of fish survival during experiment.

Subsequently, three fish individuals were placed in each of the four experimental aquaria. The time scale of exposure of fish to eDNA (macrozoobenthos and algae) was: 24, 48 and 72 h. Such a time scale was set to detect when the eDNA appears on the fish skin mucus for the first time. After 24 h, the fish skin mucus was wiped with two consecutive smears (the same procedure as for the fish from Ee aquarium, see above). The wiped fish were subsequently placed into the aquarium Ee, and new specimens were taken from Room 2 and placed into the four experimental aquaria for 48 h. The same procedure was applied after 72 hours (Fig. 1). The fish were not fed during the experiment (*i.e.* their 24, 48 and 72 h stay in the four experimental aquaria). Once placed in the Ee/Fe aquarium (now used as the control of the fish survival), fish were fed again.

All persons involved in the experiment (including molecular analysis) wore full body protective laboratory clothing and surgical gloves. All surfaces in the fish laboratory were cleaned with bleach and denatured ethanol several times a day. Also, the molecular laboratory was frequently cleaned with bleach and denatured ethanol, and irradiated with high-intense UV light for one hour every morning. Samples for molecular analysis were isolated separately and handled under sterile UV hoods.

To identify whether foreign DNA can be captured on the fish skin mucus, only the first smears with eDNA were taken into account. To find out if the foreign DNA can be removed by repeated wiping of the skin mucus, first smears, as well as the first and the second smears with eDNA were taken into consideration. The presence of foreign DNA on the second smear was considered additional contamination only. Molecular analyses of actual contamination of the fish faeces by the mucus were not performed.

### 2.3 Molecular analysis: DNA extraction

Using sterile tweezers, filters were transferred to DNA-free Eppendorf tubes (2 ml) containing 96% molecular grade ethanol. Fresh, TNES preserved smears (Forensics swab in transport tube with ventilation membrane, DNA-free) from the fish were cut into DNA-free Eppendorf tubes (1.5 ml). Before DNA extraction, the filters were dried overnight in sterile Petri dishes and ripped into small pieces using sterile tweezers. DNA was extracted from both types of samples (filters, smears) in a PCR-free laboratory using a modified salt extraction protocol (Sunnucks and Hales, 1996; Weiss and Leese, 2016) with an increased proteinase K digestion time of 12 hours. After extraction, DNA was treated with 1 µl of RNase A (10 mg/ml, Canvax Biotech) to 30 µl of eluted DNA solution. Afterwards, samples were cleaned using the Nucleospin® gel and PCR Clean-up kit (Macherey-Nagel).

### 2.4 Molecular analysis: Concentration/presence of eDNA in aquaria

Purity (260 nm/280 nm) and total DNA concentration (ng/µl) in aquaria were determined by optical density spectrophotometry (BioDrop® Touch Duo Spectrophotometer) and quantified by using Qubit™ 4 Fluorometer (Thermo Fisher Scientific) with the 1x dsDNA HS Assay kit (Thermo Fisher Scientific). In addition to the general eDNA concentration, the presence of macrozoobenthos DNA (*i.e.* *Dugesia* sp. and Chironomidae) in aquaria was verified using the specific primer pairs and Sanger sequencing. These specific primers were designed for this experiment using Primer-BLAST (Tab. 1). GC scanning and the melting temperature (T<sub>m</sub>) were also verified in Vector NTI version 11 (Invitrogen, Carlsbad). The primers were designed according to standard criteria (Chuang *et al.*, 2013; Wu *et al.*, 2004), focusing on small fragment size (100–300 bp) and lower T<sub>m</sub>. Several genes (18S rRNA, COB, COI) were compared to find the most suitable region for the widest possible species representation. The resulting genus-specific and group specific primers were designed in the Cytochrome c Oxidase subunit I (COI) gene (Tab. 1) and the sequences of the compared sections were obtained from the NCBI database (Tab. S1).

