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Fish diversity assessment in the headwaters of the Volga River using environmental DNA metabarcoding

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Abstract

1. The headwaters of the Volga River exhibit large reaches with near-pristine conditions, and therefore long-term biodiversity monitoring of this catchment can provide rare and valuable information on a European lowland river. More specifically, freshwater fish species assemblages are a good indicator of ecosystem status, as they are particularly sensitive to environmental changes and hydromorphological alterations. Historical records show that the fish fauna of the Upper Volga has changed over time, both in species composition and in abundance. The construction of the Volga–Kama cascade (a series of large dams) has specifically affected the migration of diadromous species.
2. Environmental DNA metabarcoding offers a non-invasive approach to determine the number of species in an aquatic ecosystem, as well as their identity and distribution. This approach is especially useful for fish fauna surveys along large rivers and long-term biomonitoring, with the advantage of having no impact on the species and their habitats.
3. To infer the current fish species diversity and the spatial distribution of each species in the free-flowing section of the Upper Volga River, as well as in selected tributaries, an environmental DNA metabarcoding approach was applied, using three mitochondrial DNA markers. This method allowed the positive identification of 23 fish species and their respective distributions in the headwaters of the Volga.
4. This assessment provides a valuable example of the application of environmental DNA metabarcoding in a large river system, and constitutes a starting point for future investigations and long-term biomonitoring in the Upper Volga system. In addition, the results can also serve as a reference for fish diversity assessments of other large European lowland rivers, and can guide future conservation and management measures in the headwaters of the Volga.

KEYWORDS

biodiversity, catchment, ecological status, lowland river, monitoring

1 | INTRODUCTION

The Volga River is Europe's longest river, with a total length of more than 3,500 km. It drains a catchment of 1.4×10^6 km^2, including over 150,000 rivers, before flowing into the Caspian Sea. The headwaters of the Volga are relatively pristine, reflected by a fauna similar to that found in other near-pristine European lowland rivers (Leummens, 2016; Schletterer et al., 2019; Schletterer & Füreder, 2010; Schletterer, Füreder, Kuzovlev, Zhenikov, & Grigorieva, 2014). Mainly forests and peatlands cover the landscape of the Upper Volga River upstream of the city of Tver, and the water quality in these reaches has been classified between 'quite pure' and 'slightly polluted' (see Schletterer, Shaporenko, et al., 2019). Monitoring the biodiversity in the free-flowing stretch of the Volga headwaters can provide valuable information on a relatively undisturbed lowland river (Schletterer, Füreder, Kuzovlev, Zhenikov, & Zhenikov, 2016), providing a reference for bioassessments of other European lowland rivers affected by multiple human disturbances.

Fish diversity surveys of the Volga River date back more than 200 years (Baer, 1855; Kessler, 1877; Pallas, 1814) and continue to this day (Behning, 1924, 1928; Butorin & Mordukhai-Boltovskoi, 1978; Litvinov et al., 2009; Poddubny & Galat, 1995; Reshetnikov, 2002). A comparison with historical records shows that the fish fauna has changed in its species composition and its abundance (Górski, 2010). These changes are mainly due to human influence, such as overfishing and pollution, as well as the construction of the Volga–Kama cascade of dams, which has had drastic effects on the migratory continuity of diadromous species (Litvinov et al., 2009; Schletterer et al., 2018). The Volga River is mainly populated by eurytopic cyprinids, as well as common piscivorous fishes, with a total of 79 fish and lamprey species from 23 families occurring in this river (Schletterer, Kuzovlev, et al., 2018). Among these species, 60 are considered native to the Volga. Certain species are broadly distributed throughout the river system, whereas the occurrence of others is reach specific (Leummens, 2016). Fish species diversity and dominance change throughout a river depending on hydromorphological conditions and physico-chemical properties. This phenomenon is reflected in the concept of fish regions or fish zonation (Aarts & Nienhuis, 2003; Huet, 1959).

Freshwater fish assemblages can be a good bioindicator of ecosystem status owing to their vulnerability to environmental stressors and human disturbances (Dudgeon, 2010). Indeed, knowing the species composition of fish communities—that is, the presence or absence of particular species and their distributions—can provide valuable information for the protection of endangered species and vulnerable habitats (Arponen, Heikkinen, Thomas, & Moilanen, 2005). This can also help in the identification of invasive species (Didham, Tylianakis, Gemmell, Rand, & Ewers, 2007), which can have adverse effects on the ecosystem.

Environmental DNA (eDNA) is the DNA left by organisms in their environment, mainly through skin shedding or production of mucus, secretions, gametes, or even faeces (Deiner et al., 2017; Taberlet, Coissac, Hajibaei, & Rieseberg, 2012). Such DNA can persist in aquatic environments for days or even weeks (Dejean et al., 2011; Thomsen et al., 2012), depending on a number of abiotic and biotic conditions, such as oxygen level, light exposure, pH, and salinity, as well as enzymatic and microbial density (Barnes et al., 2014). In rivers, eDNA can be captured up to several kilometres downstream from the location of the donor organisms (Deiner & Altermatt, 2014; Jane et al., 2015). eDNA was initially used to detect one or a few species (Keskin, 2014; Nathan, Simmons, Wegleitner, Jerde, & Mahon, 2014; Takahara, Minamoto, & Doi, 2013; Wilcox et al., 2013), especially for conservation purposes (Atkinson et al., 2018; Boothroyd, Mandrak, Fox, & Wilson, 2016; Jerde, Mahon, Chadderton, & Lodge, 2011; Stoeckle, Kuehn, & Geist, 2016). However, more recently, eDNA has been used to investigate and describe the species composition of entire communities through metabarcoding, combined with high-throughput sequencing (Evans et al., 2016; Hänfling et al., 2016; Olds et al., 2016; Shaw, Weyrich, & Cooper, 2016; Thomsen et al., 2012). Implementing eDNA metabarcoding from water samples provides a non-invasive method to determine the number of species, their identity, and their distribution in an aquatic environment, which in turn provides information on ecosystem health. This approach has already proved to be an effective method to assess freshwater fish diversity for specific communities (Civade et al., 2016; Evans et al., 2017; Hänfling et al., 2016; Valentini et al., 2016), constituting a powerful non-invasive tool for biodiversity assessments and conservation projects in freshwater ecosystems (Hänfling et al., 2016; Thomsen & Willerslev, 2015).

The aim of this study was to survey the fish diversity of the free-flowing headwater reaches of the Volga River and selected tributaries, using eDNA. This study exemplifies the application of this method in a large river network—that is, from a small stream down to a large lowland river, and under different physico-chemical conditions. This biodiversity assessment of the Volga headwaters can provide a starting point for long-term eDNA monitoring of these stretches. In addition, the present species composition and the longitudinal distribution of particular species can be compared with historical data, providing valuable insights on ecosystem status, as well as evaluation of the potential to use the Volga headwaters as a reference for investigating other European lowland rivers.

