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Using eDNA to simultaneously detect the distribution of native and invasive crayfish within an entire country

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ABSTRACT

The introduction of invasive crayfish has led to a decline of many European native species of crayfish across their range. In this study, novel duplex assays for all crayfish occurring in Switzerland were developed. We aimed to identify the distribution of the seven species using a traditional trap surveillance method as well by collecting water samples to detect eDNA by species-specific quantitative real-time PCR. We reveal our overall experience in finding optimal field and laboratory techniques to discover the distribution and abundance of native and invasive species in order to enhance knowledge of early invasive species invasion and highlight important pockets of populations where native species remain, for implementation of conservation strategies. Using eDNA, important populations of native noble and white-clawed crayfish were revealed in multiple waters across various cantons. The successful identification of native and invasive crayfish species in Switzerland using eDNA can be applied to future nationwide projects. This method which has the ability to detect all species simultaneously across an entire country, will allow an improvement in freshwater crayfish conservation management.

1. Introduction

The continuous decline in biodiversity is one of the most critical challenges of the 21st century (Butchart et al., 2010). Growing numbers of invasive species are one major reason for this loss of biodiversity worldwide (Mathers et al., 2020). In 2015, nearly a third of the world's crayfish species were threatened with extinction (Richman et al., 2015). Since the 1970s, native crayfish in Europe have declined between 50% and 80% across their range with many local populations becoming extinct (Richman et al., 2015). The European crayfish decline was in correlation with the occurrence of invasive crayfish species, namely signal crayfish, *Pacifastacus leniusculus* (Dana, 1852). The signal crayfish carries, while being mostly immune to, the infectious water mould; crayfish plague, *Aphanomyces astaci* (Schikora, 1906) (Thomas et al., 2020), to which the European native species are not resistant (Rezinciuc et al., 2016).

In the 1920s, only three native species - noble crayfish, *Astacus astacus* (Linnaeus, 1758), white-clawed crayfish *Austropotamobius pallipes* (Lereboullet, 1858), and stone crayfish *Austropotamobius torrentium* (Schrank, 1803) were present in Switzerland (Stucki and Zaugg, 2006). Like all European, native crayfish species, the remaining populations within Switzerland are now under severe threat of extinction. They are endangered by habitat destruction, water pollution and introduced invasive crayfish which originate from deliberate or accidental, past or present, stocking (Hefti and Stucki, 2006; Holdich et al., 2009). Four exotic crayfish, which appeared during the 1970s, can now be found in the waters of Switzerland; three invasive crayfish - signal, spiny-cheek, *Faxonius limosus* (Rafinesque, 1817), and red-swamp crayfish, *Procambarus clarkii*, (Girard, 1852) which are all vectors of the crayfish plague - as well as the non-indigenous species, narrow-clawed crayfish *Astacus leptodactylus* (Eschscholtz, 1823) (Barbaresi and Gherardi, 2000; Kouba et al., 2014).

Across Europe various protection and control measures have already been implemented to counteract the threats of invasive crayfish and in Switzerland these are listed in the Swiss Crayfish Action Plan (Hefti and Stucki, 2006). In order to be able to implement appropriate measures, knowledge of the distribution of both native and non-native crayfish species is vital. In addition to the traditional methods, hand-catch and trapping, there is now an ever advancing and promising way of detecting organisms using water samples to detect environmental DNA (eDNA) (Jerde et al., 2011). Monitoring of aquatic systems using eDNA promises to improve species conservation management of threatened species by providing precise information about distribution and population size (Bohmann et al., 2014; Takahara et al., 2013). eDNA also shows

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potential for identification of invasive species, even when they are at low densities, at any life stage and throughout the year (Kelly et al., 2014; Strand et al., 2014; Thomsen and Willerslev, 2015; Walker et al., 2017). Water samples allow identification of both micro- and macro-organisms, through broad approaches including; metabarcoding (Baird and Hajibabaei, 2012; Shaw et al., 2016; Valentini et al., 2016) or species-specific, targeted methods including; quantitative real-time PCR, qPCR or droplet digital PCR, ddPCR (Doi et al., 2015a, 2015b; Thomsen and Willerslev, 2015; Vrålstad et al., 2014). Freshwater crayfish species are among many macro-organisms where eDNA has been used to test for their occurrence (Agersnap et al., 2017; Dougherty et al., 2016; Tréguier et al., 2014).

Many factors influence the detectability of eDNA from water samples (Stewart, 2019). Firstly, the waterbody and its characteristics as well as external environmental conditions, are some of the reasons for the varying results of eDNA analysis (Buxton et al., 2017b). These abiotic factors include; water flow rate, temperature and pH, changes in weather including high rainfall or drought, and distance between collected samples. eDNA travel distance increases in correlation with higher river flow rates but this in turn increases degradation (Matthew A. Barnes, Cameron R. Turner et al., 2014). Weather shapes certain characteristics of waterways: high rainfall induces a dilution effect of eDNA (Buxton et al., 2017b; Harper et al., 2019), whereas drought creates low water levels which can consequentially also lead to lower species detection (Deiner et al., 2016; Sales et al., 2019).

Secondly, crayfish variables can affect eDNA detection, these biotic factors include species density and activity (Dunn et al., 2017). Throughout the year, during changing seasons, crayfish moult their exoskeleton; this occurs most frequently from summer until September when temperatures are warmest. Increased activity of crayfish reflect annual rising water temperatures (Reeve, 2004), therefore, increasing eDNA concentration in water (Troth et al., 2020).

Although various species of crayfish have been detected using eDNA in several countries (Chucholl et al., 2021; Rusch et al., 2020) and a multiplex assay has been used to determine presence of native and invasive crayfish (Robinson et al., 2018), a method has not yet been concisely established to simultaneously test for all native and invasive crayfish occurring across an entire country using several multiplex assays. Alternative approaches, including metabarcoding from a single sample, may be more concise but previous studies have shown that PCR provides a greater detection accuracy compared to metabarcoding techniques (Harper et al., 2018a, b), hence why this approach was selected in this paper. Additionally, there are few studies which use eDNA results to apply management strategies for native and invasive species (Cristescu and Hebert, 2018) and to our knowledge, those which apply eDNA to implement conservation plans across an entire country do not yet exist.

This study compares the use of traditional trap and hand-catch monitoring with eDNA methods using newly developed species-specific qPCR duplex assays to simultaneously detect crayfish populations across a whole country. We highlight our experience in relation to finding optimal techniques (field sampling, laboratory extraction and PCR) in which to show distribution and abundance of native and invasive species to apply relevant conservation management. We aimed to define the occurrence of the seven native and invasive crayfish species in 14 out of 26 cantons (political districts) across Switzerland. Here we focus on two cantons, *Lucerne* and *Vaud*, which were highlighted from eDNA analysis as significant for implementation of native species protections measures due to the fact that many populations of native species of crayfish were found to still exist here. This knowledge was used to provide relevant crayfish management strategies including native species arc site establishment and application of containment, suppression, and eradication techniques for invasive species. Both lotic and lentic sample sites were included to verify eDNA detectability differences and detection distance in various types of waterbody.

2. Material and methods

2.1. Validation sites

Several lotic and lentic sites (flowing and still waters, respectively) were selected to validate the eDNA method and compare its detection at sites where trapping (using crayfish pot PIRAT traps, 61 × 31,5 × 25 cm, with 2 funnel sections, bait box and bait needle) or hand-catch (using nets, stone turning and night-time torch surveys), had shown presence of specific species of crayfish (Table 1). Sites were sampled between May and September 2018 and again in the same months of 2019. For the purpose of this study, large rivers and lakes were not selected for validation as they are affected by the dilution effect due to increased volumes of water. Additionally, in larger waterbodies eDNA sinks down to deeper areas which also disturbs eDNA sample collection. In Switzerland, all large lakes and rivers are populated by invasive crayfish species. Therefore, in the field of crayfish conservation within Switzerland, the eDNA method is of particular interest in order to detect new occurrences of indigenous and immigrating invasive crayfish species in small and medium sized water bodies.

2.1.1. Lotic waters

Eleven sites were selected for the validation for lotic waters (Table 1). At *Riedbächli*, 8 traps (Bock-Ås Ky, Finland) were set at the respective eDNA sampling sites for one night, with dry dog food used as bait (See Fig. 1). The traps were checked daily and left for a maximum of two consecutive days. If a species of crayfish was caught in a trap, it was removed and the non-catch traps were left in the water for another night. At all other lotic sites, trapping was not carried out, instead, crayfish were found by hand-catch. Surveying for crayfish at these sites was carried out for 20 min at which point crayfish absence or presence was noted.

Riedbächli is home to a large population of white-clawed crayfish, present downstream in the watercourse. The last reliable evidence for their presence was in 2015 (personal communication). The well-structured and rather small stream has a width of 0.5 m–2 m and flows into the *Frenke* near *Bubendorf*. White-clawed crayfish also occur in the same catchment area, with evidence of their presence in the streams *Bennwilerbach* and *Flue Bach*.

2.1.2. Lentic waters

Two sites, *Mellinger Tanklagerweiher* (2 hectares in size) and *Mauensee* (surface area of 55 hectares), were chosen for lentic water validation (Table 1). At *Mellinger Tanklagerweiher* (See Fig. 2) and *Mauensee*, crayfish were identified by trap and hand-catch, respectively. *Mellinger Tanklagerweiher* is a pond with a large population of invasive red swamp crayfish. In 2015, a drainage system was installed in order to prevent the further spread of this species into the outflow of the pond. In September 2019, for validation of this study, samples were taken in the outflow drainage of the pond (VAL-Mell-F1+2), as well as in the outlet of the pond (VAL-Mell-F3+4) and in the main waterbody on the south side of pond (VAL-Mell-F5+6). *Mauensee* is a small lakefound in canton *Lucerne which* is home to populations of native noble crayfish (Bott, 1972).

2.2. eDNA sampling sites

After validation, we selected 153 streams, rivers, ponds and lakes within 14 of 26 cantons of Switzerland, to further test the detection of crayfish using eDNA (Fig. 3). Each canton was offered the opportunity to participate in the study. In this paper, we focus on waterways in cantons *Lucerne* and *Vaud*. Water samples were taken in these cantons from July until September 2019.

2.3. Field work – water sampling

Field work was adapted from Strand (Strand et al., 2019). For lotic

Table 1
Lotic and lentic water validation sites with confirmed crayfish occurrence by trapping or hand-catch.

