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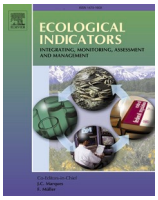

Targeted non-invasive bioindicator species detection in eDNA water samples to assess and monitor the integrity of vulnerable alpine freshwater environments

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ABSTRACT

Due to a high number of specialized species and unique environmental conditions, alpine spring ecosystems are particularly vulnerable to environmental change and human impact. Therefore, the assessment of ecosystem integrity through habitat monitoring over long periods of time is of particular importance, especially in protected areas. Bioindication by conventional ecosystem assessment and monitoring includes sampling whole communities and morphological species identification. This approach, however, brings along major drawbacks such as high invasiveness, low reproducibility, low specificity and is relatively time-consuming. To overcome these issues, we developed a targeted bioindicator species eDNA detection approach for representative freshwater macroinvertebrate species and compared the results with detection through conventional sampling. Macroinvertebrates of 15 springs, located in the Swiss National Park and the UNESCO biosphere reserve Engiadina Val Müstair, were sampled using a hand-net and species were morphologically identified. We selected six spring-bound species: *Hygrobates norvegicus*, *Partnunia steinmanni*, *Dictyogenus fontium*, *Protonemura lateralis*, *Lithax niger,* and *Wormaldia occipitalis* and designed novel, species-specific qPCR primers and hydrolysis probes. Spring eDNA was collected by filtering 1.5 l water through cellulose nitrate filter funnels and DNA extracts were screened by qPCR for the selected bioindicator species. Results showed congruence between conventional and eDNA qPCR-based species detection. The assay targeting *L. niger* was less sensitive and qPCR performance in eDNA samples was decreased compared to the other species, indicating the necessity for careful indicator species choice and evaluation. The newly developed eDNA-based qPCR protocols allow detecting indicator species in alpine springs and represent a non-invasive, sensitive and specific, cost- and time-effective alternative to conventional biomonitoring approaches. Particularly in protected areas such as the Swiss National Park, the implementation of indicator species detection in eDNA filtered water samples can be beneficial and fosters sustainable freshwater ecosystem monitoring and assessment.

1. Introduction

Alpine ecosystems are highly susceptible to environmental change (Beniston, 2006; Fait et al., 2020; Gobiet et al., 2020; Körner, 2003; Mastrotheodoros et al., 2020; Rogora et al., 2018), and the biota of small water bodies are considered to be vulnerable (Oertli et al., 2007; Robinson and Oertli, 2009; Rosati et al., 2017; Rosset et al., 2010). Highly adapted species such as spring-dwelling crenobionts and crenophiles that require oligotrophic and relatively stable environmental conditions are endangered due to global climate change and anthropogenic impacts (Cantonati et al., 2020; Glazier, 2014; Reiss et al., 2016; von Fumetti et al., 2017). The loss of such unique species communities (Cantonati et al., 2020; Pascual et al., 2020; Pozojević et al., 2020) would severely decrease biodiversity and lead to degradation of ecosystem integrity (Eisenhauer et al., 2019; Fanin et al., 2018). Assessment and monitoring of vulnerable ecosystems become increasingly crucial for estimating the impact of environmental change and informing management strategies (Gerecke et al., 2011; Harvey et al., 2020; Küry et al., 2016; Reiss et al., 2016). The development of rapid, cost- and time-effective instruments to assess ecosystem integrity is, therefore, a critical prerequisite advancing environmental monitoring efforts.

Bioindication by recording the presence or absence of species that

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indicate environmental integrity is widely used as tool to examine the status quo of ecosystems (e.g., Gieswein et al., 2019; Lencioni et al., 2020; Thomsen et al., 2012; Więcek et al., 2013). Particularly freshwater habitats are assessed on a regular basis with standardized bioindication methods that have proven to be reliable and reproducible (Gerecke et al., 2011; Hering et al., 2004; Kuefner et al., 2020; Küry et al., 2016; Morinière et al., 2017; Robinson and Oertli, 2009). Furthermore, national governments (e.g., Switzerland: Lubini et al., 2014; Stucki et al., 2019) and the European Water Framework Directive (European Commission, 2000) have admitted bioindication in freshwater environments as an official tool to assess environmental integrity. However, conventional protocols based on direct sampling, preservation and morphological identification of organisms often require laborious field and laboratory work and are therefore time and cost-intensive (Mächler et al., 2014; Stein et al., 2014; van der Loos and Nijland, 2020; Watts et al., 2019). Furthermore, morphological identification is limited to relatively large specimens in late larval stages (Deiner et al., 2013; Stryjecki et al., 2016; Sweeney et al., 2011), which leads to the inclusion of by-catch species without indicative value that do not contribute to the final ecological assessment (Piper et al., 2019; van der Loos and Nijland, 2020). In conjunction with sampling methods, as e.g., Surber and kick sampling, which disperses large amounts of sediment to retrieve as many species as possible, such techniques can be rather invasive. In small habitats like freshwater springs, invasive sampling methods cause significant disturbances and, when performed regularly or over more extended periods of time, may lead to habitat destruction (Beng and Corlett, 2020; Bossley and Smiley, 2019; Goldberg et al., 2016). Furthermore, undisturbed habitats without direct human impact, and suitable for environmental monitoring, can often be found in protected areas like National Nature Reserves (NNRs) (Robinson and Oertli, 2009; von Fumetti and Blattner, 2016). Paradoxically, invasive and disruptive sampling techniques are common practice and used to assess ecosystems in such areas despite their potential negative influence on natural environments.