2 | MATERIAL AND METHODS

2.1 | Sampling locations and eDNA extraction

The Volga River headwaters can be divided into three distinct hydromorphological reaches: the source region, the Upper Volga lakes, and the free-flowing section (Schletterer & Füreder, 2010). Based on previous records (e.g. from angling catches), the free-flowing section of the Volga is divided into two main fish regions: hyporhithral and epipotamal (Kuzovlev & Schletterer, 2006). In the hyporhithral region, the fish fauna is mainly dominated by brown trout (*Salmo trutta*), European grayling (*Thymallus thymallus*), Volga undermouth (*Chondrostoma variabile*), chub (*Squalius cephalus*), burbot (*Lota lota*)

and gudgeon (*Gobio gobio*), whereas in the epipotamal region the fish fauna is typically dominated by freshwater bream (*Abramis brama*), roach (*Rutilus rutilus*), rudd (*Scardinius erythrophthalmus*), asp (*Leuciscus aspius*), European perch (*Perca fluviatilis*) and northern pike (*Esox lucius*).

Sampling was conducted in August 2017, in the free-flowing section of the Upper Volga and selected tributaries, such as the Tvertsa and Tudovka rivers (Table 1). Most sampling locations correspond to those from the long-term research and monitoring programme 'REFCOND VOLGA' (Schletterer et al., 2016). The hydrochemistry of the Volga headwaters is influenced by the mires and peat bogs, whose high humic acid content can adversely affect eDNA quality (Stoeckle et al., 2017). Therefore, to provide a control, samples were also collected in a subcatchment with contrasting geochemistry, the Moksha River, a tributary of the Oka River, the largest right-hand tributary of the Volga (Figure 1).

In total, 60 eDNA samples were collected in 11 locations, across five rivers (Figure 1; Supporting information in Appendices A and B). In each location, four or eight samples were collected (amounting to either 1 or 2 L), depending on river size (see Supporting information in Appendix B), and a number of physico-chemical properties were measured (Table 1; Supporting information in Appendix C). One single sample corresponds to 250 ml of water, which was filtered from each sampling point, within a location, using a sterile microfibre filter GF/F (nominal pore size of 0.7 μm). All equipment involved in the sampling and filtering process (hand-driven filter assembly, i.e. syringe and filter housing) was carefully cleaned and decontaminated using bleach. In addition, 11 negative controls of double-distilled water (ddH_2O) were processed on site, in the same way for each sampling location. Each sample was filtered and subsequently placed in a tube with 700 μl of Longmire lysis buffer (Longmire, Maltbie, & Baker, 1997) for DNA preservation (Renshaw, Olds, Jerde, McVeigh, & Lodge, 2015), and stored for the first 2 weeks at room temperature and afterwards at −20°C until extraction.

In the laboratory, genomic DNA was extracted from the filters following, a modified phenol–chloroform–isoamyl alcohol protocol (Sambrook, Fritsch, & Maniatis, 1989) with ethanol precipitation (Renshaw et al., 2015). The tubes containing the filters and preservation buffer were first incubated for 10 min at 65°C, then 900 μl of phenol–chloroform–isoamyl alcohol (25 : 24 : 1) was added to each tube and mixed by short vortexing. The tubes were centrifuged at 15,000 *g* for 5 min and 700 μl of the upper aqueous layer was carefully transferred to a new tube for each sample, to which 700 μl of chloroform–isoamyl alcohol (24 : 1) was added, followed by a short vortexing. An additional centrifugation at 15,000 *g* for 5 min was performed, and 500 μl of the upper aqueous layer was transferred to a new tube for each sample, and then a mix of 1.25 ml of 100% ice-cold ethanol and 20 μl of 5 M sodium chloride was added to each tube. The tubes were gently mixed, and the DNA was precipitated overnight at −20°C. The tubes were then centrifuged at 15,000 *g* for 10 min and the liquid was carefully removed. Ethanol was eliminated by evaporation. Each DNA pellet was then dissolved individually in 100 μl of TE buffer 1X× (low ethylenediaminetetraacetic acid). The presence of genomic DNA and the DNA integrity were assessed on a TapeStation 2200 (Agilent) using Genomic ScreenTape (Agilent). Each sample was treated with the OneStep™ PCR Inhibitor Removal (Zymo Research).

2.2 | Two-step polymerase chain reaction-based amplicon amplification, Illumina library preparation, and MiSeq sequencing

For each sample, three partial mitochondrial genes were amplified: cytochrome *b* (Cyt *b*), 12S, and 16S genes, the latter two encoding

TABLE 1 River names, sampling locations, sampling date, and selected physico-chemical properties (water temperature, pH, oxygen level (O_2), conductivity, colour based on the chromium–cobalt scale)

River	Location	Sampling date (2017)	Water temp. (°C)	pH	O_2 (%)	Conductivity (μS cm^{-1})	Colour (° Cr–Co)	August mean flow discharge (m^3 s^{-1})	eDNA	Coordinates
Nochnaya	Nochnaya	Aug 16	14.8	7.36	97	111	339	<0.5	3	56°31′47.6″N 33°07′37.8″E
Tudovka	Istok	Aug 15	14.6	7.12	84	113	708	<0.5	3	56°26′17.9″N 33°05′19.5″E
Tudovka	3 Trubi	Aug 14	17.1	6.90	40	101	461	<0.5	3	56°28′57.9″N 33°05′59.8″E
Tudovka	Krasny Stan	Aug 16	15.1	7.26	75	121	339	<1	3	56°31′43.6″N 33°07′42.5″E
Tudovka	Redkino	Aug 17	17.5	7.60	130	187	n/a	<1.5	3	56°22′52.2″N 33°17′39.8″E
Tudovka	Molodoy Tud	Aug 13	19.3	8.52	146	304	153	1.65	2	56°25′16.9″N 33°36′28.4″E
Volga	Rzhev	Aug 13	21.4	8.60	133	223	119	58	7	56°15′31.7″N 34°19′12.2″E
Volga	Staritsa	Aug 13	22.2	8.19	133	241	128	90	8	56°30′44.1″N 34°55′33.2″E
Volga	Tver Migalovo	Aug 17	22.0	8.42	150	275	n/a	>100	8	56°50′53.7″N 35°46′40.9″E
Tvertsa	Mel'nikovo	Aug 18	20.6	8.34	101	218	n/a	>25	6	56°56′35.0″N 35°47′00.1″E
Moksha	Georgiyevskiy	Aug 20	n/a	n/a	n/a	n/a	n/a	n/a	3	53°34′18.9″N 44°22′15.6″E

For each sampling location, the mean flow discharge at the time of sampling (in August) is provided based on hydrological estimates compiled by Shiklomanov (1999), as well as the number of environmental DNA samples typed using high-throughput metabarcoding (for each location, one negative control was also processed together with the samples) and coordinates of the sampling locations.