Water type	Canton	Waterbody	Location	X-Coordinate	Y-Coordinate	Field indentification	Species detected
Lentic	Aargau	Mellinger Tanklagerweiher	Drainage	662′648	253′272	Trap	*P. clarkii*
Lentic	Lucerne	Mauensee	Neuschlosshof	648′143	224′319	Hand-catch	*A. astacus*
Lotic	Basle-Countryside	Riedbächli	Bubendorf	622′227	256′279	Trap	*A. pallipes*
Lotic	Basle-Countryside	Riedbächli	Shooting range	621′860	256′014	Trap	*A. pallipes*
Lotic	Lucerne	Schlimbach	Wiggehof	665′110	208′250	Hand-catch	*A. astacus*
Lotic	Lucerne	Rotbach	Rothenburg	669′866	218′886	Hand-catch	*A. astacus*
Lotic	Lucerne	Waldibach	Eschenbach	668′012	218′378	Hand-catch	*A. astacus*
Lotic	Schwyz	Chräbslibach	Talweid downstream	702′683	227′968	Hand-catch	*A. torrentium*
Lotic	Schwyz	Chräpsbach	Rüenzel	713′546	228′494	Hand-catch	*A. torrentium*
Lotic	Solothurn	Ibach	Chessiloch	609′783	252′458	Hand-catch	*A. pallipes*
Lotic	Solothurn	Verenabach	Wengistein	607′760	229′908	Hand-catch	*P. leniusculus*
Lotic	Vaud	Ruisseau du Crêt	Bois de l'Étange	533′899	183′030	Hand-catch	*A. pallipes*
Lotic	Vaud	Riau Gresin	Frémont	550′927	170′455	Hand-catch	*A. pallipes*

water sampling, 5 L of filtered water were collected on three separate filters for each location. On the other hand, at lentic sites, eDNA was collected by means of sub-samples where 1 L of water was taken at five locations evenly spread over a sampling site (distance between samples therefore varied depending on the size of the sampling site). These 5 L taken from various locations were collected in a bucket and pumped through each filter. This was carried out three times, once for each filter. The remainder of the procedure was the same for both lotic and lentic sites. At all sites, water was pumped with an Alexis® 12-V peristaltic pump (Bradenton Florida) directly from the sample site through a glass microfiber filter (Grade GF/B 1.0 μm, Whatman™, Maidstone, United Kingdom) (Fig. 4). If the filters clogged before the desired 5 L were reached, the volume was recorded and filters were changed, with a maximum of three additional filters taken, even if 5 L were not reached. A plastic funnel with a 100 μm polyamide mesh (Sefar AG, Heiden, Switzerland) helped prevent suspended particles from clogging the filter and a 1 kg plastic-coated lead weight (Best Divers, Rezzato, Italy) ensured the funnel was at the necessary water depth so that eDNA in the water would not be collected from any sediment, which could contain DNA preserved from potential historical populations of crayfish. The funnel was placed in the water at a location with moderate to strong current. At each location, 5 L of ultrapure water was filtered through the entire kit before the sampling began. This acted as a field negative control by ensuring that the sampling material used was not already contaminated with DNA, which would be visible after laboratory extraction and analysis of the corresponding filter.

New sterile sampling kits were assembled and used for each site to prevent false positive results by contamination. Silicon tubing, filter holders, funnels, weights, and tweezers were washed to remove excess visible sediment, disinfected with 2.5% bleach for 10 min, rinsed with nanopure water and thoroughly dried before being re-used. Plastic hoses, gloves and ropes were disposed of and replaced at each site. Between sampling sites, wellies and buckets were treated with Virkon® (Antec International - A DuPont Company, Sudbury, United Kingdom), according to instructions. This served to prevent the spread of aquatic animal diseases, including crayfish plague spores, between waterbodies as well as to try to avoid false positive results.

After filtering a maximum of 5 L of water through the filter, it was transferred using sterile tweezers into a bead tube from a PowerWater DNeasy kit. Tubes were transported from the sampling site to the laboratory at −18 °C in a portable freezer (CoolFreeze, CF, CF26, Dometic Group, Solna, Sweden) and there, stored in the freezer at −80 °C until extraction.

2.4. Laboratory work

2.4.1. Development of the qPCR primers and multiplex assays

Primers and probes were developed at Microsynth AG (Switzerland, Balgach) for each species occurring in Switzerland. They were designed as described below in the paper and then tested using real-time PCR against DNA samples obtained from tissue extractions of the seven different crayfish species. An in-silico search was carried out on the NCBI (National Center for Biotechnology) nucleotide database. The gene sequences of cytochrome oxidase subunit I, 16 S as well as 28 S were selected, which are known to be suitable for differentiating between species (Thomsen, Kielgast, Iversen, Wiuf, et al., 2012). From this, accession numbers (Table 2) were gathered and were used to produce oligonucleotide sequences. Bioinformatic software was used to find out which region of this sequence was conserved within each species. Known sequences from all closely related species (crustaceans and gammarids) were filtered-out so that sequence-specific primers could be developed. The resulting set of sequences was used to design primers and probes for the real-time PCR assays with proprietary software. To improve specificity of the designed oligonucleotides, several chemical modifications, known to increase the affinity of the specific sequence, were introduced to the primers and probes during synthesis (Table 2). An additional real-time PCR system (inhibition control) was designed to monitor potential inhibition in the reaction. This system contained primers and probes as well as an artificial DNA template at a defined concentration and was designed to not interfere with the crustacea assays or interact with other DNA in the samples in any kind of way. The specificity, efficiency and linearity of each assay were tested with the available biological material. For this purpose, parts of the body (pereopods, pleopods, uropods, antennae or muscle) of the seven species of crayfish were extracted using sterile scissors. In detail, nine different individuals of noble crayfish (five pereopods, three pleopods and one uropod) which all originated from *Steffetsmöösli*, *Wohlen*; five different individuals of white-clawed crayfish (two pereopods) which all originated from *Bennwilerbach*, *Hölstein*; seven different individuals of stone crayfish (two muscle and five pleopods) of which two originated from *Etzgerbach*, *Etzgen* and *Chellenbach*, *Hohfrist* and one from *Rätscherenbach*, *Schutzmühlebach* and *Grafenaubächli*, *Kaltbrunn*; seven different individuals of signal crayfish (four pereopods, two pleopods and one gonopod) of which three originated from *Eisweiher*, *Basle*, one from *Alter Teich*, *Riehenteich*, *Aubach* and *Amphibienweiher Autäli*; seven different individuals of narrow-clawed crayfish (three pereopods, one pleopod, one muscle and one antennae) which all originate from *Wenkenweiher*; seven different individuals of red swamp crayfish (four pleopods, two pereopods and one muscle) which all originate from *Tanklagerweiher*, *Mellingen* and eight different individuals of spiny cheek crayfish (five pereopods, two pleopods and one muscle) of which two originate from *Inseli*, *Rheinfelden* and six from *Limmat*, *Wettingen*. were used for DNA extraction and subsequent assay development. DNA was extracted in house using a NucleoSpin Tissue kit (Machery-Nagel, Switzerland) according to manufacturer's protocol with minor adaptations. Briefly, a 5 mm stainless steel bead (Qiagen, Germany), 180 μl lysis buffer and 0.5 μg proteinase K were added to the crayfish tissue and homogenised using a TissueLyser II (Qiagen, Germany) for two 1-min--cycles at 1/20s. Following this, the mixture was left at room temperature for 1 min and incubated at 56 °C for 1.5 h at 600 rpm. After washing,

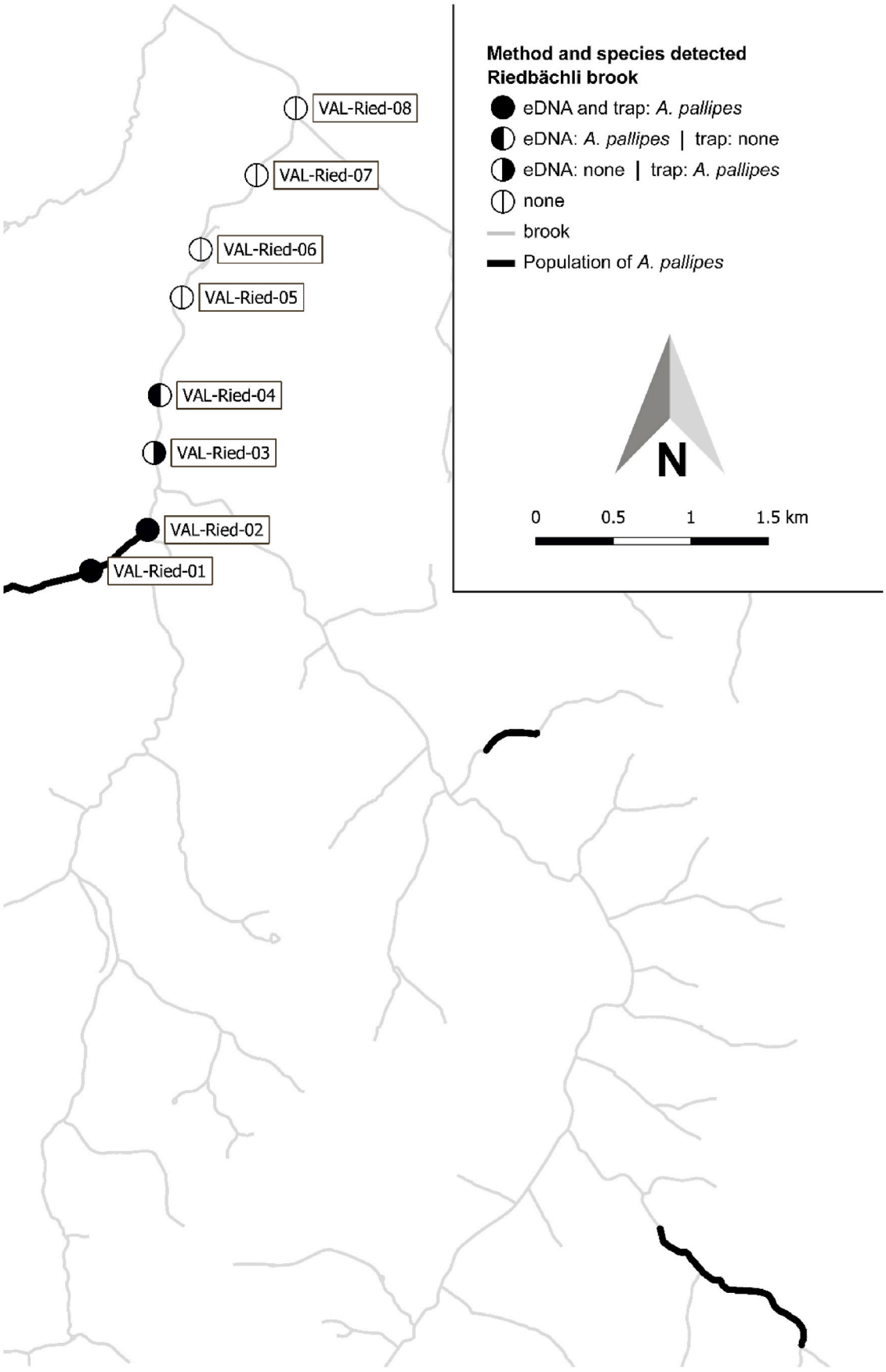

Fig. 1. Validation Site – *Riedbächli.* Results from eDNA sampling and trapping in the validation site, *Riedbächli*. Each circle represents where traps were placed and eDNA samples collected. VAL = Validation, Ried = *Riedbächli* and 01–08 = filter collection number.