Each PCR reaction consisted of illustra PuReTaq Ready-to-go PCR beads (GE Healthcare), 0.5 µM of primers and 10 ng of DNA. A range of annealing temperatures (ATs) similar to the calculated T<sub>m</sub> were tested in a gradient PCR (45 °C – 55 °C) and subsequently all PCRs were run with the following program: 95 °C for 5 min; 38 cycles of 95 °C for 30 s, 45 °C for 30 s (Duge primers) / 52 °C for 30 s (Chiro primers), and 72 °C for 1 min; and final extension at 72 °C for 5 min

**Table 1.** Primers developed and used for the quick verification of the target invertebrates' DNA (Chironomidae, *Dugesia* sp.) presence in the experimental aquaria.

Primer	Sequences (5'→3')	$T_m$	Expected product length
Chiro_for	GTAGAAAATGGAGCAGGAAC	45.6 °C	209 bp
Chiro_rev	GATCAAACAAATAGAGGTATTCGATC	51.8 °C	
Duge_for	TTTGTCTTTACATATTGCTGGT	46.5 °C	170 bp
Duge_rev	AARCAGCAGCTAAAACAGGAAGAG	49.7 °C	

(C1000 Touch™ Thermal Cycler). Selected PCR products were purified using a Thermosensitive Alkaline Phosphatase (FastAP; ThermoFisher) and Exonuclease 1 (Exo 1; ThermoFisher) according to manufacturer's instructions. For a quick verification, the selected samples were sequenced via conventional Sanger sequencing in a commercial laboratory (Eurofins Genomics GmbH). Alignments and the final matrix were completed in MEGA-X (Kumar *et al.*, 2018). Sequences were identified with BLAST searches (Basic Local Alignment Search Tool).

## 2.5 Molecular analysis: Presence of eDNA on the fish mucus

The smears were pooled for aquaria Ae + Be and Ce + De due to similar eDNA concentrations in these aquaria, respectively. Thus, higher concentrations of eDNA in the samples, as well as reduced sequencing costs were achieved. A total of 13 selected smear samples were sent for MiSeq sequencing (sample pooling scheme in Tab. S2). The COI gene region was amplified using the universal primers mCOLintF and jgHCO2198 (Geller *et al.*, 2013; Leray *et al.*, 2013). The PCR was performed using the HotStarTaq Plus Master Mix Kit (Qiagen) under the following conditions: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min, after which a final elongation step at 72 °C for 5 min was performed. Then, all amplicon products were mixed in equal concentrations and purified using calibrated Agencourt AMPure XP beads. The pooled and purified PCR product was used to prepare Illumina MiSeq DNA library according to the manufacturer's guidelines. Sequencing was performed at MR DNA ([www.mrdnalab.com](http://www.mrdnalab.com)) on a MiSeq following the manufacturer's guidelines.

Sequence data were processed using MR DNA analysis pipeline. Sequences were depleted of barcodes and primers, then sequences <150 bp were removed, as well as sequences with ambiguous base calls and with homopolymer runs exceeding 6 bp. Sequences were quality filtered using a maximum expected error threshold of 1.0 and dereplicated. The dereplicated or unique sequences were denoised; unique sequences identified with sequencing or PCR point errors were removed, followed by chimera removal, thereby providing zOTU (zero-radius Operational Taxonomic Units). In the case of zOTUs, all correct biological sequences were identified. This level of specificity is not achievable when using a 97% identity threshold as with traditional zOTUs. Final zOTUs were taxonomically classified using BLASTn against a curated database derived from NCBI and compiled into each taxonomic level into both "counts" and "percentage" files.