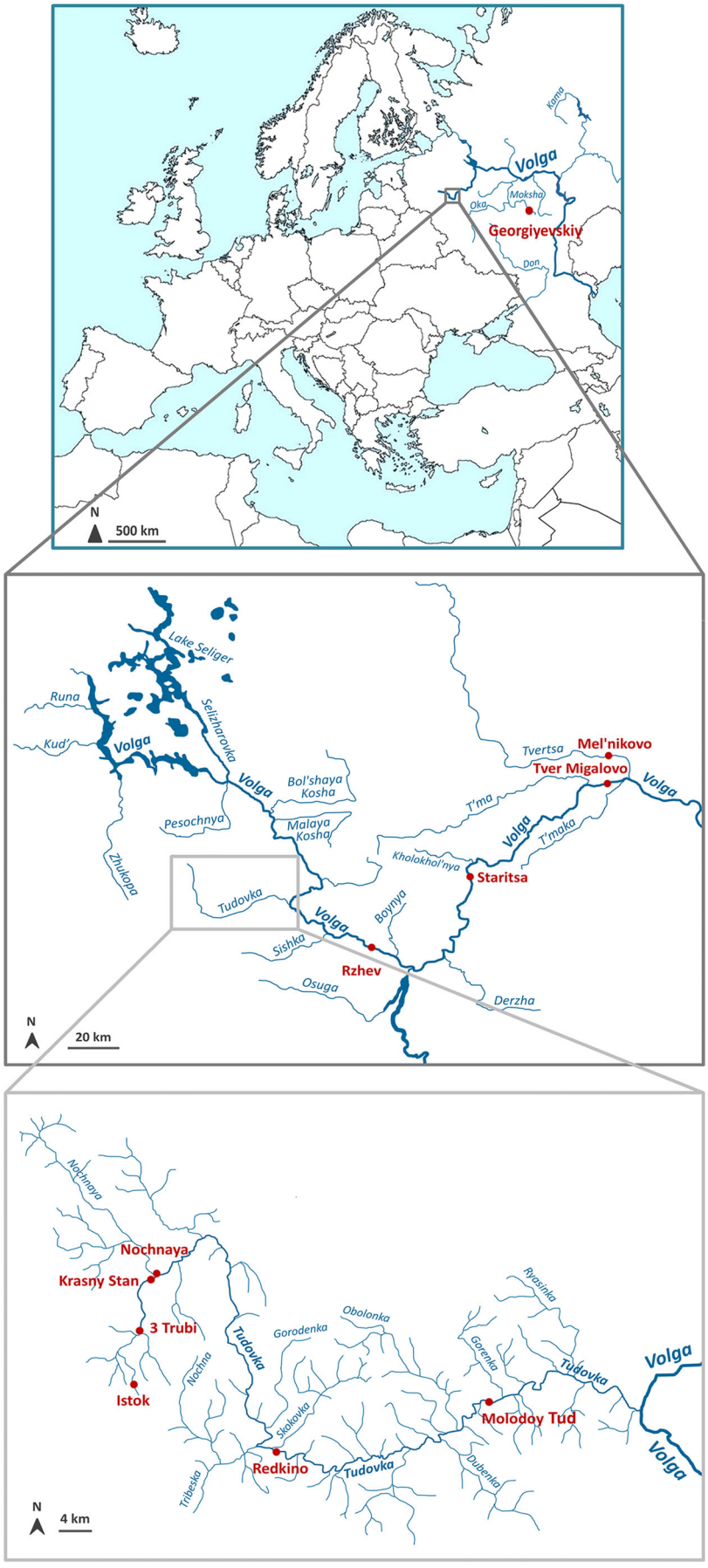

FIGURE 1 Maps of the sampling locations (red dots) included in this study. Rivers and lakes are depicted in blue, country borders are delineated in black, and seas are represented in light blue

for ribosomal RNA. These genes are among the most frequently used for vertebrate metabarcoding. For species identification, the polymerase chain reaction (PCR) primers used should bind in highly conserved regions of the genes, whereas the amplified sequence should exhibit adequate species-specific variability, and these three specific markers are known to have these characteristics. Using several markers increases the reliability of species identification, while minimizing taxonomic bias due to varying mismatches (Evans et al., 2016; Hänfling et al., 2016; Harper et al., 2018; Valentini et al., 2016). All three primer sets, used for the first-step PCR, were previously described: primers for Cyt *b* (L14735/H15149c) were designed by Burgener and Hübner (1998) (Table 2), and primers for 12S and 16S partial genes were developed by Evans et al. (2016) (Table 2). To incorporate the Nextera dual index (Illumina) in the second-step PCR, primers were modified by adding a sequence on the 5′ end of each primer (Table 2) (Olds et al., 2016). For PCR amplification, AccuStart II PCR ToughMix (Quantabio) reaction mix was used, which improves reproducibility, reduces the risk of contamination, and performs well in the presence of PCR inhibitors, which are often present in crude extracts from environmental samples.

The total volume for each PCR reaction was 25 µl, consisting of 12.5 µl of AccuStart II PCR ToughMix, 0.75 µl of each primer (Table 2), 6 µl of high-performance liquid chromatography (HPLC)-grade H_2O, and 5 µl of DNA template. Cycling conditions for the amplification of Cyt *b* gene consisted first of a denaturation step for 1 min at 95°C, then 20 cycles of 10 s at 95°C, 20 s at 55°C, and 30 s at 72°C, followed by an additional 30 cycles of 10 s at 95°C, 20 s at 53°C, and 30 s at 72°C, and a final extension step of 10 min at 72°C. Cycling conditions for the amplification of 12S and 16S amplicons consisted of a denaturation step for 1 min at 95°C, then 10 cycles of 10 s at 95°C, 20 s at 63°C, and 30 s at 72°C, another 10 cycles of 10 s at 95°C, 20 s at 60°C, and 30 s at 72°C, followed by an additional 30 cycles of 10 s at 95°C, 20 s at 58°C, and 30 s at 72°C, and a final extension step of 10 min at 72°C. To assess potential contamination (Schloss, Gevers, & Westcott, 2011), a positive control, in the form of a mock community sample, was also amplified for each marker, as suggested by Olds et al. (2016). The mock community was composed of six Indo-pacific marine fish species: clown anemonefish (*Amphiprion ocellaris*), two-spined angelfish (*Centropyge bispinosa*), bicolor blenny (*Ecsenius bicolor*), yellowspotted wrasse (*Macropharyngodon negrosensis*), peach fairy basslet (*Pseudanthias dispar*), and jewelled blenny (*Salarias fasciatus*).

PCR products were run on 2% agarose gel with peqGREEN dye (PEQLAB). The gel was visualized on an ultraviolet light plate, and each successfully amplified product was manually cut from the gel with sterilized razor blades. This procedure was repeated for negative controls, at the position in the gel where the expected size fragment would occur. Gel fragments were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega), following the provided protocol. The positive and negative controls were processed through the entire library preparation and sequenced, in the same manner as the eDNA samples.

For each sample, the purified PCR products of each gene were measured using a NanoDrop and then pooled in the following manner: 9 ng from Cyt *b*, 8.7 ng from 12S, and 7.3 ng from 16S, to obtain a total of 25 ng of DNA. The final volume was brought to a total of 5 µl by adding HPLC-grade H_2O. Adjusting the amount of DNA input for each marker helps compensate for the sequencing bias toward smaller fragments (Olds et al., 2016). Samples for which one of the markers, or more, could not be successfully amplified were discarded.

The second-stage PCR enables the incorporation of a unique index combination for each sample being processed and sequenced, allowing multiplexing. The total volume for each PCR reaction was 25 µl, consisting of 12.5 µl of AccuStart II PCR ToughMix, a unique combination of 2.5 µl of Nextera index 1 i7 (10 nmol ml^{-1}; Illumina) and 1 µl of Nextera index 2 i5 (10 nmol ml^{-1}; Illumina), 4 µl of HPLC-grade H_2O and 5 µl of the previous pooled amplified markers for each sample. Cycling conditions for the incorporation of the Nextera dual index consisted first of a denaturation step for 1 min at 95°C, followed by eight cycles of 10 s at 95°C, 20 s at 55°C, and 30 s at 72°C, and a final extension step of 10 min at 72°C.