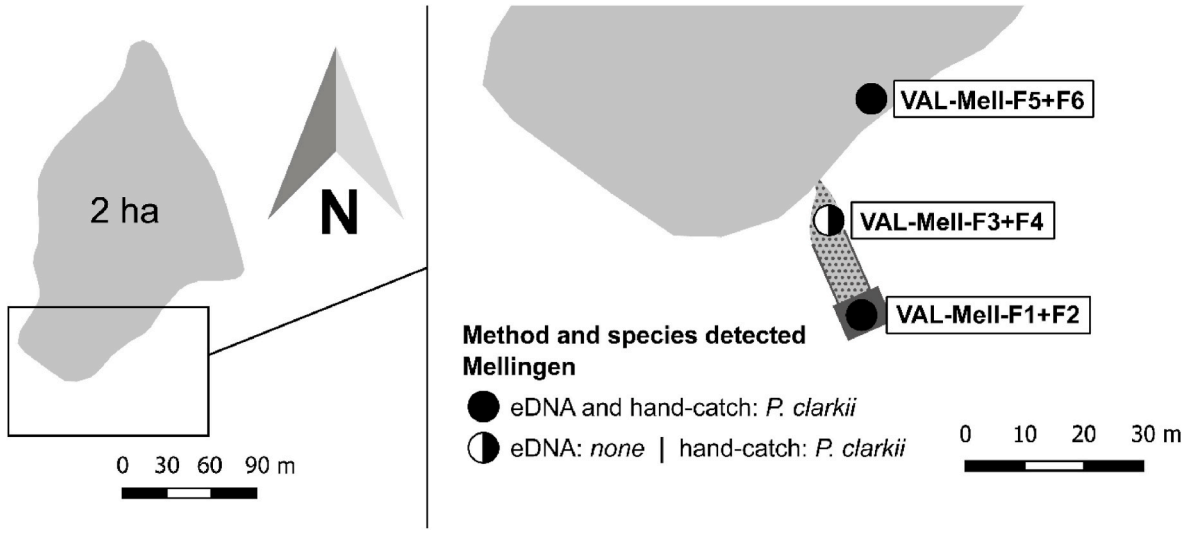

Fig. 2. Validation Site – *Mellinger Tanklagerweiher*, Results of eDNA sampling and trapping in the validation site, *Mellinger Tanklagerweiher.* Each circle represents where traps were placed and eDNA samples collected, VAL = Validation, Mell = *Mellinger Tanklagerweiher* and F1 – F6 = filter collection number.

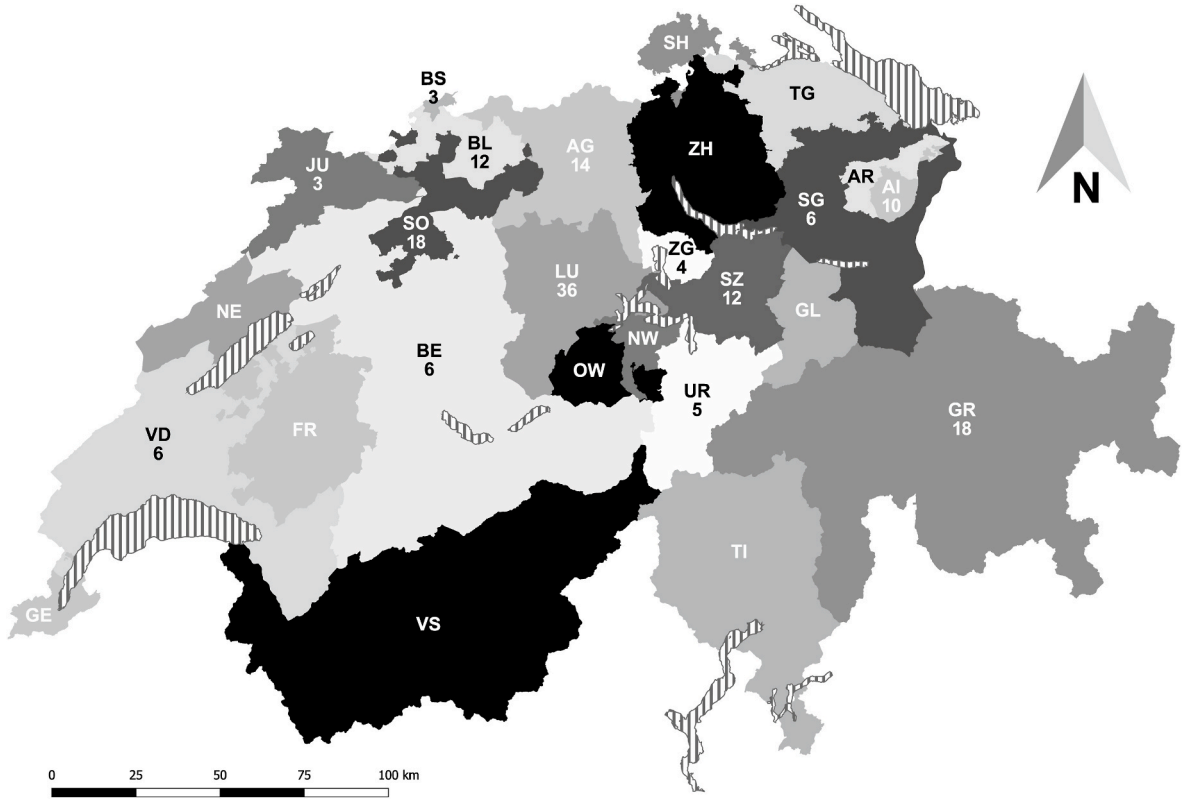

Fig. 3. Map of Switzerland showing an overview of all cantons and number of sites sampled in each. Hashed areas indicate large lakes. Ag = *Aargau*, AR = *Appenzell Ausserrhoden*, AI = *Appenzell Innerrhoden,* BL = *B*asle-Countryside, BS = *Basel*-City, BE = *Bern*, FR = *Fribourg*, GE = *Geneva*, GL = *Glarus*, GR = *Grisons*, JU = *Jura*, LU = *Lucerne*, NE = *Neuchâtel*, NW = *Nidwalden*, OW = *Obwalden*, SH = *Schaffhausen*, SZ = *Schwyz*, SO = *Solothurn*, SG = *St. Gallen*, TG = *Thurgau*, TI = *Ticino*, UR = *Uri*, VS = *Valais*, VD = *Vaud*, ZG = *Zug*, ZH = *Zurich.*

DNA was eluted with 100 μl elution buffer which had previously been heated at 70 °C and the concentration was measured at 260 nm using a Nanodrop 2000 spectrophotometer (ThermoFisher, Switzerland). Ten-fold serial dilutions of isolated DNA from each of the single species ranging from 5 to 0.00005 ng/μl, specific pools (which contained DNA from six of the seven species, for each of the seven species-specific tests) and a negative control template (NTC) ensured primers and probes were species-specific and could robustly quantify target species in an acceptable range of detection. The seven PCR systems were grouped in four duplex assays based on the melting temperature of the designed oligonucleotides: noble and red swamp, stone and white-clawed, signal and spiny cheek and narrow-clawed and the inhibition control. The probes of the different PCR systems which had been selected and grouped in one duplex assay, were labelled with different fluorophores (FAM and YYE) to differentiate signals from the specific PCR systems. The duplex assays were additionally tested for their ability to detect only

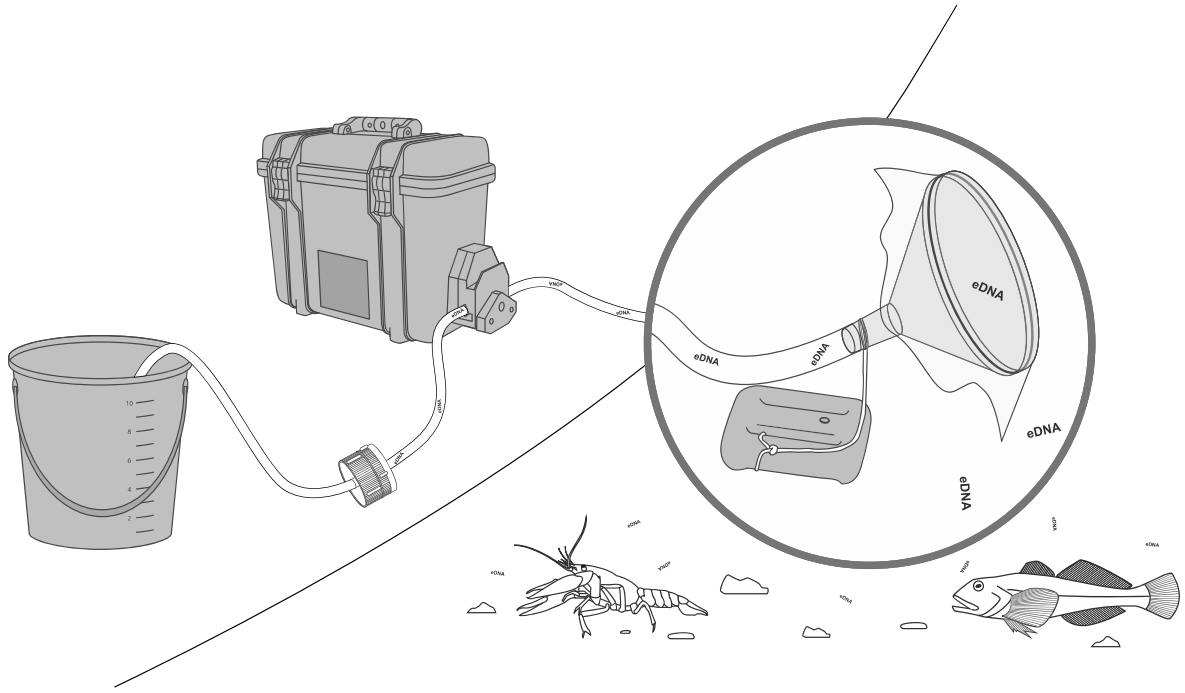

Fig. 4. Sampling setup indicating different components. Showing funnel with 100 μm mesh, suspended above sediment held down with a 1 kg weight. PVC tubing connects the funnel to the peristaltic pump. Water is pumped through the filter holder before being directed into the silicon tubing into the bucket to measure 5 L of filtered water. A rope is tied to the weight for retrieval of equipment at the end of sampling and for gentle lowering and placement of the setup at the beginning.