The development of alternative methods that overcome these drawbacks have gained increasing attention in studies applying molecular genetic approaches. Collectively, these techniques have multiple advantages compared to conventional bioindicator sampling (Beng and Corlett, 2020; Elbrecht et al., 2017; Liu et al., 2020; Weigand et al., 2019; Zizka et al., 2020). DNA metabarcoding, i.e., high throughput sequencing of PCR amplified DNA extracted from environmental or bulk organismal samples and matching resulting sequences against reference sequence databases of preidentified specimens (Liu et al., 2020), can be used to identify many species in parallel, including life stages that are not distinguishable by morphological species identification (Elbrecht et al., 2017). When applied to environmental DNA (eDNA) samples obtained from filtered water, soil, or sediment instead of bulk macroinvertebrate samples, no living organisms need to be captured, and sampling invasiveness is kept minimal (Beng and Corlett, 2020; Hernandez et al., 2020; Senapati et al., 2019). However, challenges like inconsistent species detection efficiencies due to amplification bias or index jumps (Krehenwinkel et al., 2017; Zinger et al., 2019), incomplete or misleading sequence databases (Elbrecht et al., 2017; Weigand et al., 2019), difficulties to reliably quantify species abundances (Beng and Corlett, 2020; Elbrecht and Leese, 2015), and the need of demanding expert knowledge can diminish the feasibility and implementation in applied environmental monitoring projects.

In recent years, targeted quantitative real-time PCR (qPCR) detection has become a key competence in applied environmental sciences and has found applications in various fields such as, e.g. pathogen detection in wastewater (Langone et al., 2020), monitoring the expansion of invasive species (Adrian-Kalchhauser et al., 2016; Thomas et al., 2019) or quantifying gene expression and adaptation of species to their environment (Evans and Vis, 2020; Zhang et al., 2020). Furthermore, the detection of rare (e.g., Hernandez et al., 2020; Lor et al., 2020) and bioindicator taxa (Senapati et al., 2019) through species and environment-specific qPCR assay establishment gains increasing importance in nature management strategies. Targeted species detection through species-specific qPCR assays can overcome methodological difficulties of metabarcoding, i.e., primer bias, sequencing costs, and laborious data analysis and interpretation. Moreover, amplicon sequencing libraries are usually generated with 30 – 35 PCR cycles (e.g., Gleason et al., 2020; Krehenwinkel et al., 2017; Leese et al., 2021), whereas qPCR assays are commonly conducted with $\pm$ 40 cycles. This increases the amplification performance of low copy number target DNA and consequently the detection sensitivity of low abundand taxa. Therefore, it can be considered an advantageous approach when aiming to detect specific species in freshwater environments.

Given the drawbacks of conventional bioindication methods and the challenges of metabarcoding techniques, we aimed to develop and implement an operational approach to detect macroinvertebrate bioindicator species in protected alpine spring ecosystems. We focused on a set of six representative species and non-invasive eDNA sampling, thus avoiding the necessity to sample living individuals or entire spring communities. The development of a highly specific and sensitive qPCR approach to detect indicator species in filtered water samples circumvents the invasiveness of conventional and laboriousness of metabarcoding techniques without losing the ability to assess bioindicator species presence quantitatively.

2. Materials and methods

2.1. Study sites

The studied springs are located in the southern Swiss Alps as part of the Engiadina Val Müstair UNESCO biosphere reserve (UBEVM) and the Swiss National Park (SNP), which is the biosphere core area and classified as a strict nature reserve (IUCN category Ia). All 15 investigated springs (Fig. 1, Supplementary data 1: Table A1, and Supplementary data 7: KML file) are part of a long-term monitoring program that started in 2019, intending to observe the integrity of spring ecosystems over time and identify possible impacts of climate change. Therefore, spring species communities are sampled and assessed annually in summer, and physicochemical conditions, as well as substrate composition, are documented by using standardized methods according to Hotzy and Römheld, 2008 and Lubini et al., 2014.

2.2. Conventional sampling and indicator species selection

Spring macroinvertebrate communities were sampled in July 2020 by using a hand net (200 μm). Samples were taken at the spring mouth and not >10 m downstream, including all substrate types according to their percental occurrence to account for high habitat diversity. Subsequently, bulk samples containing sediment and organic material were separated into coarse- and fine-grained fractions using a wide-meshed hand net (1 mm). Coarse-grained samples were sorted directly in the field, and large macroinvertebrates preserved in 100% Ethanol. Both fractions were separately transferred to a collecting vessel and preserved with Ethanol (100%) to be transported to the laboratory and sorted under a stereomicroscope. This procedure allows for optimal preservation of intact large specimens suitable for morphological identification and the possibility to screen sediment samples for smaller organisms that can easily be overlooked, such as small Hydrachnidia species.

Morphological identification was done with a focus on the most abundant taxa by using widely applied identification keys (Hydrachnidia: Bartsch et al., 2007; Di Sabatino et al., 2010; Gerecke et al., 2016, Plecoptera: Lubini et al., 2012 and Trichoptera: Waringer and Graf, 2011). Target species with $\leq$ 1 individuals were considered as not detected to compensate for the influence of stochasticity and accidental detection (see Supplementary data 4 Tables D.1 and D.6). The resulting species list reporting presence or absence in all 15 springs was subsequently screened for suitable indicator species by aiming at spring-dwelling species representing the spring fauna of the study area. Due