The PCR products were cleaned by adding 50 µl AMPure XP beads (Beckman Coulter), incubating for 5 min, then 2 min on a magnetic stand, removing the supernatant, and washing the beads twice with 200 µl of 80% ethanol. The beads were dried and thoroughly resuspended in 27.5 µl HPLC-grade H_2O, incubated for 2 min and another

TABLE 2 Custom primer sets for the amplification of each marker in the first-stage polymerase chain reaction amplification (modified for the subsequent incorporation on Nextera dual index): primer sequence with the part section of the sequence binding to the target gene in bold, amplicon length and reference

Name	Marker	Primer sequence (5′ → 3′)	Amplicon length (bp)	Reference
NGS-L14912	**Cyt *b***	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**AAAAACCACCGTTGTTATTCAACTA**	413	Burgener & Hübner, 1998
NGS-H15149c		GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GCCCCTCAGAATGATATTTGTCCTCA**		
NGS-Ac12S-F	**12S**	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**ACTGGGATTAGATACCCCACTATG**	385	Evans et al., 2016
NGS-Ac12S-R		GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GAGAGTGACGGGCGGTGT**		
NGS-Ac16S-F	**16S**	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTTTTGCATCATGATTTAGC**	330	Evans et al., 2016
NGS-Ac16S-R		GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CAGGTGGCTGCTTTTAGGC**		

2 min on a magnetic stand, before transferring 25 µl of the supernatant to a new plate. The concentration of each sample was measured, and 100 ng of each sample was pooled into a single tube. In total, 61 samples were pooled, including 11 negative controls and one positive control. A final clean-up step was performed using the DNA Clean & Concentrator™-5 (Zymo Research), following the provided protocol. The sequencing of the finished library was performed by the Next-Generation Sequencing (NGS) Facility at Vienna Biocenter Core Facilities (Austria) on an Illumina MiSeq (reagent kit v3), to produce paired-end sequences with a length of 250 bp each. To mitigate the effects of sequencing a low-complexity library, and to improve the clustering on the MiSeq, 10% PhiX was added to the library by the sequencing facility.

2.3 | Species list and reference database construction

Prior to bioinformatic analysis, a list of all fish species known to occur between the source of the Volga in the Valdai Hills, and the Gorky dam, was compiled based on Schletterer, Kuzovlev, et al. (2018). This amounted to 43 species, among which 40 are native to the Volga (Table 3). For each species, additional information was included, such as their International Union for Conservation of Nature (IUCN) Red List status (IUCN, 2017), flow preference, pelagic zone inhabited, migration type, temperature range tolerance, as well as their tolerance to common environmental stressors, based on data compiled by Holzer (2008) (Table 3). In order to build a database of reference sequences, multiple sequences from GenBank (Clark, Karsch-Mizrachi, Lipman, Ostell, & Sayers, 2016) were retrieved for each of these species and each marker. Multiple sequences provide more reference diversity, and therefore facilitate taxonomic assignment in downstream analysis. Reference sequences of Cyt *b* were retrieved for every species, but no sequences could be found of the partial 12S and 16S markers for four species: blue bream (*Ballerus ballerus*), white-eye bream (*Ballerus sapa*), Siberian spiny loach (*Cobitis sibirica*), and asp. Sequences were trimmed using BioEdit (Hall, 1999) to retain only the sequence between the two primers used for each marker. The database was curated for each marker based on phylogenetic inferences performed in R (R Core Team, 2015), which allow the identification and removal of mislabelled or misidentified sequences from GenBank (Clark et al., 2016). The final reference database contained 300 sequences for Cyt *b*, 206 sequences for 12S, and 173 sequences for 16S (Supporting information in Supplementary file 1).

2.4 | Bioinformatic analysis and species detection

The raw reads were demultiplexed by the sequencing facility, based on the unique Nextera index combination for each sample. The BAM files were converted to FASTQ files using Bam2fastq tool v1.1.0 (Dexheimer, 2010). The overall quality of the reads for each sample was analysed with FastQC (Andrews, 2010). Subsequently, the reads were processed using a custom pipeline, specifically designed for metabarcoding data analysis and taxonomic assignment: metaBEAT v0.97.11 (metabarcoding and eDNA analysis tool, https://github.com/HullUni-bioinformatics/metaBEAT). Within the pipeline, sequences of each marker were extracted and analysed independently. Quality trimming of the reads was performed using Trimmomatic v0.32 (Bolger, Lohse, & Usadel, 2014). Reads shorter than 100 bp were discarded, and subsequently paired-end reads were merged, using FLASH v1.2.11 (fast length adjustment of short reads; Magoč & Salzberg, 2011). When forward and reverse reads could not be merged, only the forward reads were retained, as they typically have higher quality. Reads were clustered based on 100% identity using the software VSEARCH v1.1 (Rognes, Flouri, Nichols, Quince, & Mahé, 2016). Only clusters with coverage of at least five reads were kept for subsequent analyses. For each marker, the sequence of each cluster was compared with all sequences in the reference database using BLAST (Zhang, Schwartz, Wagner, & Miller, 2000). Taxonomic assignment followed a lowest common ancestor approach (Huson, Auch, Qi, & Schuster, 2007). Clusters were retained when the sequence had at least 80% identity, across at least 80% of the read length, with any sequence of the reference database. Sequences that did not meet these criteria were considered non-target sequences, and therefore discarded. For each retained cluster, only the most significant BLAST matches to the reference sequences database (top 5% bit scores) were considered, and the taxonomic assignment was made based on these matches. Species assignment was only made when a single species was among the best matches. If the query sequence matched to more than one species in its top matches, the sequence was assigned to the lowest shared taxonomic level among the most significant matches.

To avoid false-positive species detection through cross-contamination or barcode misassignment, the level of false-positive species detection in the positive control sample for each marker (any species other than the six species constituting the mock community) was used as a minimum threshold for considering species detection valid and real in the eDNA samples tested. To ascertain the presence of a species at a location, the species had to be detected with at least one marker with a higher number of reads than the minimum threshold. Only the presence or absence of species was determined. Deriving quantitative estimates from an eDNA metabarcoding approach remains problematic owing to a number of conditions influencing eDNA in the aquatic environment (Barnes et al., 2014; Jane et al., 2015; Lawson Handley, 2015), as well as various potential biases that can occur during sampling, laboratory processing, sequencing, and throughout the bioinformatic analysis (Ficetola et al., 2015; Yu et al., 2012). These biases affect the final read numbers, limiting a direct relationship between these numbers and the actual biomass or species density in the ecosystem.