Table 2
Four duplex assays developed for the detection of crayfish. Indicating probe and forward and reverse primer sequences for the four duplex assays, relative chemical modifications and accession numbers so sequences can be found within the database (https://www.ncbi.nlm.nih.gov/). AA = *Astacus astacus*, PC = *Procambarus clarkii*, AT = *Austropotamobius torrentium*, AP = *Austropotamobius pallipes, PL = Pacifastacus leniusculus, OL = Orconectes limosus, AL = Astacus leptodactylus, IC = inhibition control.*

Assay	Oligoname	Oligosequence 5'-3'	Chemical modifications	Duplex-Assay	Accession Number
AA	Probe	CGAACACAGCACCTATTGAA	FAM/MGB-IQ500	AA + PC	AY151515.1
	Forward primer	GGGGTTTAACAGGAGTGGTTTTAG			
	Reverse primer	TCCCGTGAATAAAGGAAATCAATGA			
PC	Probe	AACTACATCACGCCACCATT	YYE/MGB-IQ530		KX268742.1
	Forward primer	AGACCTTGACCTTTAACTGGTTCA			
	Reverse primer	CAGCCCCAGTATG6AA6CCTTGA	6 = LNA-T		
AT	Probe	CCAATACCACGCCGCTGCTT	FAM/MGB-IQ500	AT + AP	KX268734.1
	Forward primer	CGGACTCTATTTACGGGTCAAC			
	Reverse primer	GAGGAA7AGTCACACTACATCTACA	7 = 2AdA		
AP	Probe	CAGTTCATCCTGTGCCAACC	YYE/MGB-IQ530		JX446632.1
	Forward primer	GGGGTTTGG7AATTGATTAGTTCC	7 = LNA-G		
	Reverse primer	GCTGATGCTAGAGG5GGATAA	5 = LNA-C		
PL	Probe	TGAATCACCGCAACAGCAGT	YYE/MGB-IQ530	PL + OL	KX268740.1
	Forward primer	GT5CGATTAGCTGCAAATATAATTG	5 = pdC		
	Reverse primer	CTTCCCTAGCATATAAAGTTCT5AA	5 = pdC		
OL	Probe	ATCCTCCTCTCGCTTCTGC	FAM/MGB - IQ500		MK439896.1
	Forward primer	GGAGTTGGGACAGGGTGAA			
	Reverse primer	GGCATTCGATCCATAGT5ATTCC	5 = pdC		
AL	Probe	TGATGC5TA5A5AAC5TGAA	FAM/BHQ-1	AL + IC	KX279350.1
	Forward primer	ATGG5ATACCTCGTCGGTA	5 = LNA-C		
	Reverse primer	GCCTCCCAAACAATAATAACAAACC			
IC	Probe	ACTCCGGTGAGACTCTCTCTACAGGGG	YYE/BHQ-1		
	Forward primer	CATCTTGCCTGAGTGACAATATTTTG			Synthetic construct
	Reverse primer	TTGTCCACGTCGTACAAGACC			
	DNA	TTCATCTTGCCTGAGTGACAATATTTTGTTCCCCTGTAGAGA GAGTCTCACCGGAGTTTGGTCTTGTACGACGTGGACAATT			

specified species. This was performed experimentally again by testing specific pools as described above. No cross-reactivity was observed (Table 6, Supplementary Information). By separating them into duplex reactions, we were sure, that if we got a signal from a specific species, it was indeed for that species of crayfish, therefore specificity of the duplex was given.

2.4.2. eDNA extraction

Various extraction methods (DNeasy® PowerWater® Kit and DNeasy® Blood & Tissue kit) were examined with samples containing genomic extractions from the seven species. Following this, qPCR revealed the optimal extraction kit, which was chosen for use in further extractions. Consequently, DNA was extracted from three of sampled

filters and the field negative control using DNeasy® PowerWater® Kit from Qiagen (Hilden, Germany), within one month of field collection. In the case of sites where additional filters were taken because it was not possible to filter 5 L of water, the three filters with the highest volume of water pumped were selected for extraction and subsequent analysis. However, for validation site samples, all filters were extracted and evaluated. The extraction was performed according to the manufacturer's protocol (Qiagen, Hilden, Germany) with the following adjustments: solutions PW1 and PW3 were heated for 10 min at 65 °C (preparation); after addition of PW1 into the tube, it was heated at 65 °C for 10 min (step 3); the tubes were centrifuged at 8000×*g* for 2 min (step 7) and IRS was incubated at 4 °C and 200 μl of it used (step 11). The extraction took place under a fume hood in a laboratory away from PCR amplification processes.

2.4.3. qPCR setup and eDNA analysis

The PCR was performed in a separate laboratory to where the extraction took place in order to prevent sample contamination. Duplex mixes were made up of relative amounts of the two species specific probes as well as the forward and reverse primers. For the duplex involving narrow-clawed crayfish and the inhibition control, DNA was included in the duplex mix (Table 7, supplementary information). A Qiagen pipette robot (Hilden, Deutschland) was used to set up 384 well plates for qPCR analysis. For each filter examined, two replicates were included. The plate was set up with each well containing relative amounts of specific primer and probe mix (duplex mix), Taqman mastermix buffer, 5x HOT FIREPol® Multiplex qPCR Mix and individual filter or negative control extraction. Each plate also included eight pipetting negative controls (water) which were additionally used as negative controls within the PCR process. These served to detect contamination during pipetting and PCR processes.

The different assays were performed in the LightCycler® 480II (Roche, Basel, Switzerland), with various thermocycling programmes optimal for each. For each species, a standard 10-fold serial dilution series (ranging from 5 to 0.00005) was prepared in every qPCR run to generate a standard curve for quantification of eDNA in the samples with respective cycle threshold (CT) values, which were used to calculate the DNA concentrations in each filter and determine the limit of detection (LOD) for each species. The presence or absence of qPCR inhibition was identified by calculating the difference in CT values, between the PCR negative control (water) and corresponding DNA filter replicates. A sampling site was regarded as positive if at least one of the two PCR replicates of a filter was above the species-specific LOD. If a positive detection of a target species was present in the field negative control filter or in the pipetting or PCR negative control for any site, the site results were not considered valid as the sample was deemed to be contaminated during field sampling or during pipetting or PCR processes, respectively.

After analysis of the qPCR results, both narrow-clawed and signal crayfish had a high LOD, therefore, we decided to try to improve the detection limit for these species. Using the same primers and probes that were used for qPCR, ddPCR was carried out for both species. The ddPCR analysis was performed for narrow-clawed and signal crayfish at Microsynth AG (Switzerland, Balgach). Here, 5.5 μl of the DNA sample was combined with 2xSupermix for the corresponding primers (0.9 μM final concentration) and probe (0.25μM final concentration) and the volume was increased to 21 μl with water. 20 μl of the mixture was used to generate droplets with the Bio-Rad Droplet Generator. Cycling conditions can be seen in Table 8 in the supplementary information. Droplet reading was performed with Bio-Rad QX200 droplet reader. Quality control of the ddPCR and data analysis was performed in QuantaSoft analysis Pro. All ddPCR reactions resulted in over 10,000 droplets per reaction. Populations of the positive and negative droplets were clearly distinguishable using amplitude differences greater than 2000 fluorescent units.

3. Results

3.1. Optimising laboratory results

3.1.1. Development of the qPCR primer and duplex assay

Four duplex assays for the seven species of crayfish and the inhibition control were developed (Table 2) with various programmes optimal for each (Table 9, supplementary information), which allowed the simultaneous detection of two species at one time. After in-vitro testing was carried out efficiency of each assay was found to be close to 100%, meaning it could be concluded that each assay performed well and proved that each did not detect non-target species (Table 10, supplementary information).

3.1.2. LOD determination

The LOD for qPCR was determined for each species (Table 3); it was theoretically one DNA copy for noble, white-clawed, stone and red swamp crayfish; 100 copies for signal crayfish and 1000 copies for narrow-clawed crayfish. DdPCR allowed clearer identification of signal and narrow-clawed crayfish at lower densities, with only 20 copies (0.0006 ng/reaction) needed for a positive detection of both species.

3.2. Trapping and eDNA detection at validation sites

3.2.1. Lotic waters

Crayfish were found in traps or by hand-catch in all 11 of the lotic validation water sites. All three native species (white-clawed, noble and stone crayfish) were detectable by eDNA in a total of 9 out of 11 lotic water validation sites (Table 4). Signal crayfish at *Verenabach* and white-clawed crayfish at *Ibach* were found by hand-catch but undetected by eDNA methods.

At the locations, VAL-Ried-01 and VAL-Ried-02, within the validation site *Riedbächli*, white-clawed crayfish were detected with both traps and by eDNA analysis. At VAL-Ried-03, only a single, white-clawed crayfish was found in traps. The most downstream location identified using eDNA was VAL-Ried-04, which was 900 m downstream of the known population in the *Riedbächli* (Fig. 1).

3.2.2. Lentic waters

Crayfish were found in traps or by hand-catch in both lentic validation sites, *Mellinger Tanklagerweiher* and *Mauensee*. eDNA from red swamp and noble crayfish was detectable at the respective sites (Table 4). At *Mellinger Tanklagerweiher*, two adults and one juvenile red swamp crayfish were discovered in the drainage of the outflow of the water at VAL-Mell-F1-2. In the outflow of the pond, VAL-Mell-F3 and F4, two further adults were found and in the pond itself, VAL-Mell-F5 and F6, two moult were discovered. Using eDNA, it was possible to detect red swamp crayfish in both filters from the drainage system and in the main waterbody of the pond (Fig. 2).

3.3. Nationwide eDNA monitoring

White-clawed crayfish could be detected with eDNA in eight cantons,

Table 3
qPCR LOD, detection limits, for each individual species and ddPCR LOD for P. leniusculus and A. leptodactylus.

Species	qPCR LOD DNA concentration [ng/Reaction]	ddPCR LOD DNA concentration [ng/Reaction]
A. astacus	0.00005	–
P. clarkii	0.0000025	–
P. leniusculus	0.001	0.0006
O. limosus	0.0000025	–
A. torrentium	0.0000025	–
A. pallipes	0.000003	–
A. leptodactylus	0.003	0.0006

Table 4
Results of eDNA detection at validation sites where there was a confirmed crayfish occurrence. Including the number of filters where the species was detected, seen in brackets, and the number of crayfish found in the field (+= crayfish exoskeleton, ++ = single finding, +++ = 2–5 crayfish, ++++ = >5 crayfish). PC = *Procambarus clarkii* , AA = *Astacus astacus* , AP = *Austropotamobius pallipes* , AT = *Austropotamobius torrentium* .