## 3 Results

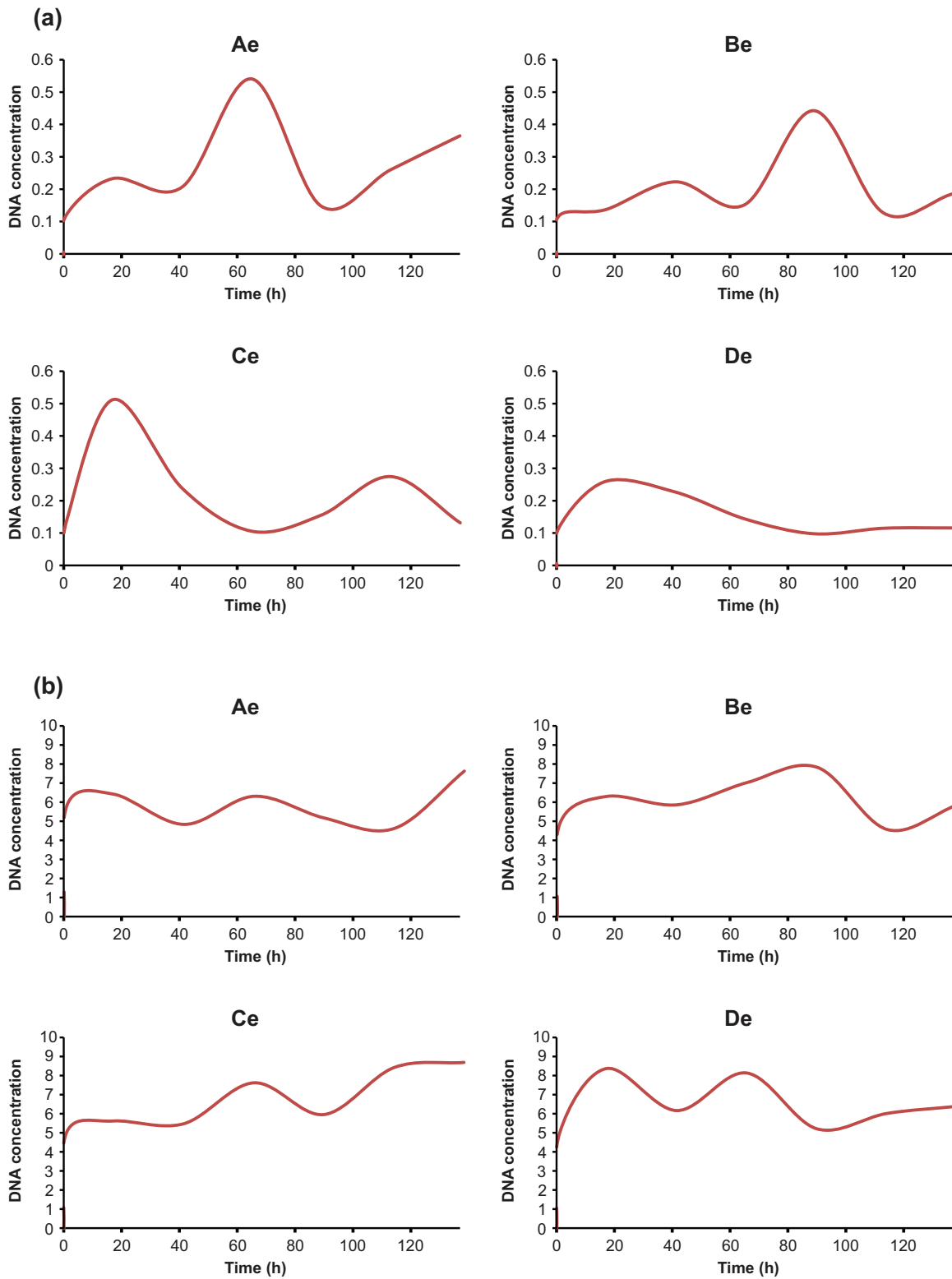
### 3.1 Concentration/presence of eDNA in aquaria

The concentration of DNA in all aquaria fluctuated over time mostly around 0.2 ng/μl and 6.0 ng/μl, depending on the method used, Qubit™ and BioDrop® respectively (Figs. 2a,b). The BioDrop® was used to identify purity of the samples and the difference between BioDrop/Qubit numbers indicates presence of contaminants. The Qubit™ showed final DNA concentrations that are not affected by the presence of other contaminants. However, the difference was observed in the time of maximum concentration per aquarium. Based on the Qubit™ numbers, aquaria Ae and Be reached the highest concentration on days 3 and 4, whereas Ce and De had their maxima immediately after 20–24 h. The highest measured concentration (Qubit™) was 0.55 ng/μl for aquarium Ae, 0.45 ng/μl for aquarium Be, 0.51 ng/μl for aquarium Ce and 0.27 ng/μl for aquarium De (Fig. 2a).