3 | RESULTS

The eDNA library yielded good quality reads with a total of 12 M paired-end reads in the raw data. On average, each sample, including the mock community, had 260,000 paired-end reads, and negative

TABLE 3 List of the native and non-native fish of the Upper Volga River, based on Schletterer, Kuzovlev, et al. (2018), with species name, description reference, common name, IUCN status, flow preference, inhabited pelagic zone, migration type, temperature range, as well as tolerance to low oxygen concentration, pollution, and habitat degradation, based on data compiled by Holzer (2008)

								Tolerance to:[f]		
Species	Authority	Common name	IUCN status[a]	Flow guild[b]	Pelagic zone[c]	Migration type[d]	Temp. range[e]	Low [O_2]	Pollution	Habitat degradation
Native species										
Abramis brama	Linnaeus, 1758	Freshwater bream	LC	EURY	BP	POTA	EU	T	T	T
Acipenser ruthenus	Linnaeus, 1758	Sterlet	VU	RH	D	POTA	EU	M	M	M
Alburnus alburnus	Linnaeus, 1758	Bleak	LC	EURY	BP	POTA	EU	M	M	T
Ballerus ballerus	Linnaeus, 1758	Blue bream	LC	RH	BP	POTA	EU	M	M	IN
Ballerus sapa	Pallas, 1814	White-eye bream	LC	RH	BP	RESI	EU	T	T	T
Barbatula barbatula	Linnaeus, 1758	Stone loach	LC	RH	D	POTA	EU	M	M	M
Blicca bjoerkna	Linnaeus, 1758	White bream	LC	EURY	D	POTA	EU	T	T	T
Carassius carassius	Linnaeus, 1758	Crucian carp	LC	LIMNO	D	POTA	EU	T	T	T
Chondrostoma variabile	Yakovlev, 1870	Volga undermouth	LC	RH	BP	RESI	EU	IN	IN	IN
Cobitis sibirica	Gladkov, 1935	Siberian spiny loach	NE	EURY	D	RESI	EU	M	M	M
Cobitis taenia	Linnaeus, 1758	Spined loach	LC	RH	D	POTA	EU	M	M	M
Coregonus albula	Linnaeus, 1758	Vendace	LC	LIMNO	BP	ANA	ST	IN	M	IN
Coregonus maraenoides	Berg, 1916	Peipsi whitefish	NE	LIMNO	BP	RESI	ST	IN	M	IN
Coregonus megalops	Widegren, 1863	Lacustrine fluvial whitefish	LC	LIMNO	D	RESI	ST	IN	M	IN
Coregonus vessicus	Dryagin, 1932	Beloye cisco	NE	LIMNO	BP	RESI	ST	IN	M	IN
Cottus gobio	Linnaeus, 1758	European bullhead	LC	RH	D	POTA	ST	IN	IN	IN
Esox lucius	Linnaeus, 1758	Northern pike	LC	EURY	D	POTA	EU	M	M	T
Gobio gobio	Linnaeus, 1758	Gudgeon	LC	RH	BP	POTA	EU	IN	M	T
Gymnocephalus cernua	Linnaeus, 1758	Ruffe	LC	EURY	D	POTA	EU	M	M	T
Lampetra planeri	Bloch, 1784	Brook lamprey	LC	RH	D	POTA	ST	IN	IN	IN
Leucaspius delineatus	Heckel, 1843	Belica	LC	LIMNO	P	POTA	EU	M	M	M
Leuciscus aspius	Linnaeus, 1758	Asp	LC	RH	BP	POTA	EU	M	M	M
Leuciscus idus	Linnaeus, 1758	Ide	LC	RH	BP	POTA	EU	M	M	M
Leuciscus leuciscus	Linnaeus, 1758	Common dace	LC	RH	BP	POTA	EU	M	M	M
Lota lota	Linnaeus, 1758	Burbot	LC	EURY	D	POTA	ST	IN	M	M
Misgurnus fossilis	Linnaeus, 1758	Weatherfish	LC	LIMNO	D	POTA	EU	T	T	IN
Neogobius melanostomus	Pallas, 1814	Round goby	LC	EURY	D	AMPHI	EU	M	T	T
Osmerus eperlanus	Linnaeus, 1758	European smelt	LC	EURY	N	ANA	ST	M	M	M
Pelecus cultratus	Linnaeus, 1758	Sichel	LC	EURY	P	ANA	EU	IN	M	M
Perca fluviatilis	Linnaeus, 1758	European perch	LC	EURY	D	ANA	EU	M	M	T
Phoxinus phoxinus	Linnaeus, 1758	Common minnow	LC	RH	D	POTA	ST	IN	M	IN
Rutilus rutilus	Linnaeus, 1758	Roach	LC	EURY	BP	POTA	EU	T	T	T
Salmo trutta	Linnaeus, 1758	Brown trout	LC	RH	N	ANA	ST	IN	IN	IN
Sander lucioperca	Linnaeus, 1758	Pike-perch	LC	EURY	P	POTA	EU	M	M	M
Sander volgensis	Gmelin, 1789	Volga pike-perch	LC	EURY	D	RESI	EU	M	M	M
Scardinius erythrophthalmus	Linnaeus, 1758	Rudd	LC	LIMNO	BP	POTA	EU	T	M	M
Silurus glanis	Linnaeus, 1758	Wels	LC	EURY	BP	RESI	EU	M	M	T
Squalius cephalus	Linnaeus, 1758	Chub	LC	EURY	BP	POTA	EU	M	M	T

(Continues)

TABLE 3 (Continued)

Species	Authority	Common name	IUCN status[a]	Flow guild[b]	Pelagic zone[c]	Migration type[d]	Temp. range[e]	Tolerance to:[f]		
								Low [O_2]	Pollution	Habitat degradation
Thymallus thymallus	Linnaeus, 1758	European grayling	LC	RH	BP	RESI	ST	IN	IN	IN
Tinca tinca	Linnaeus, 1758	Tench	LC	LIMNO	D	POTA	EU	T	T	IN
Non-native species										
Anguilla anguilla	Linnaeus, 1758	European eel	CR	EURY	D	CATA	EU	IN	IN	M
Carassius auratus	Linnaeus, 1758	Goldfish	LC	LIMNO	BP	POTA	EU	T	T	T
Coregonus peled	Gmelin, 1789	Peled	LC	LIMNO	D	ANA	EU	T	M	T

[a]LC: Least Concern; VU: Vulnerable; CR: Critically Endangered; NE: Not Evaluated.

[b]RH: rheophilic; EURY: eurytopic; LIMNO: limnophilic.

[c]N: neritic; P: pelagic; BP: benthopelagic; D: demersal.

[d]RESI: resident; POTA: potamodromous; CATA: catadromous; ANA: anadromous; AMPHI: amphidromous.

[e]EU: eurythermal; ST: stenothermal.

[f]IN: intolerant/low tolerance; M: medium tolerance; T: tolerant; ?: unknown.

controls had on average 5,000 paired-end reads each (refer to the detailed summary in Supporting information, in Appendix D). The highest level of false-positive detection in the mock community was higher than the highest level of false detection of species from the eDNA samples, and therefore this level was used as a minimum threshold for valid species identification in each sample. For the Cyt *b* marker, the highest number of reads corresponding to a false-positive identification was 28, representing 0.05% of the reads in the mock community sample. For the 12S marker, a maximum of 650 reads corresponded to a false positive (1.3% of the total reads), and for the 16S marker, 10 reads were associated with a false positive (0.08% of the total reads).