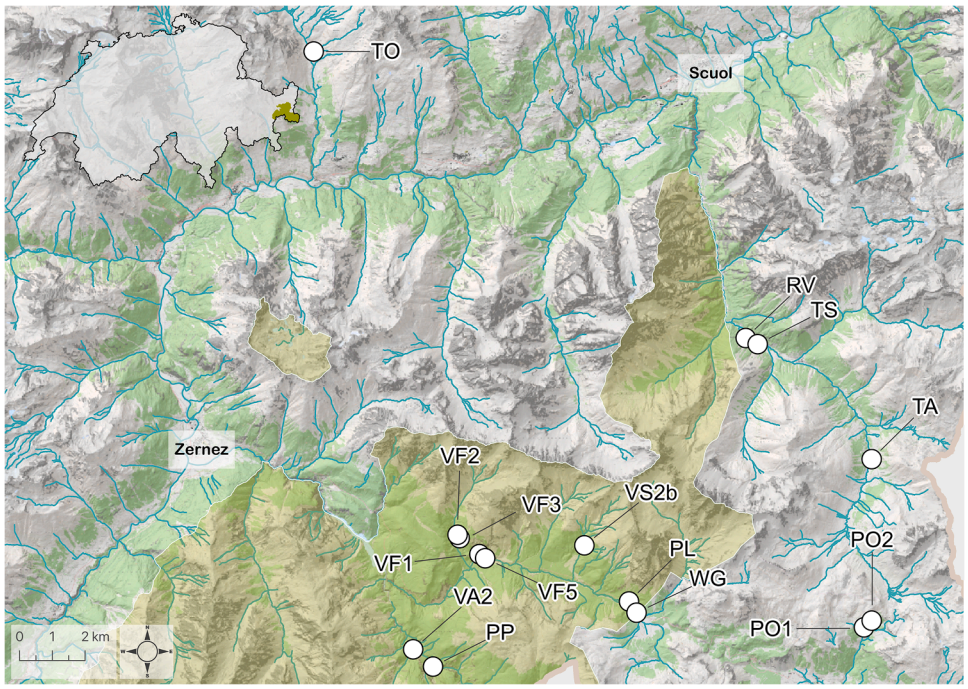

Fig. 1. Map of the study area showing the different springs. The location of the Engiadina Val Müstair UNESCO biosphere reserve (UBEVM) within Switzerland is shown in green colour in the overview that can be found in the top left part of the map. The highlighted part of the map shows the Swiss National Park, which is the core area and most protected part of the biosphere reserve. The map was created in QGIS V3.18 with data of the Swiss Federal Office of Topography, swisstopo, freely available on https://www.swisstopo.admin.ch. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to relatively high species diversity, a high number of spring-bound species, and generally high abundances, we focused mainly on Trichoptera, Hydrachnidia and Plecoptera (Cantonati et al., 2012, 2006; Küry et al., 2016). Furthermore, Trichoptera and Plecoptera species are among other taxa usually targeted in freshwater ecosystem bioindication methodologies (Blackman et al., 2019; Bush et al., 2019; Jourdan et al., 2018; Küry et al., 2016) and Hydrachnidia are known to be exceptionally diverse, with many species that are strictly bound to springs (Blattner et al., 2019; Gerecke et al., 2018). Therefore, these species can potentially be used as powerful bioindicators (Di Sabatino et al., 2003; Gerecke and Lehmann, 2005; Goldschmidt, 2016; Miccoli et al., 2013). Suitable species were chosen according to different criteria, namely: High degree of spring specialization, spring type preference, wide distribution range in the study area, co-occurrence in springs and indicative value according to existing published (e.g. Blattner et al., 2019; Nadig, 1942; von Fumetti and Blattner, 2016; Von Fumetti and Felder, 2014) and unpublished data of the study area. The resulting subset of species: *Lithax niger* (Hagen, 1859), *Wormaldia occipitalis* (Pictet, 1843), *Partnunia steinmanni* Walter, 1906 genotype A and *Hygrobates norvegicus* (Thor, 1897) genotype B sensu Blattner et al., 2019, *Dictyogenus fontium* (Ris, 1896) and *Protonemura lateralis* (Pictet, 1836) were considered as suitable indicator species and candidates for the development of the eDNA-based qPCR assay.

2.3. Sequence library construction

Because of the sparsity of spring species DNA sequences in preexisting databases, we build a custom sequence library to provide the genetic basis for qPCR primer and probe design. Due to the advantages of targeting mitochondrial marker regions for eDNA species detection (Tsuji et al., 2019), mainly because of higher molecule abundance and mutation rate compared to nuclear DNA (Goldberg et al., 2016; Handley, 2015), genetic sequence libraries containing Cytochrome *c* oxidase I (MT-CO1), mitochondrial cytochrome *b* (MT-CYB), mitochondrial 12S (MT-RNR1) and 16S rRNA (MT-RNR2) gene fragments of the target indicator species and as many co-occurring species as possible were generated. Several individuals of different sampling locations per target species were included to address the relatively high variability of mitochondrial DNA and the possibility of geographic genetic separation, which could lead to false negative detection (Arabi et al., 2012; Bergsten et al., 2012; Boumans et al., 2016; Ketchum et al., 2009; Toews and Brelsford, 2012; Weiss and Leese, 2016). Crenobiont species are generally not well studied by molecular methods, and sequence information is scarce or nonexistent. Therefore, a new set of taxon-specific primer pairs (Table 1) targeting the four regions of interest were designed by aligning in-house generated genetic data and sequence information available on NCBI GenBank, including published Plecoptera (Accession no.: KF484757, MG910457, MH085453, MK290826 and MN400756), Trichoptera (Accession no.: KX385010, MG201852, MG669125 and MG980616), and Hydrachnidia (Accession no.: EU856396, LC552026, HQ386015 and MG701313) mitochondrial genomes. The alignments were computed by the MAFFT (Katoh and Standley, 2013) algorithm implemented in Geneious Prime 2021.0.3 (https://www.geneious.com), and all PCR primer pairs were designed by hand, synthesized, and HPLC purified by Microsynth AG (Balgach, Switzerland). To be able to use universal sequencing primers for all reactions, amplification primers were tailed with modified M13 sequences (M13: 5′-TGT AAA ACG ACG GCC AG-3′ and M13r: 5′-CAG GAA ACA GCT ATG AC-3′) (Messing, 1983).

Subsequently, genomic DNA (gDNA) of sampled target and co-occurring species (Supplementary data 2: Tables B.1 and B.2) was extracted by using the magnetic bead-based DNAdvance Kit (Beckman Coulter Live Sciences, Indianapolis USA) according to manufacturer protocol, with 100 µl elution buffer. All PCR amplifications were performed with 0.25 µl of Phusion™ High-Fidelity DNA Polymerase [2 U/µl] (ThermoFisher Scientific, Massachusetts, USA), 5 µl of 5X Phusion™ HF Buffer (ThermoFisher), 0.5 µl of 10 mM dNTPs (SigmaAldrich, Switzerland), 1.25 µl of forward and reverse primers [10 µM each], 5 µl template DNA and molecular grade H_2O to a final reaction volume of 25 µl. PCRs were run with an initial denaturation of 30 s at 98 °C; 35 cycles of 10 s at 98 °C, 30 s at primer pair-specific annealing temperatures (Table 1), and 30 s at 72 °C followed by a final elongation step at 72 °C for 2 min. PCR products were purified enzymatically with ExoSAP-IT™ (Affymetrix Inc., USA) according to manufacturer protocol, and Sanger sequenced bi-directionally with the mentioned M13 primers by Microsynth AG. After sequence retrieval, raw bi-directional reads were

Table 1
Mitochondrial DNA library generation primers designed and used in this study. Oligonucleotide types are abbreviated as F = Forward primer and R = Reverse primer.