Canton	Water	Field	eDNA
Aargau	Mellinger Tanklagerweiher	PC+++	PC (2)
Lucerne	Mauensee	AA+++	AA (1)
Basle-Countryside	Riedbächli	AP+++	AP (2)
Basle-Countryside	Riedbächli	AP++++	AP (3)
Lucerne	Schlimbach	AA++++	AA (3)
Lucerne	Rotbach	AA++	AA (2)
Lucerne	Waldibach	AA+	AA (3)
Schwyz	Chräbslibach	AT++++	AT (3)
Schwyz	Chräpsbach	AT++++	AT (2)
Solothurn	Ibach	AP+++	–
Solothurn	Verenabach	PL+	–
Vaud	Ruisseau du Crêt	AP+++	AP (3)
Vaud	Riau Gresin	AP++++	AP (3)

stone and noble crayfish in four cantons, and red swamp crayfish in one canton. Signal, spiny-cheek, and narrow-clawed were not detected at any site in any of the cantons sampled (Fig. 5). In canton *Lucerne*, 13 sampled sites showed noble crayfish detection by eDNA and one site was positive for white-clawed crayfish with this method. Noble crayfish were only found at 5 of the 36 sites sampled in canton *Lucerne* using the traditional hand-catch technique; however, using eDNA it was possible to detect noble crayfish at an additional 8 sites (Figs. 6 and 7 and Table 11, supplementary information). In canton *Vaud*, eDNA revealed a positive detection of white-clawed crayfish in five of six sites, whereas they were only found in two sites by hand-catch (Fig. 8 and Table 12, supplementary information). For the purpose of this study, positive and negative eDNA results and the presence and absence of crayfish observed in traps or during hand-catch at all sites in canton *Lucerne* and *Vaud* as well as at validation sites were used in a Bayes test to calculate the prevalence estimative (Table 5). From Bayes tests, the false negative rate was calculated by dividing the total number of sites where eDNA showed a negative result by the total number of sites where the species were actually found with hand-catch or in traps, this equalled a conditional probability, P (eDNA negative | present in trap and hand-catch) = $2/(2+13)$, of 0.13. On the other hand, the false positive rate probability was calculated at P (eDNA positive | absent in trap and hand-catch) = $12/(12+23) = 0.34$ based on the total number of sites where a species was positive with eDNA but absent in traps or with hand-catch.

4. Discussion

4.1. Application of methods and results to other countries and species

4.1.1. eDNA as a technique

After evaluating the usefulness of both traditional hand-catch methods and the eDNA method, we found eDNA to be a reliable, non-invasive tool (Strand et al., 2019; Thomsen and Willerslev, 2015; Troth et al., 2020) which can be used in addition to traditional monitoring methods (trapping and hand-catch) for the successful detection of endangered species (Thomsen et al., 2012b) as well as for simultaneous multiplex detection of various species of crayfish across an entire country (Robinson et al., 2018, 2019). We found that by taking eDNA samples in combination with using traditional methods of sampling we were able to increase detection of species, as seen in sites where species were detected with just one of these methods (for example at the sites VAL-Ried-03 and VAL-Ried-04, within the validation site *Riedbächli*, crayfish were found only in traps or only with eDNA, respectively). eDNA has previously been considered a more sensitive tool for the detection of crayfish in ponds, in one study it showed 73% accuracy compared to 65% with traditional trap surveys (Tréguier et al., 2014).

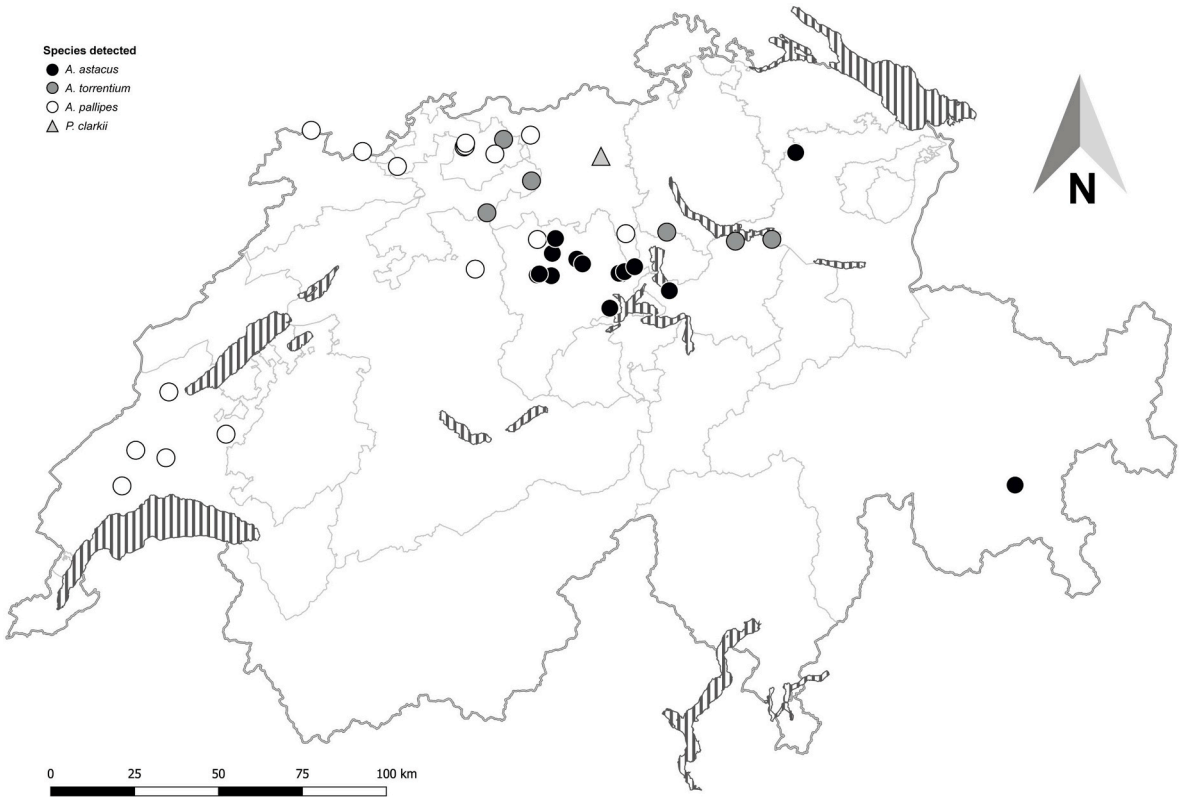

Fig. 5. Summary of the cantonal occurrence of the four species of crayfish found using eDNA within Switzerland. Hashed areas indicate large lakes. There were no positive results from eDNA analysis for *P. leniusculus*, *O. limosus* or *A. leptodactylus*.

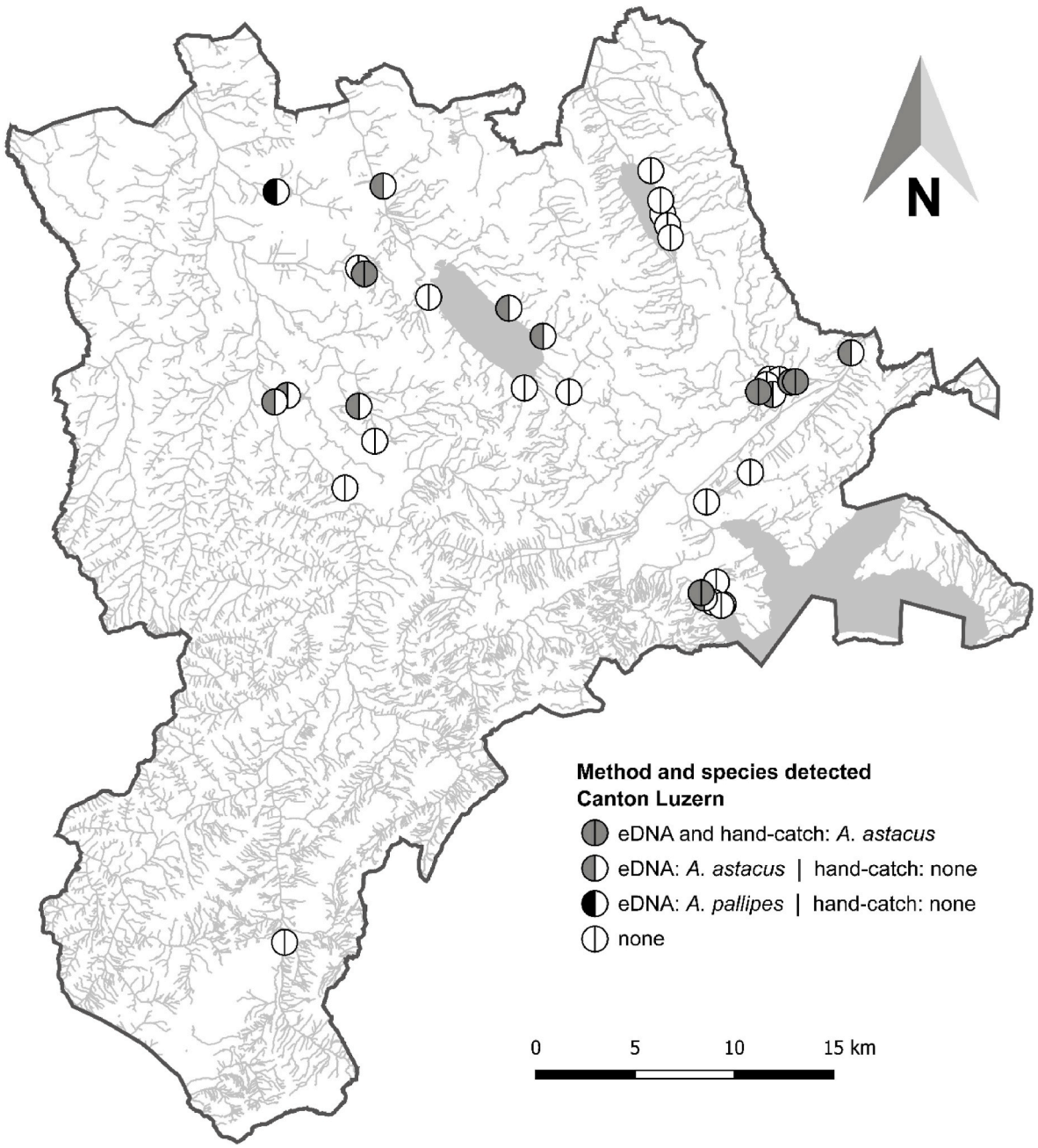

Fig. 6. Overview of results from eDNA sampling in Canton *Lucerne*. Left half of the circle: eDNA results; right half of the circle: detection during sampling.