3.1 | Species detected

Out of the 43 native and non-native fish species historically known to occur in the Volga River headwaters (Table 3), 23 were successfully and unambiguously detected when combining results from all sampling locations (Table 4). All 23 detected species were native to the Volga, and all are listed as Least Concern in the IUCN Red List database (Table 3; IUCN, 2017). One species classified as Vulnerable, sterlet (*Acipenser ruthenus*), and one Critically Endangered species, European eel (*Anguilla anguilla*), introduced to the Volga, as well as three species for which the status has not been evaluated by the IUCN—Siberian spiny loach, Peipsi whitefish (*Coregonus maraenoides*) and Beloye cisco (*Coregonus vessicus*)—were not detected, whereas the remaining undetected species (N = 16) are also listed as Least Concern.

Among the 23 identified species, 12 belong to the order Cypriniformes, nine of which are from the family Cyprinidae. Among the detected species, 10 are listed as eurytopic, defined by their ability to tolerate a wide range of ecological conditions, 10 are rheophilic, reflecting their preference to live in fast-flowing water, and only three species are limnophilic, indicating their association with lentic or slow-moving water (see Table 3). Most of the identified species in the samples are potadromous (migration within fresh water), two species are considered non-migratory residents, and three species are diadromous (one amphidromous and two anadromous) (see Tables 3 and 4), which is only one-third of the number of diadromous species historically known to occur in the Volga headwaters. Only six of the detected species are stenothermal; however, the ratio of stenothermal species to eurythermal species is similar to what is known from historical records (see Table 3), indicating that stenothermal species present the same likelihood of being detected through eDNA metabarcoding. Thirteen identified species are demersal, seven are benthopelagic, two are pelagic, and one is neritic. Eight species known to be intolerant to habitat degradation were detected, as well as eight species intolerant to low levels of oxygen and five species intolerant to pollution. In total, 10 species are intolerant to one or more of these environmental stressors and five are intolerant to all (Table 3).

3.2 | Species distributions

The combined results revealed an average of about 11 species per sampling location (Table 4). In the Nochnaya River, a tributary of the Tudovka River, 13 species were detected. In the Tudovka River itself, the number of species increased from six at the uppermost location (Istok) to 11 at the lowermost location (Molodoy Tud) (Table 4). In the Tudovka River, 14 species were detected when combining all sites. The highest number of species detected at a single site (N = 17) occurred in the uppermost sampling site of the Volga River (Rzhev), with the river gradually losing one species per site going downstream (Table 4). In total, 21 species were detected in the Volga River, by combining the results of all sampling locations. In the Tvertsa River, which enters the Volga at the end of the free-flowing section of the Upper Volga (Tver), 13 species were identified. Eight species were found in the Moksha River (Table 4). The pattern of

TABLE 4 Summary list of the species detected (when combining the results from all markers) using environmental DNA samples from the Volga River headwaters[a]

	Nochnaya	Tudovka					Volga			Tvertsa	Moksha	
Species	Nochnaya	Istok	3 Trubi	Krasny Stan	Redkino	Molodoy Tud	Rzhev	Staritsa	Tver Migalovo	Mel'nikovo	Georgiyevskiy	Total
Abramis brama				✓	✓		✓	✓	✓	✓		6
Alburnus alburnus		✓	✓		✓	✓	✓	✓	✓	✓	✓	9
Barbatula barbatula	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11
Blicca bjoerkna	✓						✓	✓	✓	✓		5
Chondrostoma variabile							✓		✓			2
Cobitis taenia							✓	✓		✓		3
Cottus gobio	✓				✓	✓	✓	✓	✓	✓		7
Esox lucius	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11
Gobio gobio	✓				✓	✓	✓	✓	✓	✓	✓	8
Gymnocephalus cernua	✓			✓	✓	✓		✓	✓	✓		7
Lampetra planeri	✓										✓	2
Leucaspius delineatus	✓						✓		✓			3
Leuciscus idus			✓			✓	✓	✓	✓			5
Lota lota	✓	✓	✓	✓	✓		✓					6
Misgurnus fossilis								✓				1
Neogobius melanostomus									✓			1
Perca fluviatilis	✓			✓			✓	✓	✓	✓	✓	7
Phoxinus phoxinus	✓				✓	✓						3
Rutilus rutilus	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11
Salmo trutta	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	11
Sander lucioperca								✓				1
Thymallus thymallus						✓	✓					2
Tinca tinca							✓	✓		✓		3
Total	13	6	7	8	11	11	17	16	15	13	8	

[a]The species name, the name of the river and location in which the species was detected (when combining all the samples of one location) are shown, as well as the total number of locations in which each species were detected, and the total number of species identified in each location. River names and sampling locations are arranged from left to right, following an upstream to downstream direction.

the results was very similar across all markers (Supporting information in Appendix E).

Based on the combined results, the most widespread species in the free-flowing section of the Volga headwaters are brown trout, roach, northern pike, stone loach (*Barbatula barbatula*), bleak (*Alburnus alburnus*) and gudgeon, being detected in 70–100% of the sampling locations (Table 4). Although the seven predominant fish species in the Upper Volga are expected to be freshwater bream (*Abramis brama*), roach, blue bream, white bream (*Blicca bjoerkna*), sichel (*Pelecus cultratus*), European perch, and pike-perch (*Sander lucioperca*) (Litvinov et al., 2009), neither blue bream nor sichel were detected, and pike-perch was only identified in one location. Within the Volga, three of the species identified were only detected at one sampling location: weatherfish (*Misgurnus fossilis*), round goby (*Neogobius melanostomus*), and pike-perch (Table 4); however, weatherfish was detected in multiple samples within this location (Supporting information Appendix F). Volga undermouth and brook lamprey (*Lampetra planeri*) were only detected with 12S, but they were both detected in two different locations. On the other hand, 16S allowed the detection of Ide (*Leuciscus idus*), which was detected in five separate locations and in 16 different samples.

3.3 | Marker and sample efficiency

Individually, none of the three markers detected unambiguously all 23 species. However, 12S detected 21 of the 23 species and an average of about nine species per site. 16S detected 18 species, with an average of seven species per site, and Cyt *b* allowed the detection of 16 species, with an average of six species per site. Cyt *b* was therefore the least efficient at species detection, and 12S was the most successful. However, all markers identified important parts of the biodiversity,

and combined they provided a more complete picture of the fish fauna and its distribution in the Volga River headwaters. Moreover, when comparing the total number of species identified per sample, for each location (Supporting information in Appendix F), and the total number of species identified per location (Supporting information in Appendix E), it clearly shows that the species diversity is rarely fully described with one sample. The overall diversity of a sampling location is determined by combining the results of several samples, taken at the same general location, but in different specific places of the river: close to the bank or 10–15 m away (on the right or left side) or slightly upstream or downstream of the sampling location. These multiple samples act as near-replicate samples for one location, while optimizing the potential of eDNA recovery.

3.4 | Environmental conditions

During the sampling period, the prevailing ionic composition of the Volga headwaters was hydrocarbonate–calcium. The mean water temperature at the sampling sites of the Volga and Tversa rivers was 21.5°C, and 16.4°C for the Tudovka River. The mineralization of water increased downstream owing to an increase in depth and drainage of more mineralized groundwater. The upper reach of the Tudovka River is characterized by a high concentration of peat bogs, resulting in acidity and low mineralization, as well as saturation with humic substances. During summer, in the middle and lower reaches of the Tudovka River, the water is oversaturated with oxygen, resulting from intensive photosynthesis of aquatic plants (Table 1). The lowest levels of oxygen and pH values occurred in waters flowing from marshes.