Locus	Taxon	Oligonucleotide	Sequence [5′ – 3′]	App. Fragment Length [bp]	Annealing [°C]
MT-CO1	Hydrachnidia	CO1_Hy_nF	CMAAYCAYAAAGAYATTGGAAC	1000	53
		CO1_Hy_R	GCGAAWACWGCTCCTATWG		
	Plecoptera	CO1_Pl_F	ACAAAYCAYAARGAYATTGGAAC	1500	54
		CO1_Pl_R	GRGCTTAAATCCATTGCAC		
	Trichoptera	CO1_Pl/Tr_F	MAAAGAAACTAATGAAYAAACC	1100	52
		CO1_Tr_R	GCRAAWACWGCYCCTATWG		
MT-CYB	Hydrachnidia	CYB _Hy_F	CAAATATCYTTYTGAGGRGC	900	53
		CYB _Hy_R	ATGTACATATCGCCCGTC		
	Plecoptera	CYB _Pl_F	MAAAGAAACTAATGAAYAAACC	1500	52
		CYB _Pl_R	CTTATGYTTTCAAAACATATGC		
	Trichoptera	CYB _Tr_F	AGGWCAAATATCHTTTTGAGG	900	53
		CYB _Tr_R	TTAAGTTTTCAAAACAAAWGCT		
MT-RNR1	Hydrachnidia	12S_Hy_F	GYGACGGGCGATATGTAC	400	60
		12S_Hy_R	AGCAGTTGCGGTTATACG		
	Plecoptera	12S_Pl_F	YCTACTATGTTACGACTTATCTC	400	53
		12S_Pl_R	ATTTGGCGGTGTTTTAGTC		
	Trichoptera	12S_Tr_F	CTACTWTGTTACGACTTRTYTY	500	53
		12S_Tr_R	ARACTRGGATTAGATACCC		
MT-RNR2	Hydrachnidia	16S_Hy_F	TTAYGCTGTTATCCCTTARG	1100	53
		16S_Hy_R	AGGTATGARCCCRTTAGC		
	Plecoptera	16S_Pl_F	TCTATAGGGTCTTCTCGTC	1000	53
		16S_Pl_R	GAGATAAGTCGTAACATAGTAG		
	Trichoptera	16S_Tr_F	AGATAGAAACCAACCTGGC	500	53
		16S_Tr_R	GGTYTGAACTCAGATCATG		

assembled, remaining primer sequences trimmed and final contigs checked for the presence of low quality or ambiguous base calls in Geneious Prime. All sequences are stored and available on NCBI GenBank under accession numbers listed in Supplementary data 2: Tables B.1 and B.2.

2.4. In silico development of qPCR primers and probes for selected bioindicator species

Due to the high specificity and possibility to multiplex several targets in a single reaction (Rodríguez et al., 2015; Tsuji et al., 2019, 2018), a hydrolysis probe-based approach was developed by designing indicator species-specific primer/probe sets. To avoid false positive detections the sequences generated during the sequence library development (Section 2.3) containing target species sequences (Supplementary data 2: Table B.1) as well as sequences of co-occurring macroinvertebrates (Supplementary data 2: Table B.2) were aligned and screened for regions that are highly specific to the target indicator species but as distinct as possible from non-target species. The oligonucleotides were designed according to general recommendations (e.g. Bustin et al., 2020, 2009; Rodríguez et al., 2015) and with at least two of the oligonucleotides having at least two mismatches with non-targets as recommended by Currier et al. (2018). In addition, primers with multiple polymorphisms at the 3′ end between sequences of target and non-target species were preferred as this significantly reduces the possibility of unwanted primer annealing and elongation (Stadhouders et al., 2010; Wilcox et al., 2013). We aimed at theoretical melting temperatures (T_m) of primers as close as possible to 60–65 °C and probes 6–8 °C higher to be able to amplify different templates with standardized qPCR conditions. Furthermore, an amplicon length of < 200 bp was preferred because of the high level of DNA degradation that can be expected from eDNA samples (Mächler et al., 2014; Tsuji et al., 2019; Wood et al., 2020). Several possible primer and probe sets were designed using Primer3 v4.1.0 (Kõressaar et al., 2018; Koressaar and Remm, 2007; Untergasser et al., 2012) and adjusted by hand if necessary. All hydrolysis probes were synthesized with 6-FAM fluorescein at the 5′ end and double quenched with BMN-Q535 between the 8th and 9th base and at the 3′ end, respectively. Final qPCR oligos were ordered with HPLC purification and manufactured by Biomers GmbH (Ulm, Germany).

2.5. In vitro oligonucleotide efficiency and specificity evaluation

Evaluation of possible primer/probe sets was performed in several steps following MIQE guidelines (Bustin et al., 2009), general qPCR recommendations (Taylor et al., 2019), and suggestions from eDNA-specific studies (Blackman et al., 2020; Klymus et al., 2020b; Mauvisseau et al., 2019; Wilcox et al., 2013). First, synthetic standard DNA templates were generated by ordering the target DNA fragments as gBlocks™ (Integrated DNA Technologies Inc., Iowa, USA) (Supplementary data 6 Tables F.1), allowing for precise copy number calculation based on the molecular weight of the DNA fragments supplied by the manufacturer. A 6-fold 1:5 dilution series of these DNA templates was prepared to generate standard curves to test the performance of the designed primer/probe sets. Primer/probe combinations with amplification efficiencies < 90% and showing $r^2 \leq 0.98$ were considered suboptimal and were therefore excluded.