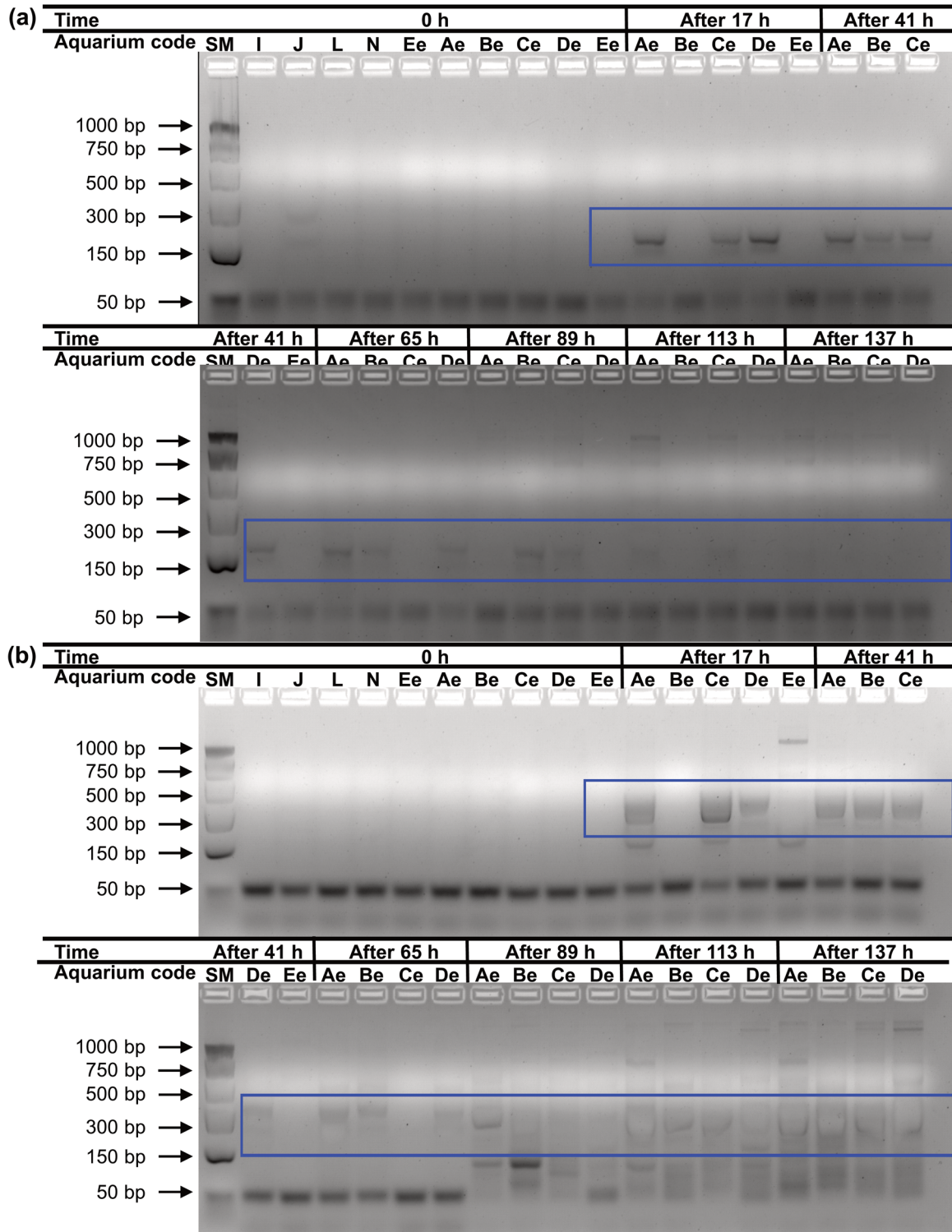
Genus-specific primers designed for *Dugesia* sp. (Duge\_for/rev) appeared to be effective to detect not only the tissue samples of this target macrozoobenthos (Fig. S1) but also the eDNA of *Dugesia* sp. from filter samples (Fig. 3a). Selected PCR products (Ae after 17 h, and Ae after 65 h sample – Fig. 3a) matched with the sequence of *Dugesia* sp. at 99% (Tab. S1). However, group specific primers designed for Chironomidae (Chiro\_for/rev) were problematic. These primers worked for the tissue samples (Fig. S1); however, they failed to detect eDNA of Chironomidae from filter samples (Fig. 3b). It was not possible to obtain a sufficiently good-quality sequence from Sanger sequencing (due to unclear and overlapping peaks) before using an Illumina MiSeq platform. Thus, the eDNA presence of Chironomidae in aquaria could only be assumed based on the expected length of the fragments on the agarose gel (Fig. 3b). In both cases, genus-specific primers and group specific primers were able to detect presence of target macrozoobenthos in the aquaria right after 17 hours (Fig. 3a, b).