4 | DISCUSSION

This study provides the first assessment of the diversity of the fish assemblage, in the headwaters of the Volga River, using eDNA metabarcoding. This provides a partial baseline for future long-term monitoring in this system and a valuable example of the application of this molecular-based method in a large lowland river system.

4.1 | Relevance for conservation and management

Freshwater river systems are among the most threatened ecosystems worldwide (Dudgeon et al., 2006), and legislation such as the European Water Framework Directive (Council of the European Communities, 2000) has been created with the aim of protecting these systems from further degradation, as well as of improving the ecological health of systems that have already become degraded. Ecological monitoring is a key component in the implementation of such long-term legislative goals. Thus far, in Europe, the qualitative status of so-called 'biological quality elements', such as fish (Geist, 2014) or macrozoobenthos (Hering, Feld, Moog, & Ofenböck, 2006), plays a key role in assigning both the current ecological status of an aquatic system, and predicting and documenting degradation or improvement in the future (Birk et al., 2012; Gieswein, Hering, & Lorenz, 2019). The development of a sound methodology to achieve these goals in large rivers has proved to be a major challenge, especially because of the lack of baseline data or so-called 'reference states', as degradation is almost always defined in terms of the deviation from an expected non-degraded state (Kelly, Chiriac, Soare-Minea, Hamchevici, & Birk, 2019; Pardo et al., 2012). The use of an eDNA metabarcoding approach to improve the bioassessment of aquatic habitats is considered to have great potential (Carew, Miller, & Hoffmann, 2011; Lefrançois et al., 2018; Stein et al., 2014). The non-invasive sampling, the reductions in cost, and the potential ease of standardization and repeatability, and thus the increase in objectivity, have drawn the interest of a broad range of bioassessment stakeholders. In this regard, an eDNA metabarcoding survey of the least-disturbed Upper Volga River, upstream of the city of Tver, is particularly interesting, if the results are sufficiently accurate for the goals of long-term monitoring. In this light, the results of this survey highlight both strengths and suggestions for improvement focusing on methodological limitations.

4.2 | Species detected and their distribution

The 23 species detected represent more than half of the fish species presumed to occur in the Upper Volga River, based on historical records (Behning, 1924; Butorin & Mordukhai-Boltovskoi, 1978; Reshetnikov, 2002; Schletterer, Kuzovlev, et al., 2018). All of the species detected are native to the Volga system, and are largely consistent with the expected species composition of this specific reach (Litvinov et al., 2009; Poddubny & Galat, 1995; Schletterer, Kuzovlev, et al., 2018; Schletterer, Shaporenko, et al., 2019). The highest number of species identified was in the Volga River itself, compared with lower numbers in its tributaries. The total number of species increased from upstream to downstream for each river, when all sampling locations of a river are combined, as well as within the Tudovka River when looking at the total species number for each sampling location; however, the higher number of species in the Volga could also result from the transport and aggregation of eDNA from nearby upstream tributaries. Similarly, this could partly explain the increasing number of species in the Tudovka River, following a downstream gradient. For instance, brown trout is known to occur throughout the headwaters of the Volga River, but mainly in its tributaries (Viktorov, Kirillov, Nezdolii, & Sokolov, 2002); therefore, the constant signal of the species along all sampling sites is most likely the result of brown trout eDNA entering the main channel of the Volga via tributaries.

When considering the entire Upper Volga area, the predominant fish species are expected to be freshwater bream, roach, blue bream, white bream, sichel, European perch, and pike-perch (Litvinov et al., 2009). From this list, roach was indeed found at all sites, and white bream, freshwater bream, and European perch were found at five, six, and seven of the 11 sampled sites, respectively, and in all three sampling sites of the Volga. Pike-perch was only found in one of the three sampling location of the Volga and is not expected to occur in tributary habitats. Sichel, although listed in Litvinov et al. (2009) as

common in the Upper Volga, would in reality either occur in the uppermost reservoir (the semi-natural Upper Volga lakes, several hundred kilometres upstream from the study area), or could appear in river reaches during spawning migrations (May/June), and thus are not likely to be present in August, when the sampling was carried out for this study. Thus, among the dominant species reported in Litvinov et al. (2009), only the blue bream was unexpectedly not detected. Within the main channel of the Volga, European grayling was not detected downstream of Rzhev, which indicates a change in habitat conditions, as well as in the overall biocoenosis. In the reaches surveyed, Volga pike-perch is known only from sites near Tver, which is the most downstream sampling location of this study; hence, the downstream transport of eDNA may explain the non-detection of this species at the sampling site of Tver.

Of the 18 fish species known to occur in the Tudovka River (Zheltukhin, Avdanin, & Istomin, 1995), 15 were confirmed together with two additional species, freshwater bream and white bream. At Istok on the Tudovka River, brown trout, burbot, and roach were detected, although the stream is very small and unlikely to provide permanent habitat for these species. However, burbot is known to occur in small and cold mountain streamlets with slow-moving waters in summer (Kottelat & Freyhof, 2007), and they especially move toward shallow waters at night for feeding (Scott & Crossman, 1973), which makes the presence of eDNA from this species in Istok plausible. Otherwise, for these species, the presence of juveniles or allochthonous eDNA might explain their detection. This means that eDNA could have been transported to the site, from nearby locations of the Tudovka River or ponds connected to the river, via faecal deposition or carcass displacement by predators, such as piscivorous birds, or scavengers (Goldberg et al., 2016; Mahon et al., 2013; Merkes, McCalla, Jensen, Gaikowski, & Amberg, 2014). Allochthonous eDNA can indeed be a potential source of false-positive detection in biomonitoring studies of aquatic environments (Darling & Mahon, 2011; Guilfoyle, Dorr, Hanson-Dorr, Fischer, & Friona, 2017).

All species detected in the Moksha River are known to occur there (Artaev & Ruchin, 2017), with the exception of brown trout and brook lamprey. Ukrainian brook lamprey (*Eudontomyzon mariae*) has been recorded a few kilometres from the sampling location (Artaev & Ruchin, 2017), so it is plausible that both lamprey species occur at this location; however, traditional surveys (Artaev & Ruchin, 2017; Dushin, 1978) and communication with local anglers do not support the occurrence of brown trout in the main river. Therefore, the detection of brown trout in the main channel of this river is most likely the result of allochthonous eDNA, potentially originating from escaped fish or water released by an aquaculture site near Kamenka.