Data from canton *Lucerne* and canton *Vaud* collected during our main sampling campaign also showed this trend, with 42% and 83% detectability using eDNA compared to 14% and 33% using traditional methods, respectively. However, based on our results from validation sites, we uncovered the presence of crayfish species with traditional methods at 100% of the sites compared to only 85% of time with eDNA methods. Additionally, previous studies state that a single sampling event can be sufficient to detect crayfish using eDNA (where crayfish are known to occur) (Cai et al., 2017); whereas, others have suggested eDNA monitoring cannot substitute catch per unit effort (CPUE) data (Johnsen et al., 2020). With such discrepancies, we recommended that both hand-catch and eDNA methods are used in combination for validation of positive occurrences, especially at locations where the presence or absence of species is unknown. Consequently conservation management strategies can be appropriately applied (Darling and Mahon, 2011; Jerde, 2019; Sepulveda et al., 2020).

4.1.2. Success of assays and methodology

Our designed assays will allow large-scale eDNA method application to improve nationwide native and invasive species assessments and management which was previously lacking (Langlois et al., 2020). In this study, we were able to detect four out of seven of the species occurring in Switzerland: noble, white-clawed, stone, and red swamp crayfish but not spiny-cheek, signal or narrow-clawed crayfish. This indicates that the first four assays are effective in detecting the presence of the species in various waterbodies and at differing population densities. The lack of detection of spiny-cheek, signal or narrow-clawed crayfish was, in this case, due to the low population abundance in the field, high LOD during qPCR or because of biotic or abiotic characteristics discussed in further detail below in section 4.3; however, these were resolved with use of ddPCR. The choice of protocols used for capture and extraction of eDNA from water can strongly affect species detection (Deiner et al., 2015). After testing various extraction kits, we

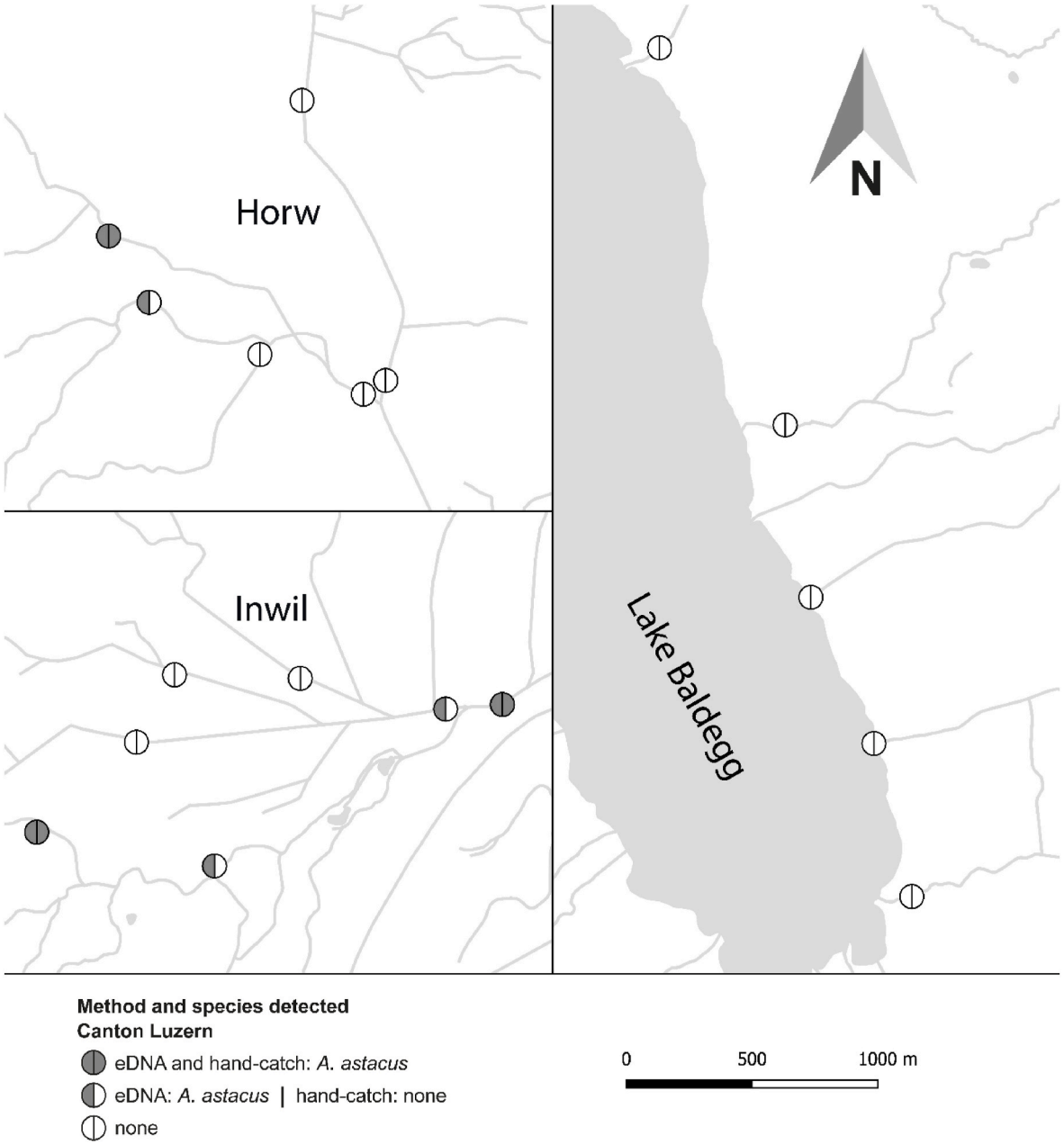

Fig. 7. Detailed results from the eDNA survey in Canton *Lucerne*. Left half of the circle: eDNA results; right half of the circle: detection during sampling.

were able to optimise extraction using DNeasy® PowerWater® Kit from Qiagen (Hilden, Germany), to detect eDNA by sub-sampling in lentic waters and by fixed-location sampling in lotic sites.

In-silico and in-vitro tests did not reveal any matches with non-target species indicating that the primers and probe were highly species-specific. Optimal mismatch number, mismatch type, GC content, and annealing temperature were applied when designing species-specific primers and probes so as to improve primer specificity (So et al., 2020). To minimise potential sources of error with specificity (incorrectly detecting non-target species) and sensitivity (detecting very low concentrations of target species) an extremely robust in-silico assay design was carried out, which was reliant on having plenty of genomic data from target as well as non-target organisms with which to design extremely discriminative assays (Us et al., 2019). Further details regarding the DNA extractions are given in the methods under section 2.4.1. The mid-regions of the sequences selected to produce crayfish primers and probes are specific to each species so the assays developed in our study should be repeatable in other regions with the same species because unlike in viruses (Domingo and Perales, 2019), animal genetic change is rather low (Allendorf, 2017). Therefore, our duplex assays as well as the methods and analysis using multiplex in this study can be applied to other countries to survey multiple species across an entire country.

Various methodological aspects, namely accuracy, repeatability and detection probability have been previously found to influence the reliability of the eDNA assays. Variability between natural replicates (the accuracy) is highly influenced by the amount of replicates needed to reliably detect and quantify species in the field (Mauvisseau et al., 2019). It could be argued that multiplex reactions reduce sensitivity and can lead to increased error probability; therefore, an increased number of replicates would increase results validity (Mauvisseau et al., 2019); however, the risk of false positives might increase with increased number of replicates (Ficetola et al., 2015). Previous studies were successful when only using two replicates (Willerslev et al., 2014). During

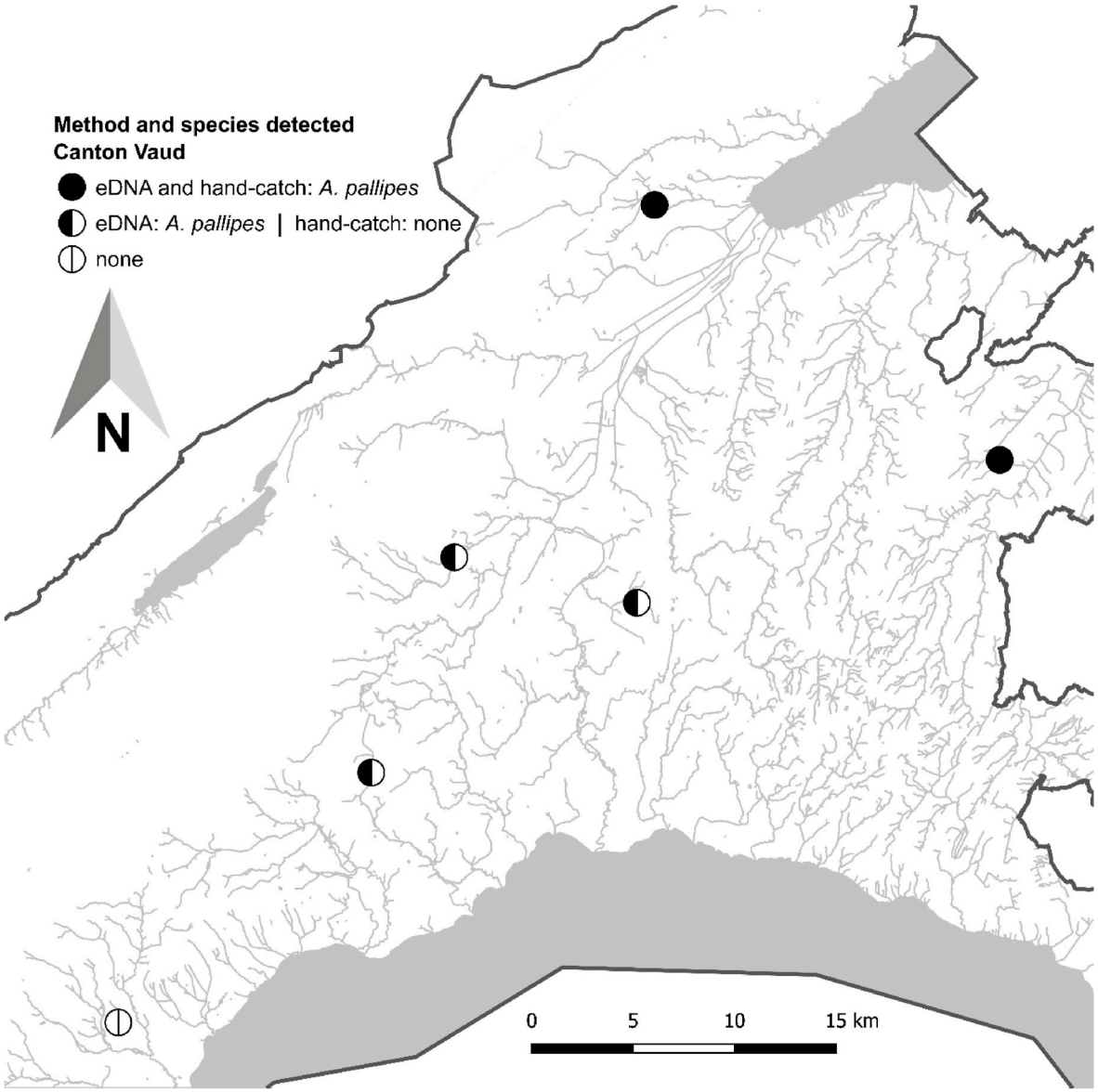

Fig. 8. Results from eDNA sampling in canton *Vaud*. Left half of the circle: eDNA results; right half of the circle: detection during sampling.