### 3.2 Presence of eDNA on the fish mucus

The eDNA was present on smears after all three time periods (*i.e.* 24, 48, 72 h; Tab. 2). Sequencing of amplified DNA from fish smears generated from 92146 to 245944 raw reads per sample (Tab. S3) with an average read length of 300 bp (299–301 bp). Post filtering reads were mapped to 7 phyla and 32 genera assigned to the 53 sequence zOTUs, of which the target contaminants represented 5 or 6 zOTUs (zOTU\_1–zOTU\_6; Tab. S4). The DNA quality or partial degradation on



**Fig. 2.** DNA concentration in experimental aquaria over time (hours). (A) Qubit™ 4 Fluorometer (ng/μl). (B) BioDrop® Touch Duo Spectrophotometer (ng/μl).



**Fig. 3.** The results of 3% agarose gel electrophoresis with the target fragments bounded by a rectangle: (A) Results of the eDNA filters for *Dugesia* sp. (B) Results of the eDNA filters for chironomid larvae. Abbreviations as in [Figure 1](#); also SM – DNA Ladder (Promega).

**Table 2.** Relative abundance (%) of zOTUs with the most relevant taxonomic level identified on the first or on the first and second fish smear. Reduction in the number of DNA copies from the first to the second smear is marked in green. Increase in DNA copies from the first to the second smear is marked in pink. Abbreviations: AeBe-1s – pooled sample from experimental aquaria Ae and Be, first smear; AeBe-2s – pooled sample from experimental aquaria Ae and Be, second smear; CeDe-1s – pooled sample from experimental aquaria Ce and De, first smear; CeDe-2s – pooled sample from experimental aquaria Ce and De, second smear;

zOTU	Taxonomy (class)	% Homology	Smears after 24 hours				Smears after 48 hours				Smears after 72 hours			
			24AeBe- 24AeBe- 24CeDe- 24CeDe-		48AeBe- 48AeBe- 48CeDe- 48CeDe-		72AeBe- 72AeBe- 72CeDe- 72CeDe-		48AeBe- 48AeBe- 48CeDe- 48CeDe-		72AeBe- 72AeBe- 72CeDe- 72CeDe-			
			1s	2s	1s	2s	1s	2s	1s	2s	1s	2s	1s	2s
zOTU_1	Insecta	77.82	0	0.250*	0	0	0.163	0.085	0	0	1.823	0.776	0	0
zOTU_2	Insecta	100	0	0	0.115	0	0	0	0	0	0	0	0	0
zOTU_3	Chrysophyceae	84.11	0	0	0	0.014	0.010	0	0	0.709	0.041	0.193	0	0
zOTU_4	Phaeophyceae	79.47	0	0	0	0.068	0	0	0	0.077	0.215	0	0	0
zOTU_5	Chrysophyceae	84.97	0.109	0	0	0	0	0	0	0	0	0	0	0
zOTU_27	Aves	100	0.046	0	0	0	0	0.020	0	0	0	0	0.190	0
zOTU_33	Agaricomycetes	73.33	0	0	0	0	0	0.242	0	0	0	0	0	0
zOTU_35	Oomycetes	85.47	0.049	0.175	0	0	0	0	0	0	0	0	0	0
zOTU_36	Oomycetes	85.33	0.181	0	0.106	0.232	0	0	0	0	0	0	0	0
zOTU_38	Hydrozoa	84.35	0	0	0	0	0	0	0	0	0	0	0.267	0
zOTU_39	Hydrozoa	81.36	0	0	0	0	0	0	0	0	0	0	0.218	0
zOTU_40	Protura	100	0.400	0.486	0.273	0.109	0	0	0.032	0.094	0	0	0.098	0.239
zOTU_45	Insecta	82.19	0.248	0.212	0	0	0	0	0	0	0	0	0	0
zOTU_46	Insecta	83.87	0	0	0.063	0.089	0	0	0	0	0	0	0.020	0.015
zOTU_49	Insecta	91.67	0.003	0	0.006	0	0	0	0.043	0	0	0	0	0
zOTU_50	Insecta	82.98	0.020	0.003	0.011	0.003	0	0.005*	0.109	0	0.055	0	0	0
zOTU_52	Insecta	80.45	0.084	0	0	0	0	0	0	0	0	0	0	0