Specificity was evaluated by designing mock communities consisting of gDNA of co-occurring species (Supplementary data 2: Table B.2) in equimolar ratios, resulting in an artificial DNA template containing 0.5 ng DNA of each species. All oligonucleotide sets were tested by amplifying these artificial communities with and without the target species DNA to test for false positive and false negative detection. Only primer/probe sets that did not fail to comply with these specificity tests were considered suitable for the final qPCR eDNA bioindication assay. The sensitivity of each oligonucleotide set was evaluated by calculating the limit of detection (LOD) and the limit of quantification (LOQ). For this, eight replicate amplifications of the 6-fold dilution series were established. LOD was considered as the lowest initial amount of target sequence copies per reaction with 95% detection probability, whereas LOQ was calculated as the lowest amount of sequence copies per reaction with a coefficient of variation (CV) < 35%, which are commonly used thresholds (Conte et al., 2018; Klymus et al., 2020a, 2020b). Target species-specific LOD and LOQ values were calculated using the LOD/LOQ calculator script published by Klymus et al. (2020a) in R (R Core Team, 2017).

All qPCR reactions were run on a MIC magnetic induction cycler (Bio Molecular Systems, Upper Coomera, Australia), using the SensiFAST™ Probe No-ROX Kit (Meridian Live Science, Inc., Tennessee, USA) and identical amplification conditions. The 20 µl final reactions consisted of 10 µl SensiFAST Probe No-ROX Mix [2X], 800 nM (0.8 µl) forward and

reverse primer [20 μM each], 100 nM (0.2 μl) hydrolysis probe [10 μM], 5 μl DNA standard template and 3.2 μl molecular grade H_2O. Reactions were run using the standard SensiFAST two-step amplification protocol, with an initial polymerase activation at 95 °C for 5 min, followed by 40 cycles of 10 s denaturation at 95 °C and 30 s of combined annealing and extension at 60 °C, with fluorescent signal acquisition at the end of each cycle. No template controls (NTC) containing molecular grade water instead of template, negative controls with mock communities lacking DNA of target species, as well as positive controls containing gDNA of the target species were included as duplicates in each run. The qPCR baseline threshold was calculated with the MIC software by implementing dynamic baseline correction with a fluorescence cutoff level of 5% and ignoring the first 5 amplification cycles.

2.6. Environmental DNA (eDNA) sampling

Spring eDNA was collected in July 2020, parallel to the conventional hand-net sampling. At each spring, 1.5 l water per sample was filtered through 250 ml Nalgene™ Single Use Analytical Filter Funnels with 0.45 μm cellulose nitrate membranes (ThermoFisher Scientific) by using a Nalgene™ Polypropylene Vacuum Flask (ThermoFisher Scientific) and a hand-operated vacuum pump. Each sample was composed of six 250 ml sub-samples taken directly at the stream surface between the spring mouth and 10 m downstream. Subsequently, filters were transferred to 2 ml safe-lock tubes (Eppendorf AG, Hamburg, Germany) with tweezers and directly put on dry ice for optimal DNA preservation before storage at − 20 °C in the laboratory until further processing. We considered all material that came into contact with the filtered water as contaminated and discarded it after each sampling. Tweezers were cleaned before and after each filter transfer by flame sterilization with 100% Ethanol and NaOCl (13%) treatment. We sampled three biological replicates per spring and included a negative field sample by filtering tap water brought to the field to test for possible false positive detection caused by cross-contamination during fieldwork.

2.7. eDNA extraction and purification

Due to the possibility of PCR inhibitor presence in eDNA samples (Deiner et al., 2015; Jane et al., 2015; Schabacker et al., 2020), we extracted and purified the DNA captured on filter membranes with the FastDNA Spin Kit for Soil (MP Biomedicals LLC, Santa Ana, USA), which involves a powerful inhibitor removal step. To be able to perform enzymatic cell lysis, which has proven to be beneficial to extract eDNA (Deiner et al., 2015; Deiner and Altermatt., 2014; Wong et al., 2020), instead of the initial mechanical bead beating used by default during the FastDNA Spin Kit procedure, the first steps of the manufacturer protocol were modified. The filter samples were cut into small pieces and transferred to 2 ml tubes containing 900 μl ATL (Qiagen, Hilden, Germany) lysis buffer and 100 μl Proteinase K solution [> 600 mAU/ml] (Qiagen). All samples were then incubated at 56 °C for 48 h to ensure sufficient lysis before continuing with the FastDNA Spin Kit manufacturer protocol. Purified eDNA was subsequently stored at 4 °C until running the qPCR assays, and subsequently at −20 °C for long term storage.

2.8. Indicator species detection and inhibitor control

To monitor possible false-negative indicator species detection caused by qPCR inhibition present in the eDNA templates, we screened all samples by spiking-in an internal positive amplification control (IPC) directly into the qPCR reactions. A pre-established, artificial IPC template and oligonucleotide set, qPCR DNA Extraction and Inhibitor Control Cy®5-QXL®670 (Kaneka Eurogenetec S.A., Seraing, Belgium), was used, and interference of the IPC and the indicator species assays was evaluated in vitro by amplifying the previously mentioned mock community assay (Section 2.5) as negative control and target species gDNA as positive control before continuing with eDNA samples. The IPC assay was used according to the manufacturer's protocol, and a 1:10′000 diluted IPC DNA template was added directly into the reactions to aim for a quantification cycle (Cq) = 30 – 33.