Table 5
Fourfold table including positive and negative eDNA results as well as the presence and absence of crayfish observed in traps and during hand catch; which were used in Bayes tests to calculate the prevalence estimative at all validation sites and sites in canton *Lucerne* and *Vaud*.

	eDNA positive	eDNA negative	Total
Proven presence with traps/hand-catch	13	2	15
Proven absence with traps/hand-catch	12	23	35
Total	25	25	50

validation in this study, we were able to use eDNA to identify crayfish species where their presence was known from traditional methods using only two PCR replicates; hence, why we decided to only opt for two replicates for the subsequent part of the study.

It is known that the eDNA methodology is prone to false negative and false positive errors which occur during sample collection as well as in the stages of laboratory analysis. Identifying errors in occurrence due to false positive and false negative results is extremely important to improve accuracy of results; likewise, it is vital to understand the ecological drivers which cause these errors. Increasing the eDNA replicates (number of water samples) collected at a site is thought to be more important than increasing the number of visited sites (Griffin et al., 2020). The false negative rate (0.13) and the false positive (0.34) found in this study indicate the accuracy of the result and how much emphasis of truth can be placed on this result. The false negative indicates sites where crayfish were found with traditional methods but not highlighted with eDNA analysis. This prevalence estimative can be inaccurate or false because crayfish activity can vary greatly depending on where traps are placed and where traditional catching methods are carried out. Deeper holes or other out-of-reach hiding places make it almost impossible to catch certain individuals and this in turn will alter the accuracy. If the density of the population is less, there will be fewer animals and in turn less activity, meaning there is a lower chance of finding their refuge during hand-catch (Wallinger et al., 2021). In this study, hand-catch effort per site was 20 min which also means some crayfish slightly further upstream may have easily been missed and not counted. Additionally, traps have been found in one study to have a success rate of just 2.3% (Chadwick et al., 2021) indicating that the number of crayfish caught in traps are often also mis-counted.

The false positive result indicates where species of crayfish were

present with eDNA but not found with traditional methods. However, if crayfish were not found by hand-catch or in traps that doesn't mean they are not there; there may be a lower density of the species which makes it very difficult to find them with traditional methods (the few individuals could be hidden away in the river banks) but enough eDNA is still released by the individuals to be detected in the laboratory. False positive results may also occur in field surveys due to slow degradation of crayfish eDNA (Troth et al., 2021). With this said, the eDNA cannot replace trapping or hand-catch – it is just another tool with which to increase the accuracy of confidence in results.

4.1.3. New discoveries of native crayfish

In total, noble crayfish were found in 13 locations within canton *Lucerne* (Table 11, supplementary information). eDNA revealed the presence of one white-clawed and eight noble crayfish populations, where it was not possible by hand-catch. Similar to *Lucerne*, in canton *Vaud,* an additional three sites were shown to have white-clawed crayfish populations using eDNA methods compared to hand-catch (Table 12, supplementary information). At the two sites in canton *Vaud* where white-clawed crayfish were also found by traditional methods in the field, there was a high abundance of the species. At both these sites, eDNA of white-clawed crayfish was also detectable in all three collected filters. This indicates a correlation between population size and detectability (Buxton et al., 2017b). The results from these cantons reveals the importance of eDNA as a tool for identifying populations of endangered species. This information can be used to deduce that both *Lucerne* and *Vaud* are key cantons for application of conservation action. Additionally, eDNA data can be used to aid decisions in conservation re-stocking, ark site establishment and genetic studies of native species (Nightingale et al., 2017).

In this study, stone crayfish were detected in four cantons, *Basle-Countryside* (*Hemmikerbach*), as well as in the canton of *Solothurn* (*Gretzenbach* and *Schweissacherkanal*), *Schwyz* (*Chräbslibach* and *Chräpsbach*) and *Zug* (*Littibach*) (See Table 1, data not fully shown here). Previous evidence suggested that their western distribution limit was in the canton of *Aargau* (Stucki and Zaugg, 2006), which means that the detected stone crayfish in *Basle-Countryside* and *Solothurn* are further west than the previously known distribution. We show that eDNA can, therefore, provide new insights into the distribution of unknown native crayfish populations.

4.1.4. New discoveries of invasive crayfish

Our methodology also revealed new discoveries of invasive crayfish populations where management techniques can subsequently be applied. After using eDNA to build a nationwide map of the occurrence and distribution of invasive crayfish, a plan can be established which introduces protection measures against these invasive species, such as the building of crayfish barriers (Krieg and Zenker, 2020). This highlights the importance of using eDNA for invasive species detection and how the method can be used to implement successful management strategies.

The data we gathered in canton *Geneva* (data not given here), allowed successful application of conservation management strategies as a result of positive eDNA detections of both native and invasive crayfish species. After the detection of the crayfish plague and signal crayfish using eDNA techniques in the *Allondon* but not in the connected river, *Le Roulave*, which inhibits white-clawed crayfish, management strategies were consequently decided upon with the construction of a barrier to stop the spread of invasive crayfish. This highlights the effectiveness of our methodology in being able to simultaneously detect both native and invasive crayfish species and shows an example of successful conservation management application from the methodology used in this paper. Our methods and results can subsequently be applied for use in other countries, with crayfish or other species and should be combined with disease distribution in waterways to create a risk map to enforce nationwide management plans (Minett et al., 2020).

4.1.5. Improving sensitivity with ddPCR instead of qPCR

An LOD was set to ensure a positive eDNA result could be definitively communicated for management strategy enforcement (Sepulveda et al., 2020). LODs from other studies are in a similar range (Atkinson et al., 2019; Tréguier et al., 2014). During this study, we demonstrated that eDNA monitoring can reveal more accurate information about the presence of crayfish populations even at low densities compared to traditional methods. Only a single eDNA copy is required for the detection of noble, white-clawed, stone, spiny-cheek, and red swamp crayfish, which explains why they could be more easily detected in the right environmental conditions. The high LOD for signal and narrow-clawed could explain the reason for the lack of detection of these species at sample sites. We showed that ddPCR provides better estimates for abundance and biomass. It is a more sensitive technique compared with conventional qPCR thereby producing more reliable results at lower population densities (Cai et al., 2017; Doi et al., 2015a, 2015b). We found ddPCR to be a better method for obtaining clearer identification of signal and narrow-clawed crayfish compared with qPCR, with only 20 copies needed for a positive detection (See Table 3). Further analysis should now be carried out to apply the use of ddPCR to a wide range of sites and all crayfish species. Our study indicates that ddPCR should be used to detect other aquatic species where they occur in low densities or where their numbers are unknown.

4.2. Our experience - lessons learnt in the field

4.2.1. Importance of the location of the sampling site - spiny-cheek crayfish detection

Spiny-cheek crayfish are abundant in large lakes across Switzerland, including in the largest lake located entirely in Switzerland, *Lake Neuchâtel*, and in one of the largest lakes in Western Europe, *Lake Geneva*. Their presence in large waterbodies could be another major reason for the lack of detection of this species within this study. In lentic waterbodies, particularly in large lakes, there is fast settlement of DNA in the sediment as there is little water flow to suspend the DNA and carry it to various locations (Siegenthaler et al., 2019). Therefore, when sampling a lake or pond, it is crucial that subsamples are taken in order to cover a wide range of locations across the still waterbody and increase the chance of species detection. This is particularly vital if there is no previous knowledge about where populations occur.

4.2.2. A negative eDNA result does not undoubtedly indicate a negative presence

Despite a large population known at both validation sites, signal crayfish and white-clawed crayfish were found at *Verenabach* and *Ibach*, respectively, only by hand-catch and not detected with eDNA. The lack of detection at *Verenabach* was most probably due to the high LOD of the species. The water temperature of *Ibach* was 15 °C at the time of sampling so activity was relatively high. The lack of detection of species here could be explained by a localised presence of crayfish. Most of the populations occur downstream of the sample site, meaning that there was little eDNA collected on filters due to low biomass of crayfish in the area where the water was sampled (Jane et al., 2015). These lotic water validation sights highlight the fact that negative results from filters during eDNA analysis do not necessarily indicate the absence of crayfish. Previous studies have found variations in season (Troth et al., 2021) and method collection (eDNA verses traditional methods) (Johnsen et al., 2020) majorly contribute to successful species detection and careful consideration of these factors can avoid false negative results. In order to validate a negative result, we therefore recommend that additional eDNA samples from a stretch of the said waterbody should be taken throughout the year in addition to use of traditional methods, localised trapping and hand-searches. This emphasises the need to use both eDNA and traditional sampling methods to definitively indicate a positive detection. The conditional probability of Bayes tests (the false positive and false negative rate) will decrease if eDNA tests are performed more

often at various times throughout the year.

4.2.3. Travel time of eDNA in lotic waters

Our results from *Riedbächli* show that eDNA sampling can detect the presence of a large population of white-clawed crayfish when samples are collected close to the population. In addition, it is shown here that using eDNA, species could be detected up to 900 m downstream of where the population occurs in a lotic waterbody. Depending on the waterbody and its characteristics, this can vary greatly (Wilcox et al., 2016). It can be assumed that lots of DNA from white-clawed crayfish is released into the water at this site, by the fact that 5 of the 6 filters at the two *Riedbächli* locations tested positive for this species. The high release of DNA at *Riedbächli* could be explained by the large population of native crayfish living here, thereby explaining why the species could be detected at distances as far as 900 m downstream. The amount of eDNA decreases the further downstream the sample is taken from a population (Jane et al., 2015). Increasing discharge has been found to dilute eDNA, but increase transport distance (Wilcox et al., 2016). There is little consensus on the specific distance travelled by DNA and detection; the detection distance for eDNA has been reported at 2 km from the sampling point to the aquatic population (Civade et al., 2016) and even 13.2 km downstream of a population (Evans and Lamberti, 2018). In another case, eDNA of brook trout decreased by 50% every 100 m (Wilcox et al., 2016). Travel time of eDNA and retention of other cellular particles is thought to play a significant role in detection of eDNA, it is thought to take between 5 and 40 h to travel 1 km, depending on characteristics of the watercourse. Such movements are long enough to affect DNA degradation and further decrease detection over a given distance (Deiner and Altermatt, 2014).