\* excluded from evaluation of the second aim of this study, due to absence of eDNA on the first smear

the fish skin mucus were likely to lead to changes/inaccuracies in zOTU richness (zOTU\_1 and zOTU\_16–zOTU\_26). The zOTU\_1 most likely belonged to Chironomidae (78% homology, Diptera), and the zOTUs\_16–26 to *Homo sapiens* (~80% homology, Primates; Tab. S4).

The vast majority of the reads from all smears was assigned to *Rutilus rutilus* (58.8–99.6%). Invertebrates represented 0.96% on average, of which the target taxa of macrozoobenthos represented 0.27% on average. The target macrozoobenthos taxa on the smears were represented only by Chironomidae (Tab. S4), although DNA from *Dugesia* sp. was present in the aquaria during the concentration measurements (Fig. 3a). Algae represented 0.32% on average. Despite the effort to maintain a sterile environment, human DNA, as well as DNA from Aves and Fungi were also captured in the smears (Tab. S4). Based upon the percent identity for most of the gained zOTUs, the most relevant taxonomic level was class (based on the percentage of homology  $\geq 80\%$ ; Tab. S4).

Cases, when the foreign DNA occurred only on the second smear (*i.e.* when no DNA was detected on the first smear;  $n = 17$ ) were excluded from further evaluation. This contamination contained mainly Fungi and various terrestrial groups of animals (Tab. S4). The relative abundance of zOTUs decreased (or was completely reduced) between the first and the second smear in 78.8% of cases and increased in 21.2% of cases (Tab. 2). The decrease was observed in the following target taxa: order Diptera (zOTU\_1, 2) and group Algae (zOTU\_3, 4, 5), as well as in non-target taxa, *e.g.* orders Hymenoptera and Odonata (zOTU\_49, 50 respectively), and class Aves (zOTU\_27; Tab. 2).

In the negative control aquarium (Ee), no target species of macrozoobenthos and algae were found on the smears. In addition to the zOTUs of *Rutilus rutilus*, only 2 other sequences of zOTUs were present in the samples of fish skin mucus from this aquarium, namely zOTU\_48 and zOTU\_50 belonging to Hemiptera and Odonata, respectively (Tab. S4). Fish survival rate after manipulation during the experiment was 97.4%.

## 4 Discussion

Both the detectability and concentration of the organisms' eDNA depend on many variables, such as production rate of individuals, environmental conditions, density of animals, and their residence time (Barnes and Turner, 2016; Goldberg *et al.*, 2016; Hering *et al.*, 2018; Pilliod *et al.*, 2014). After the initial concentrations of target macrozoobenthos eDNA have culminated, approximately stable eDNA concentrations were observed in all experimental aquaria. Although the target organisms were placed into each aquarium at the same time, the upper aquaria (Ae and Be) reached their maximum later than the lower aquaria (Ce and De; Figs. 1, 2a). The initial rapid increase in eDNA is not uncommon in experimental aquaria, and it is caused by physiological stress of organisms (Pilliod *et al.*, 2014; Thomsen *et al.*, 2012). Such an increase was observed for Ce and De aquaria where the largest increase in eDNA concentration was around 24 hours (Fig. 2a). The postponed maximum of eDNA concentration in Ae and Be aquaria can be explained by their location closer to the light source (Fig. 1). These light conditions could have some impact on the rate of eDNA degradation (Mächler *et al.*, 2018;

Strickler *et al.*, 2015), and on the other hand, promoted algal growth, which would correspond with a delayed peak of DNA concentration in Ae and Be aquaria. Higher occurrence of algae eDNA in Ae and Be aquaria can also be seen on the smears. In smears AeBe, algae were present in the samples after 24, 48 and 72 h, whereas in CeDe, smears algae were present only in one sample – after 72 h (Tab. 2).