The IPC evaluation reactions as well as all final eDNA indicator species detection reactions were run in duplicates in 20 μl total reaction volume containing 10 μl SensiFAST Probe No-ROX Mix [2X], 800 nM (0.8 μl) forward and reverse primer [20 μM each], 100 nM (0.2 μl) hydrolysis probe [10 μM], 0.2 μl IPC template [1:10′000], 2 μl IPC mix [10X], 1 μM (1 μl) BSA [20 mg/ml] and 5 μl DNA template. Amplification cycle conditions were identical to those in section 2.5, and to monitor potential technical issues, we additionally included NTC negative and positive control reactions in duplicates by replacing the DNA template. eDNA samples that showed qPCR inhibition in the first screening were additionally treated with OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, USA) that has been shown to work well for post-extraction secondary cleanup of eDNA (Mächler et al., 2016; Sanches and Schreier, 2020; Williams et al., 2017). To ensure highly supported positive species detections, only samples with at least 2 out of 3 biological and all technical replicates showing target species amplification were considered positively detected to minimize the possibility of false positives (Supplementary data 4: Tables D.1 and D.6).

3. Results

3.1. Conventional indicator species detection

Conventionally sampled and identified macroinvertebrates (Supplementary data 3: Table C.1) showed species assemblages typical of alpine springs in the area. We recorded 52 Hydrachnidia, Plecoptera, and Trichoptera taxa and we were able to identify 36 of them morphologically to species level. Individuals that were either underdeveloped, highly damaged, or too laborious to identify were assigned to the highest taxonomic resolution possible. With 15 identified species, Trichoptera showed the highest species diversity, followed by Hydrachnidia (11) and Plecoptera (9). The indicator species chosen for the eDNA assay development showed relatively high abundances (reported in Supplementary data 4: Tables D.1 and D.2), especially *P. lateralis* (15 of 15 springs), *D. fontium* (10 springs), *L. niger* (10 springs) and *P. steinmanni* (8 springs) were widely distributed in the study area. *H. norvegicus* B and *W. occipitalis* showed the narrowest distributions and occurred in only 2 and 3 springs, respectively (Supplementary data 4: Tables D.1 and D.6).

3.2. Indicator species targeted qPCR assay development

All final indicator species-specific qPCR primer/probe sets (Table 2) showed no cross-amplification with possible co-occurring non-target species, which was tested with the artificially designed mock communities (Supplementary data 2: Table B.2 and Supplementary data 3: Table C.1). The newly developed primer/probes and amplification conditions can therefore be considered as highly specific. Mean amplification efficiencies of all primer/probe sets were $\geq$ 90% and R^2 values $>$ 0.99, indicating overall good amplification performance (Table 2). The highest LOD was measured for the *Lithax niger* oligonucleotide set, approximately seven times higher compared to *D. fontium* and *P. lateralis* (Table 2). Limits of quantification of the two Hydrachnidia species were considerably higher compared to other taxa (Table 2). All measured standard concentrations, efficiencies, and R^2 values are reported in Supplementary data 5: Tables E.1.

3.3. eDNA indicator species detection

qPCR targeted assays of the spring eDNA samples showed mean reaction efficiencies of 80% (*L.niger & W.occipitalis*), 90% (*H.norvegicus* B & *P.steinmanni* A) and 100% (*D.fontium*) (Supplementary data 4: Tables D.1 and D.6). We measured eDNA target fragment presence

Table 2
eDNA assay qPCR oligonucleotides designed and used in this study. Oligonucleotide types are abbreviated as F = Forward primer, R = Reverse primer and P = Hydrolysis Probe. All probes were labeled with 6-fam fluorophore at the 5′, BMN-Q535 internal quencher between the 8th and 9th base and BMN-Q535 quencher at the 3′ end.

Species	Oligo-nucleotide	Sequence [5′ – 3′]	Fragment Length [bp]	Efficiency [mean ± SD]	R^2 [mean ± SD]	LOD [cp/reaction]	LOQ [cp/reaction]
Hygrobates norvegicus B	Hn_CYB_F	GTTGTGGCCTTGGTCATAAG	124	1.00 ± 0.05	0.998 ± 0.001	15.01	107
	Hn_CYB_R	GGGTTCAATAGGCTTGGC					
	Hn_CYB_P	AATAACTAGTAGCTGTAAATTTAGCCCTCTT					
Partnunia steinmanni A	Ps_CYB_F	TTGGGATTACAGCTGTAG	83	0.94 ± 0.06	0.999 ± 0.006	18.75	288
	Ps_CYB_R	TGTGGATAATGACTATTATTGA					
	Ps_CYB_P	TCAGTAAGAAATGCTACCTTAAATCGT					
Dictyogenus fontium	Df_ CYB _F	GATCTCGCTTTCACCAGC	80	0.97 ± 0.03	0.998 ± 0.001	< 4.83	16
	Df_ CYB _R	CAGTGTGRAGGTAAAGGCAG					
	Df_ CYB _P	CCTCCATGCCAACGGAGCATC					
Protonemura lateralis	Pl_CO1_F	TTCGGTAACTGACTAGTTCCAT	94	0.90 ± 0.02	0.999 ± 0.001	< 6.15	9
	Pl_CO1_R	CAGCTCCATTTTCGACAAGA					
	PL_CO1_P	TGGAGCTCCAGATATGGCATTC					
Lithax niger	Ln_CYB_F	TTAGGGTTAGATTTAGTTCAGTG	154	0.96 ± 0.04	0.999 ± 0.001	38.32	51
	Ln_CYB_R	TGAAATGGGATTTTATCGGA					
	Ln_CYB_P	CTCCATCAAACCGGATCTTCTAACC					
Wormaldia occipitalis	Wo_CYB_F	TTCAGCTATTCCTTATGCA	84	0.98 ± 0.04	0.999 ± 0.001	< 5.17	20
	Wo_CYB_R	TAGCTATGATAATAAATGGAAG					
	Wo_CYB_P	TTTGCTATTGAAAATGCCACTT					

between 80 mean copies/reaction for *Lithax niger* and up to 4089 mean copies/reaction when amplifying *Protonemura lateralis* eDNA (Supplementary data 4: Tables D.3 and D.5). The inhibitor control assay showed overall good sample purity. All replicates of samples VS and VA2 showed no IPC amplification during the first screening, indicating the presence of PCR inhibition, and therefore the respective samples were purified with an additional inhibitor removal step. Subsequent IPC amplification showed successful inhibitor removal.