4.2.4. Sampling of sediment leads to false positives

DNA is expected to degrade over time but the persistence of eDNA in the water is highly variable and dependent on environmental conditions (Strickler et al., 2015). Reports of undetectable levels of eDNA after elimination of the target organism have been after a few hours (Thomsen et al., 2012a), two to three weeks (Buxton et al., 2017a; Troth et al., 2020) or even two months (Strickler et al., 2015). However, when eDNA is incorporated into water substrate, it can persist for thousands of years (Anderson-Carpenter et al., 2011; Turner et al., 2015). So although, sampling benthic environments have revealed better detection of crayfish species (Figiel and Bohn, 2015), sampling from the bottom of watercourses as well as in sediment collects DNA which could have settled and could imply a false positive of DNA from a population no longer occurring in this area. This would pose the question of whether the eDNA sampled is from a current population or from ancient populations which have been trapped and preserved in the sediment (Anderson-Carpenter et al., 2011; Buxton et al., 2018; Curtis and Larson, 2020). Therefore, water samples collected in this study were taken at a specific depth, around 5–10 cm above the ground, with avoidance of sediment collection and also not after heavy rainfall due to the dilution effect.

Different waterbodies contain various ground substrate - clay, mud, large rock or small pebbles - which in turn affect sampling and subsequent eDNA collection. Certain substrates block filters more easily during water collection; others contain more organic substances which inhibit qPCR in the laboratory and reduce detection probability (Dougherty et al., 2016; Hata et al., 2015; Jane et al., 2015; Rees et al., 2014). We emphasise the importance of considering the nature of the filtered substrate in order to correctly interpret the results from laboratory work. In this study, we aimed to filter 5 L of water three times per site, but this was not always possible in highly turbid waters or those which were fed with glacial melt. In highly turbid waters, after heavy rain fall or in watercourses with easily disturbed silt substrate, problems arise due to fast clogging of the filters (Wittwer et al., 2018a, 2018b). High amounts of suspended matter decrease the filtered water volume and increase the concentration of inhibitory substances with a potentially negative impact on subsequent eDNA analysis (Cai et al., 2017). We found a funnel mesh to be vital to help prevent the filter from being clogged and to avoid organic inhibitors.

4.2.5. Variations in field sampling can affect detectability of eDNA - abiotic

Some studies argue that detection of eDNA is generally more likely in lentic compared to lotic waterways, with higher levels of eDNA determined in lakes than in rivers (Civade et al., 2016). Others state that in lentic waters, sampling is notably more difficult, and it is harder to detect crayfish populations, especially after heavy rain events, due to loose sediments free in the water. At lentic sites, the lack of flow and variation in water being filtered creates rapid DNA settlement in sediment, therefore, decreasing the likelihood of eDNA collection (Harper et al., 2019). Consequently, in this study, at sites with still water (all ponds and lakes), fieldwork was carried out by means of sub-samples, which were taken over a transect in order to increase the chance of detecting a current population (Dougherty et al., 2016; Troth et al., 2020). Sub-sampling in this study allowed detection of crayfish species by eDNA where they were not found with traditional trapping and hand-catch methods. In canton *Vaud*, white-clawed crayfish were not found by traditional methods but could be definitively determined with eDNA after sub-samples were taken; here all three filters were positive for this species. Again, this reveals the importance of using eDNA techniques in combination with traditional methods for indicating populations of conservation importance. Despite sub-sampling in lentic waters, eDNA detection was highly variable. In Lake *Mauensee*, one sampling point was sufficient to detect noble crayfish. The more samples taken across a range of locations at a sampling site the higher the probability of detection.

There are discrepancies as to whether increased water volumes improve the likelihood of detection of crayfish by eDNA in waterways with low populations. When waterbodies flow into the sampled water, the sample is diluted and there will be a reduced collection of eDNA (Deiner and Altermatt, 2014). For this reason, sample sites selected in this study avoided areas where tributaries joined other waterways. Some studies indicate sampling higher volumes of water does increase the probability of species detection (Buxton et al., 2017b; Cowart et al., 2018; Lugg et al., 2018; Turner et al., 2015; Williams et al., 2017; Wittwer et al., 2019), but this requires more time and is sometimes not possible at sites with high turbidity. Other studies have provided evidence of detection of crayfish by eDNA with water volumes of 1 L (Cai et al., 2017; Riascos et al., 2018; Rice et al., 2018), 2 L (Atkinson et al., 2019) and 10 L (Wittwer et al., 2018a). Even with only 1 L filtered, eDNA has provided evidence of occurrence with 85.7% accuracy (Riascos et al., 2018). Varying water qualities and characteristics make each site an individual case to determine the necessary volume of water, which must be filtered to detect crayfish. In our case, when the waterway was clear we were able to collect 5 L of water per filter on average in 7 min. However, if the water was cloudy and full of sediment or glacial melt run-off, the filtered clogged quickly usually after 1 min and we were only able to collect on average 1 L of water on each filter. Several times during our study the detection of crayfish DNA in the samples was successful in only one of three filters tested. However, there was no correlation between species detection and water volume, for those sites where 5 L could not be filtered.

Differences in season, temperature and activity of crayfish can also vastly alter eDNA capture rate; therefore, careful of these factors is vital for successful eDNA collection. It is known that signal crayfish do not become fully active until May (Stucki, 2002). In this study, during field sampling in May 2019, it was unusually cold for the time of year. For this reason, it can be assumed that crayfish were still less active, produced fewer molts and therefore, there was a reduction in the amount of DNA released into their environment (Harper et al., 2018a, b; Tréguier et al., 2014; Troth et al., 2021). This could explain the lack of detection in traps and by eDNA at certain sites. In the summer, temperatures became extremely high and consequently, towards the end of the sampling period, in some streams, the water level was unusually low, which also

decreased eDNA collection on the filter. With the changing climate and vastly varying seasonal temperatures, it is necessary to consider not only the season as an indicator of optimal sampling but the actual water temperatures which trigger crayfish activity and reproductive state. This shows the impact which climate change can have on our waterways and the organism living within it.

We found that the best time to carry out field sampling is from late spring to early autumn. At this time, water temperatures are optimal, above 14 °C, and crayfish activity highest, therefore, there is an increased chance of collecting eDNA, ensuring the species that are present are detected (Bernardo et al., 2011; Buxton et al., 2017a, 2017b; Peay, 2003; Reeve, 2004; Stucki, 2002; Wacker et al., 2019; Wittwer et al., 2018a, 2018b). The detectability is said to decrease throughout autumn to a low during winter, followed by an increase from spring with peak detection levels being in summer (Buxton et al., 2017b; Peay, 2003; Wittwer et al., 2018a, 2018b). Although we should not rely solely on eDNA for density estimates (Johnsen et al., 2020), a correlation between population density and eDNA collection has been suggested (Rice et al., 2018; Troth et al., 2020; Wallinger et al., 2021), thus indicating the importance of water collection during the peak of activity. In this study, the importance of sampling during the active period was highlighted, to allow the best possible chance of detection of a current crayfish population with eDNA. This indicates that temperature and seasonal timing are crucial for valid identification of crayfish species.

4.2.6. *Variations in field sampling can affect detectability of eDNA - biotic*

The size of a population of crayfish also alters the amount of eDNA released, with water collected from streams with higher densities showing increased eDNA copy number and higher rate of detection compared to waters with very low populations estimates by traditional methods (Buxton et al., 2017b; Strand et al., 2014). This trend is also reflected in our results. The amount of eDNA present in the water changes with life history timing; eDNA production significantly increases when crayfish activity is highest, during the breeding period, while females are berried and while eggs are hatching (Dunn et al., 2017; Jane et al., 2015; Troth et al., 2020, 2021). At *Mellinger Tanklagerweiher*, a large population of red swamp crayfish was detected at two of the three sites, in four of six filters. However, in *Waldemme, Kurzenhütten, Lucerne,* even though only a single moult from a noble crayfish was found by hand-catch, it was still possible to detect the species with eDNA in all three filters. This highlights the variability amongst sites and the need to consider analysis of results from each site individually.

4.3. *Summary of findings*

The simultaneous detection of both native and invasive crayfish species by eDNA is important to indicate waters in need of protection as well as those where invasive species management strategies should be implemented. Our results show that eDNA is a reliable technique for the simultaneous detection of both native and invasive species. It should be applied with traditional methods for effective conservation action (Sepulveda et al., 2020). Application of the eDNA method carried out here and optimised sampling techniques discussed in this paper can be used for early invasive species warning and for conservation, environmental management and restocking purposes across Europe. The distribution risk maps for Switzerland created in this study identify hotspots where native and invasive crayfish occur. They can be combined with previous knowledge of waterborne disease, namely crayfish plague, in order to identify areas with potential to become ark sites as well as those waterways which should be prioritised for immediate conservation action. Fish and amphibians are generally easier to detect using eDNA compared to crayfish (Walker et al., 2017). Therefore, our method can be applied and used to simultaneously detect not only crayfish species in other countries but various endangered, native and invasive species allowing optimal aquatic eDNA sampling to create a more accurate picture of distribution (Jerde et al., 2011). It is recommended to test the effectiveness of our developed primer and probes on other populations in other countries before completing experiments.

5. Conclusions

We conclude that eDNA can provide an indication of where native and invasive crayfish populations currently occur across Switzerland and this can be applied to other countries. qPCR allowed detection of eDNA from four out of seven of the species occurring in Switzerland. Our work helps contribute to the knowledge of optimal sampling; traditional methods of hand-catch and trapping are recommended in combination with eDNA analysis, for validation of positive results. The method was altered and optimised for both lotic and lentic waters. It was possible to detect in eDNA in both types of water, showing that the technique is applicable to a wide range of sites. Each waterbody should be considered individually when assessing the best way to detect for the presence of crayfish, as many factors influence waterbody characteristics, fieldwork and subsequent laboratory analysis. It is possible that in the near future other crayfish species will also appear in Switzerland, most likely the marble crayfish, *Procambarus virginalis* (Chucholl et al., 2012) and the calico crayfish, *Faxonius immunis* (Herrmann et al., 2018), which already occur in neighbouring Germany. Therefore, it is vital to now develop primers and probes for these species. This said, other countries can use the duplex assays developed in this study to detect eDNA from native and invasive crayfish species to establish population distributions for application of relevant nationwide conservation action. We believe that the ability to simultaneously detect both native and invasive species across an entire country using eDNA will vastly improve the success of conservation management planning and action. Thereby reducing the damage caused by invasive species in the aquatic environment. The nationwide picture created will allow countries to be proactive in implementing management strategies earlier and in areas of most need.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2021.113929.

Author contributions

AZ conceived the ideas and designed the methodology; AK, AZ and RK collected the data; AK analysed the data; AW contributed to conception and design of the study, and to analysis of the data; AK and AZ led the writing of the manuscript, RK produced figures and contributed critically to the drafts and all authors gave final approval for publication.

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