Using group-specific primers, the eDNA of target organisms was captured immediately from the first filter – after 17 h. As the time progressed, a decreasing intensity of target taxa bands on the agarose gel and an occurrence of stutter bands was observed (Figs. 3a, b). This probably happened due to the increasing amount of inhibitors in aquaria or due to DNA degradation, which commonly occurs within eDNA samples (Schneider *et al.*, 2016; Stoeckle *et al.*, 2017). Although Chironomidae seemed to be an ideal group of target contaminants, because they can be obtained in large quantities, their primer design is challenging, mainly due to the limited number of sequences available in GenBank. Moreover, some species are known to hybridize (Martin, 2011).

Interestingly, eDNA from *Dugesia* sp. was not present on any smear, although it was present in the water (Fig. 3a). There are several possible explanations to this phenomenon, *e.g.* the use of non-specific primers. The mitochondrial gene COI is so variable in flatworms that the primers often have to be taxon-specific (Moszczynska *et al.*, 2009; Vanhove *et al.*, 2013). For example, when testing a new universal primer intended for eDNA monitoring of aquatic invertebrates, flatworms appeared to be a problematic group that could not be captured even after optimizing primers to a wide range of aquatic invertebrates (Leese *et al.*, 2021). Nevertheless, the development of new primers could also reveal presence of *Dugesia* sp. or other flatworms DNA on the mucus in the future. Another possible explanation is the time that had elapsed between the moments when the fish and *Dugesia* were placed into the aquaria (5.5 days). At that time, a settled layer of mucus from *Dugesia* sp. was present at the bottom of the aquaria. It is possible that the DNA remained stuck at the bottom and did not float in the water column, just like at the beginning of the experiment (Fig. 3a).

The results of our study clearly demonstrated that eDNA from other organisms is present on fish skin mucus (Tab. 2, S4), despite the mucus antimicrobial activity (Alexander and Ingram, 1992). If such contamination is found in experimental conditions, it will be probably present on the fish collected in the field, too. It is thus important to consider this finding, because such foreign DNA could be a source of bias for the results of diet analyses of faeces (Corse *et al.*, 2010; Guillerault *et al.*, 2017). However, careful wiping can be an effective way to decrease foreign DNA concentration from the surface of fish body. Indeed, the concentration of target (macrozoobenthos and algae) as well as non-target contaminant DNA decreased between the first and the second smear (Tab. 2, S4). Moreover, this procedure can be applied with minor harm to fish studied, considering the fish survival rate after experiment (97.4%). However, such a survival rate refers to experimental conditions only. In the wild the survival rate may be lower due to higher risk of infections (Svendsen and Bøgwald, 1997).

As the diet of many species is either unknown or consists of a broad array of taxa, mainly universal primers have been recently used for molecular diet analyses to allow

amplification of DNA from a wider range of eukaryotes (Elbrecht and Leese, 2017; Klymus *et al.*, 2017; Piñol *et al.*, 2015, Piñol *et al.*, 2018;2018). However, the use of universal primers brings a risk of bias to the results, and distinguishing individual diet components from the contamination may turn to be difficult. Indeed, the universal primers (Leray *et al.*, 2013) used in our study captured a high number of contaminations.

Although the rules to prevent contamination were strictly followed throughout the experiment, some degree of non-target contamination was present in the samples, *e.g.* fungi and terrestrial organisms (Tab. 2, S4). Fungi may invade living organisms and infect plants, animals, and humans (Eduard, 2009) and they are the most common contaminants found in the water and in the air (*e.g.*, Babič *et al.*, 2017; Pyrri *et al.*, 2020), and have even been detected in fish food (Namulawa *et al.*, 2020). The fungal and terrestrial organism's contamination in our study probably came from the air, being trapped on smears during sampling (these groups mostly occurred on the second smears; Tab. S4). The presence of bird DNA was also identified in the samples (zOTU\_27 *Gallus gallus* with 100% homology; Tab. S4). It probably came from the fish food, which contains egg products. Fish were not fed during the experiment, therefore the chicken DNA most likely originated from faeces (fish were fed before the onset of the experiment in the Room 2). Only one type of contamination (Odonata, zOTU\_50) found on smears from negative control aquarium (Ee) was present on almost all smears from experimental aquaria. Therefore, it is assumed that Odonata DNA came from the Room 2 aquaria (Tab. S4, Fig. 1). Cases when the relative abundance (%) of zOTUs increased between the first and second smear, as well as the presence of the foreign DNA on the second smear only were considered additional contamination (Tab. 2). This contamination arose from outside, probably during manipulation with fish.