3.4. Conventional vs. eDNA indicator species detection

Comparison between the two indicator species detection approaches showed good overall accordance (Fig. 2). The Hydrachnidia species detection of *H. norvegicus* B and *P. steinmanni* A is congruent with no differences between conventional and eDNA detection when excluding the cases were detection showed low support. For *D. fontium* and *P. lateralis,* a single spring exhibited positive eDNA detection but absence in the conventional samples. In both cases, conventional sampling recorded only single individuals (Supplementary data 4: Tables D.5 and D.6), which were detected with low support due to disagreement with our dection criterion for bioindication purposes. In two springs (PP and VF5), *W. occipitalis* was recorded in the conventional sampling but failed the criterion of >1 individual whereas the qPCR assay detection passed the validation criteria and reliably detected the species in the eDNA samples (Fig. 2). Furthermore, *W. occipitalis* was recorded by qPCR in sample TO but only one of two biological replicates showd positive

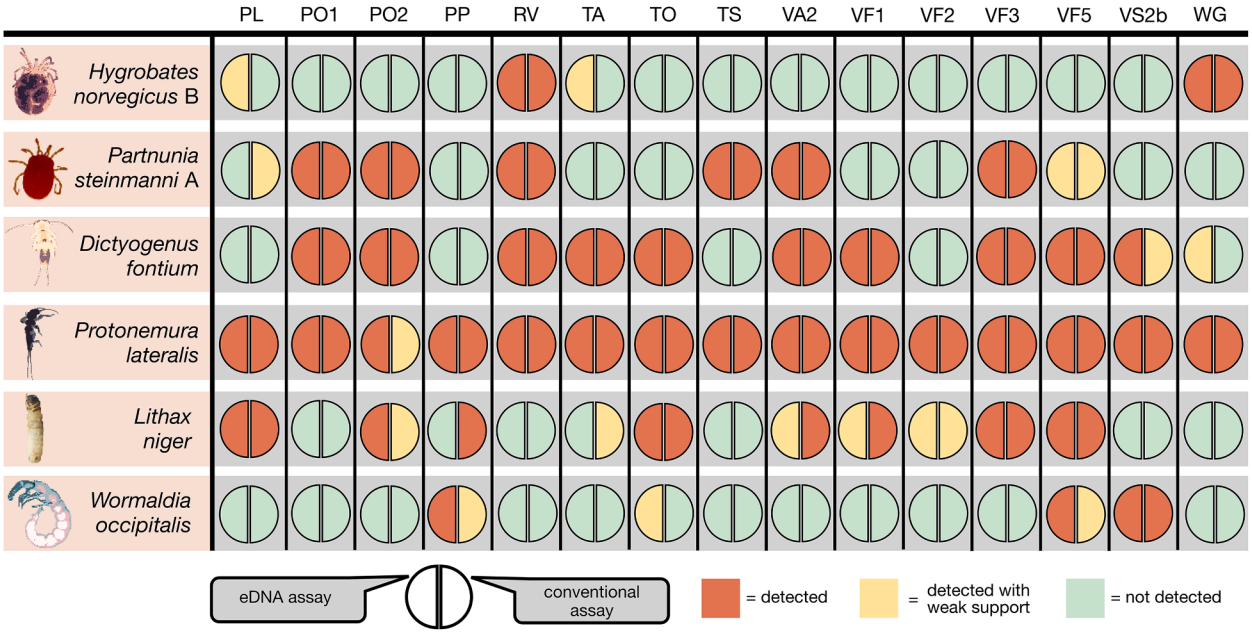

Fig 2. Comparison between conventional and eDNA qPCR bioindicator species detection for 15 freshwater springs in the Swiss National Park. Springs, where the species detection passed our limit of detection criteria for the conventional or the qPCR assay-based approaches, are indicated in red as *detected.* Cases where the species were recovered but the supported detection criteria were not met are shown in yellow as *detected with weak support.* The third category, in light green, shows cases that lack a positive detection of the respective species entirely. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

amplification (Supplementary data 4: Table D.4). *Lithax niger* detection showed the highest disagreement between conventional and eDNA qPCR assays. In one out of 15 springs, eDNA detection was positive, contradicting the conventional sampling that indicated the absence of *L. niger*. The qPCR indicator species detection in eDNA samples showed a higher rate of supported detections (41 out of 90 samples) than the conventional approach (39 out of 90 samples), indicating higher overall sensitivity.

4. Discussion

We developed eDNA detection assays for targeting six spring-bound macroinvertebrate species and compared detection rates with the conventional biomonitoring approach. The target spring species that were chosen are widely distributed in the Swiss National Park and have been recorded in previous studies (Robinson et al., 2008; von Fumetti and Blattner, 2016; von Fumetti and Felder, 2014). All investigated springs are inhabited by at least 2 of the indicator species belonging to different taxa. Given the high degree of spring specialization and the presence of multiple species per spring, we consider this species choice as suitable for bioindication and monitoring of undisturbed alpine springs, especially in and around the studied area. Recording the disappearance of these species during the ongoing spring monitoring projects would show deviation from the current natural state of the protected spring habitats and most probably indicate the influence of changed environmental conditions.

The conventional approach is based on sampling whole macroinvertebrate communities and morphologically identifying specimens to species level, which is currently seen as standard spring ecosystem assessment methodology in Europe (e.g., Germany: Hotzy and Römheld, 2008, Switzerland: Lubini et al., 2014). Furthermore, this method is traditionally applied in freshwater assessments (Elbrecht et al., 2017). Morphological species identification of many taxa is restricted to late larval stages with sufficiently developed characters and intact specimens (Deiner et al., 2013; Meredith et al., 2019), resulting in relatively high proportions of unidentifiable individuals. Therefore, our conventional sampling dataset contained specimens that were assigned to genus or family level and, especially the identification of Plecoptera species is often restricted to well-developed larvae (Lubini et al., 2012). Accordingly, specimens potentially belonging to *D. fontium* presumably have been assigned to Perloidea Superfamily, and *P. lateralis* is very likely to be included in *Protonemura* sp., resulting in false-negative indicator species detection. Furthermore, detecting singleton individuals could potentially lead to false positives due to accidental incorporation of specimens during field or laboratory work. This, e.g., can arise because of the occasional occurrence of few individuals in springs that naturally lack established populations or incorrect morphological identification. Particularly when analyzing highly abundant species with populations consisting of numerous individuals, singleton detections should be addressed and discussed carefully (Meredith et al., 2019). In our case, we decided to classify them as detected with weak support and therefore putative false positives, which we consider as appropriate when using the method for bioindication purposes that directly influence management strategies. However, the appropriateness of implementing such a criterion to validate conventional indicator species detection needs further research and should be evaluated specific to the method used and the environment under consideration.