4.3 | Limnophilic and demersal species

Overall, the results support the hypothesis that limnophilic species were less likely to be detected, which is most likely due to a lower probability of collecting the eDNA of species thriving in lentic or slow-moving water when sampling the main current of the river, stressing the importance of specifically sampling backwaters to detect such species. For instance, crucian carp (*Carassius carassius*), goldfish (*Carassius auratus*), and rudd were not detected, most probably for this reason. Although downstream drift of eDNA occurs up to several kilometres downstream of its source (Civade et al., 2016; Deiner & Altermatt, 2014; Jane et al., 2015), backwater habitats are unlikely to provide sufficient flow to the main channel of the river to transport detectable quantities of eDNA with the sampling intensity carried out in this survey. It is interesting that the results show that demersal species could be detected from the upper water column; however, brook lamprey (*L. planeri*) was only detected at one location in the Volga headwaters, but known to be present throughout the Volga system. This might be explained by the fact that they often burrow in fine sediments, resulting in their eDNA being absent or very diluted in the water column sampled.

4.4 | Undetected species

Not all the species from the compiled list (Table 3) were expected to occur in the free-flowing section that was sampled, owing to the specific hydromorphological conditions shaping the fish species assemblage. For instance, none of the *Coregonus* species were detected, which is not surprising as they are almost exclusively found in lakes Seliger and Vselug in the Volga headwaters several hundred kilometres upstream from the study area, or in the reservoirs downstream of the sampling area (Froese & Pauly, 2017). However, the absence of certain species can be due to limitations of PCR primer sets, or the incompleteness of the reference database. For instance, the reference sequences of wels (*Silurius glanis*) present mismatches with the primers of all three markers used (three mismatches with the Cyt *b* reverse primer, one insertion compared with the 12S forward primer, and one mismatch with the 3′ end of the 16S reserve primer), which very likely hindered the amplification of eDNA from this species. The absence of blue bream in the data probably results from the lack of available references for 12S and 16S, combined with the lower performance of Cyt *b* for species identification overall, making the detection of this species very challenging based on the set of markers used. The same reason could also explain the non-detection of white-eye bream, Siberian spiny loach, and asp. This highlights the fact that relying exclusively on one marker to detect certain species can prevent their detection entirely, even for prevalent species. Primer affinity bias toward certain species or genera is an important pitfall when using generic primers in eDNA metabarcoding surveys; however, the reason for the absence of sterlet and European eel in the data is currently unknown and could not be explained by primer mismatches. For some of the undetected species that have been previously recorded in the study area, further analysis using quantitative PCR (qPCR) with species-specific primers should produce more reliable results on the species' presence or absence. Alternatively, one or more of these species may have disappeared in this reach in the most recent past.

4.5 | Environmental aspects and the status of the Upper Volga

Nearly half of the species identified are either intolerant or have a low tolerance to one or more environmental stressors. This further supports the view that the Volga headwaters are a healthy and near-pristine lowland river ecosystem with little sign of degradation from human pressures (Leummens, 2016; Schletterer et al., 2016; Schletterer & Füreder, 2010; Schletterer, Shaporenko, et al., 2019). Nonetheless, the construction of the Volga–Kama cascade of dams has had considerable effects on the distribution of native diadromous species, as only two of the eight dams were built with a fish passage facility (Pavlov & Skorobogatov, 2014; Schletterer, Shaporenko, et al., 2019). This has led to significant changes in the fish community and a loss of emblematic migratory species, such as Caspian lamprey (*Caspiomyzon wagneri*), sheefish (*Stenodus leucichthys*), Russian sturgeon (*Acipenser gueldenstaedtii*), and European sturgeon (*Acipenser sturio*) (Schletterer, Kuzovlev, et al., 2018). In addition, as a consequence of the construction of these dams, rheophilic fish species are now restricted to the headwaters above these dams, as well as the downstream reaches below Volgograd (Schletterer & Füreder, 2011). The study also shows that the hydrochemical characteristics of the Volga headwaters, such as the relatively high level of humic acids, did not affect detection rates of eDNA.

4.6 | Summary, limitations, and recommendations for future bioassessments

Overall, this study presents a comprehensive list of species identified in the free-flowing section of the Volga headwaters, as well as information on the presence or absence of each species along a longitudinal gradient of the river, using eDNA metabarcoding. This fish diversity survey provides a basis for long-term monitoring of the Volga headwaters and its tributaries, based on eDNA sampling, and now constitutes a reference status for future similar assessments of other European lowland rivers. Although current environmental monitoring of the Volga River, under Russian law, primarily focuses on physicochemical parameters, some biological quality elements are being monitored in a limited number of areas (see Schletterer, Shaporenko, et al., 2019). The free-flowing section of the Upper Volga River has undergone only minor hydromorphological changes and has mostly retained its natural catchment conditions (Schletterer et al., 2014). Therefore, comprehensive and continuing biodiversity assessments of these reaches will provide valuable insights on the impact of currently implemented management and conservation strategies, as well as the potential effects of climate change. However, specific improvements should be considered to ensure the accuracy of future eDNA-based bioassessment of this river system, and of other rivers. The results of this study primarily underscore the importance of using multiple markers, which more effectively reveal ecosystem biodiversity when combined, together with a comprehensive reference database, as well as the importance of taking multiple samples at each location of interest, including unique microhabitats, in order to determine effectively and accurately the species composition of the entire ecosystem. For instance, bioassessments of large lowland river systems such as the Volga, where limnophilic species are expected in backwater habitats, need to include targeted sampling of these habitats to enable the detection of such species. In addition, missing reference sequences for species and markers of interest should be obtained via targeted sampling and sequencing. For species not detected using eDNA metabarcoding, a qPCR protocol should be developed and implemented, as this species-specific method is more sensitive and could help determine whether non-detection was due to insufficient sequencing coverage of the metabarcoding protocol or deficits in the quantity or spatial strategy of sampling. Overall, the spatial distribution and preservation of eDNA of different species is not yet fully understood owing to a number of factors involving the condition of the DNA itself and its complex interactions with numerous abiotic factors (Barnes et al., 2014; Dejean et al., 2011; Pilliod, Goldberg, Arkle, & Waits, 2014;Pont et al., 2018 ; Wilcox et al., 2016). The shedding rates of eDNA also vary across species, sexes, ages, seasons, and environmental characteristics, and a combination of biases, during library preparation and PCR, can differentially affect the amplification of eDNA across species (Harper et al., 2018; Kelly, Port, Yamahara, & Crowder, 2014). Furthermore, rare species can be more difficult to detect using an eDNA metabarcoding approach compared with a qPCR method. This is primarily the result of a reduced collection probability of eDNA and PCR amplification bias toward more abundant eDNA templates, as well as a sequencing bias toward more abundant amplicons, and ultimately constraints of sequencing depth (Adams, Amend, Taylor, & Bruns, 2013; Evans et al., 2016). These results show that the non-detection of a species does not necessarily exclude its occurrence at a particular site. Similarly, the detection of a species with eDNA metabarcoding does not necessarily mean that the species occurs at that exact location (Goldberg et al., 2016). Nonetheless, the identification of a species at a sampling location provides valuable information on its presence locally or in an area of several hundred metres, up to a few kilometres upstream, depending on the flow velocity and conditions affecting eDNA degradation (Pont et al., 2018; Wilcox et al., 2016). Since the level of eDNA dilution is thought to be correlated with the size of the river, it is important to adjust the volume of water being sampled accordingly, as was done in this study, and especially for the detection of rare species. The results of this study highlight the vast potential of eDNA metabarcoding as a method of estimating species diversity, especially in large and non-wadable streams, in which species identification using conventional survey methods would require extensive effort and could easily overlook small or rare species.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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