Since the fish skin mucus in this experiment contained eDNA from various organisms, such as Diptera, Algae, Fungi, Coleoptera, Odonata, Hydrozoa and even Aves, it is assumed that skin mucus of fish from the wild is contaminated by eDNA of diverse organisms. Although the percentage of contamination appears to be quite low in some cases, the number of contaminants is several times higher in the natural environment. Moreover, the contaminants are in constant contact with the fish body.

The potential capacity of fish skin mucus to catch eDNA of other organisms found in our study suggests its potential use for screening the environment's biodiversity. The fish skin mucus could thus become an alternative to aquatic biofilms, that act as passive eDNA samplers of aquatic communities (Rivera *et al.*, 2022). Nevertheless, the constant renewal of mucus and the high mobility of fishes should be considered.

In conclusion, this study demonstrates that DNA of other organisms can be captured on the fish skin mucus, posing a risk of contamination, especially when faeces samples are taken by gently squeezing the abdomen area. It is assumed that the fish skin mucus is exposed to a large number of contaminants (DNA of other species) especially in natural conditions, and such DNA may be incorrectly analysed as a part of the fish diet spectrum. Therefore, it is important to minimize this risk as much as possible and this can be achieved simply by careful

cleaning of the anus area, with minor harm posed to the fishes examined. This study also demonstrates that just a simple but careful manual wiping (without the use of any cleaning agent) significantly reduces or removes the amount of foreign DNA, and thus eliminates the possible bias.

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## Data availability statement

The short COI fragments from tissue samples were sequenced by our designed primers (Tab. 1) and are deposited in NCBI's Genbank database (Sample D1: MZ605406; Sample CH1: MZ605405; Sample\_K\_Dugesia\_sp.: MZ883097; Sample\_U\_Dugesia sp.: MZ883098) (<https://www.ncbi.nlm.nih.gov/genbank/>). High-throughput sequence data are in NCBI's Sequence Read Archive under BioProject ID: PRJNA755506 (Tab. S3) (<https://www.ncbi.nlm.nih.gov/sra/>).

## Ethical approval

The standards set for fish rearing in laboratory conditions according to Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes were followed carefully. The permission for sample collection was granted by Ministry of Environment of the Slovak republic (license no. 16/2018).

## Supplementary Material

The Supplementary Material is available at <https://www.kmae.org/10.1051/kmae/2023013/olm>.

**Fig. S1:** Primer tested at the tissue samples extracted from 2 randomly selected individuals from the *Dugesia* sp. biomass (D1, D2) and from the chironomid larvae biomass (CH1, CH2). Abbreviations: SM – DNA Ladder (Promega); K – negative control.

**Table S1.** Homogeneity of gained sequences from tissue and eDNA samples with the most related taxa available in NCBI GenBank. Sequences (NCBI GenBank) used for primer design for *Dugesia* sp. and Chironomidae.

**Table S2.** Scheme of pooling samples from the experimental aquariums (Ae, Be, Ce, De) and negative control aquarium (Ee).

**Table S3.** Summary table of raw reads, reads clustered to zOTUs and number of zOTUs detected at all fish smears after various number of hours. Abbreviations: Ee-1s – 3 pooled samples of the first smear from three fish from negative control aquarium; AeBe-1s – pooled sample from experimental aquariums Ae and Be, first smear; AeBe-2s – pooled sample from experimental aquariums Ae and Be, second smear; CeDe-1s – pooled sample from experimental aquariums Ce and De, first smear; CeDe-2s – pooled sample from experimental aquariums Ce and De, second smear.

**Table S4.** Summary of all identified zOTUs with their percentage reads per sample after various number of hours. Homogeneity of gained zOTUs was identified via BLAST search. Abbreviations as in Table S3. For the assignment of individual zOTUs to taxonomic levels, please see Tab. S5.

**Table S5.** Taxonomic levels of each zOTU detected from the smears of fish skin mucus during experiment.

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