We initially in silico evaluated four different mitochondrial gene fragments (CO1, CYB, 12S and 16S rRNA) for sequence library generation and subsequent qPCR primer/probe design. 12S showed high variability and degree of sequence and length polymorphisms between and within species leading to a decreased suitability for species-specific qPCR primer/probe design. In contrast, the 16S fragment dataset was relatively uniform and, therefore, likewise not ideal. CO1 and CYB, which we recommend for designing indicator species-specific qPCR assays, exhibited the best applicability. The newly designed qPCR primer/probe sets reliably amplified target species DNA with high specificity and sensitivity. Results show that the theoretical limit of detection of *Lithax niger* (38.32 copies/reaction) is higher compared to the other species (e.g., $\leq$ 4.83 copies/reaction for *D. fontium*), which could lead to false negative detection in springs with low abundances but decreases false positive detection probability. However, if qPCR is used to detect the presence of indicative species in eDNA, it is not recommended to entirely rely on theoretical LOD calculations and discard detections in samples below LOD. This is due to the high specificity of hydrolysis probe-based amplification detection, and even quantifications below LOD may be recorded as present (Klymus et al., 2020a).

We generally documented slightly decreased qPCR reaction performance applying the same amplification conditions as used during the in vitro marker development and evaluation compared to filtered water samples. eDNA is of high complexity and contains various types of reaction inhibitors, sources of DNA, and DNA degrading compounds that can influence reaction efficiency (Hunter et al., 2019; Sanches and Schreier, 2020; Wood et al., 2020). Even though we thoroughly evaluated inhibitor presence in our samples using an IPC and applied secondary inhibitor removal as recommended when processing eDNA (Sanches and Schreier, 2020), the decreased reaction performance is likely to result from remaining inhibitors, degraded, or low amounts of intact DNA. However, minor performance losses are expected in such sample types and do not affect indicator species detection (Klymus et al., 2020b). In contrast, accurate quantification is directly affected and reactions with efficiencies that are not close to 100% should be analyzed with care if quantification is the main goal (Klymus et al., 2020b; Taylor et al., 2019). This occured when amplifying *L. niger* and *W. occipitalis* eDNA. Therefore, we do not recommend using these assays if accurate quantification is of importance. Furthermore, our results show high quantification variability between sample replicates, e.g. ranging between 10^1 and 10^3 copies/reaction for *D. fontium* in the VF3 spring. Therefore, a thorough establishment of standardized spring eDNA sampling protocols would be crucial to increase the reproducibility of quantification between sample replicates (Deiner and Altermatt., 2014).

Comparison of both indicator species detection methodologies applied to the 15 investigated springs showed good overall congruence. However, as in the example of the PO2 spring, *P. lateralis* was detected solely by qPCR and the same was observed for *D. fontium* in spring VS2b. In both cases, the conventional species detection was considered negative due to singleton individuals recorded in the samples, which we considered as putative false-positives. This contradiction indicates that either the conventional sampling at this site was biased or that our criterion of classifying single individuals as false positive detections in the conventional approach needs to be reconsidered, which would be adequate for rare species (Meredith et al., 2019). Our experience shows that generally high numbers of *P. lateralis* individuals can be found in springs. Therefore, we assume a biased conventional sampling in this case. Furthermore, we also recorded the opposite situation, where the qPCR assay failed at reliably detecting an indicator species compared to the conventional approach as e.g. *l. niger* results report in VA2 and VF1. Apart from that, *L. niger* showed the most discrepancies between conventional and eDNA-based qPCR detection and the most putative false positives, indicating that the suitability of this species for bioindication purposes may need further research compared to the other spring invertebrates. The slightly decreased qPCR efficiency could be addressed by modifying the primer/probe set or testing an alternative target locus if accurate quantification is requested but with the aim of bioindication not required. Overall, qPCR detection in eDNA samples showed a generally higher detection sensitivity when excluding detections with low support compared to the conventional approach supporting the robustness of our newly developed assay.

We conclude that detecting bioindicator species through targeted qPCR in environmental DNA water samples is a an effective tool that can be used in alpine spring ecosystem assessments. Furthermore, it is a powerful supplement to conventional methodologies and can overcome

their relative drawbacks. The main advantage is the possibility to test the quality of detection rates by biological and technical replication, performance evaluation, and high specificity, enabling the prevention of false negative and false positive indicator species detection. Such bio-indication assays can similarly be developed for various ecosystems (Beng and Corlett, 2020; Liu et al., 2020; Thomsen and Willerslev, 2015). Especially in freshwater habitats located in protected areas, eDNA approaches can serve as a completely non-invasive monitoring tool to investigate ecosystem integrity over long periods of time. As shown by our results, one of the major challenges when developing targeted eDNA detection assays is the choice of suitable indicator species that should be based not only on the organism's ecological prerequisites but also on qPCR specific aspects and a thorough method evaluation.

CRediT authorship contribution statement

Lucas Blattner: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Visualisation, Writing - Original Draft, Project Administration. **Joshua Niklas Ebner:** Conceptualization, Visualization, Writing - Review & Editing. **Jakob Zopfi:** Methodology, Resources, Writing - Review & Editing. **Stefanie von Fumetti:** Investigation, Resources, Data Curation, Writing - Review & Editing, Supervision, Funding acquisition.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

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