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Vikten av provtagningskontroll vid eDNA analyser av dammlevande amphibier

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Abstract

The eDNA approach for monitoring aquatic species is already widely used. However, there are still uncertainties, for instance regarding the distribution of target DNA in ponds. These uncertainties need to be addressed when using eDNA in management. The current study uses individual capture and eDNA monitoring methods based on bottle trapping and 3x15 ml water sampling for *Triturus cristatus* in different ponds to show the importance of a carefully considered and performed study and in particular sampling design for achieving reliable results. Most of the results were received from only one pond. While here the bottle trapping resulted only in 15 catches, the eDNA method showed better results in confirming presence of the target. Examining two different primer combinations, the one with the bigger amplicon was more reliable than the one with the shorter amplicon. However, the data clearly indicated an influence of the sampling location on the eDNA results. This is also true for weather factors and / or sample time within the season. Therefore the sampling design has to be reconsidered, with respect to DNA distribution and persistence, seasonal habitation differences of the target and the primer specifics.

Key Words: eDNA, species specific markers, non-invasive monitoring, *Triturus cristatus*, bottle traps

Introduction

Almost 10 years ago, a new non-invasive tool based on short DNA fragments persisting in the environment and used as an early warning system for invasive and as a more sensitive monitoring method for rare or protected aquatic species in freshwater started to be explored (Beja-Pereira et al., 2009; Darling & Mahon, 2011; Dejean et al., 2011; Ficetola et al., 2008; Valentini et al., 2009). Since then many studies have been working on perfecting the use of environmental DNA (eDNA), extracted from water samples to receive information on presence and absence or even quantities of the target species, which can then be used for management decisions (Darling & Mahon, 2011; Eichmiller et al., 2016; Goldberg et al., 2016; Ma et al., 2016). Rees at al. (2014) wrote a review on the different attempts and trouble-shootings trying to overcome the limitations of this method. This and other studies formulated suggestions on how to standardise the use of eDNA. So far there is still no consensus about the appropriate study design for achieving reliable results, even though it is already highly investigated. This comprises the water sampling method, amount of water sampled, DNA precipitation and extraction methods, PCR composition and the way how to apply the multi-tube approach. Overall, the desired benefits are traits such as being low price, less labour intensive, non-invasive and, especially, more reliable than the traditional methods (Darling & Mahon, 2011; Thomsen & Willerslev, 2015; Valentini et al., 2009). Several studies already point out the efficiency of this method, however, the reliability, especially regarding false positives and false negatives, is still a major issue (Darling & Mahon, 2011; Jerde et al., 2011).

Despite of all the ambitious investigations made until now, further studies for certain assumptions are still missing to be able to create a reliable monitoring design. For instance regarding the distribution of eDNA throughout a pond, influencing the water sampling design to detect sometimes even rare species. The protocols mostly recommended taking 3x 15 ml water samples without giving detailed information (Ficetola et al., 2008; Sigsgaard et al., 2015; Thomsen et al., 2012). However, some assume that there is a homogeneous distribution of DNA in pond water, while other studies assume that the water samples have to be taken at different locations along the shoreline to achieve informative results due to lack of understanding about DNA distribution and persistence (Biggs et al., 2015; Davison et al., 2016; Diaz-Ferguson & Moyer, 2014; Thomsen et al., 2012).

The initial idea in this study was to make this new eDNA approach applicable for the local environmental authority of Saxony (Germany) regarding pond living amphibians under the European Habitats Directive. This should include an easy to use water sampling method, so that even unexperienced staff could be engaged and the traditional survey window could potentially be expanded (Biggs et al., 2015; Rees, Maddison, et al., 2014). Therefore, firstly new species specific and sensitive primers should be designed and tested regarding the species composition of the study area (Goldberg et al., 2016). To address the previously described sampling uncertainty, I compare the eDNA approach based on the practical three 15 ml samples with traditional trapping methods for the widely studied great crested newt (*Triturus cristatus*, (Laurenti, 1768)) in standing water bodies. The overall aims here are to check for consistency in the results within and between four trapping / sampling locations and therefore if the sampling design in terms of DNA distribution in ponds and season matters.

The study is based on the hypothesis that eDNA is not evenly distributed within a pond. Here I assume that the probability of capturing target DNA and therefore of amplification success is correlated with target activity centres interpreted from location-dependent quantitative trapping success. Based on that, I hypothesise that it does matter where and how many samples are taken to receive reliable results. The study period comprises the whole reproduction season to observe the development of the results during immigration and emigration, reproduction and hatching and presence of the larvae of the target species.

Methods

Study Area

The study took place in the borough "Leipziger Land" (Saxony, Germany) (Figure 1). Four natural monument area ponds with known occurrence of *Trituruis cristatus* were assigned by the local environmental authorities. Three were selected for bottle trapping and water sampling (eDNA), the fourth as positive control (pond PC) for the eDNA approach with low water volume and high target quantities (Table 1 and Figure 1). They were all directly surrounded by shrubbery needed for hibernation of the target species. The quantification of the study area was based on georeferenced google earth images and basemap imageries of ArcMap using GIS.

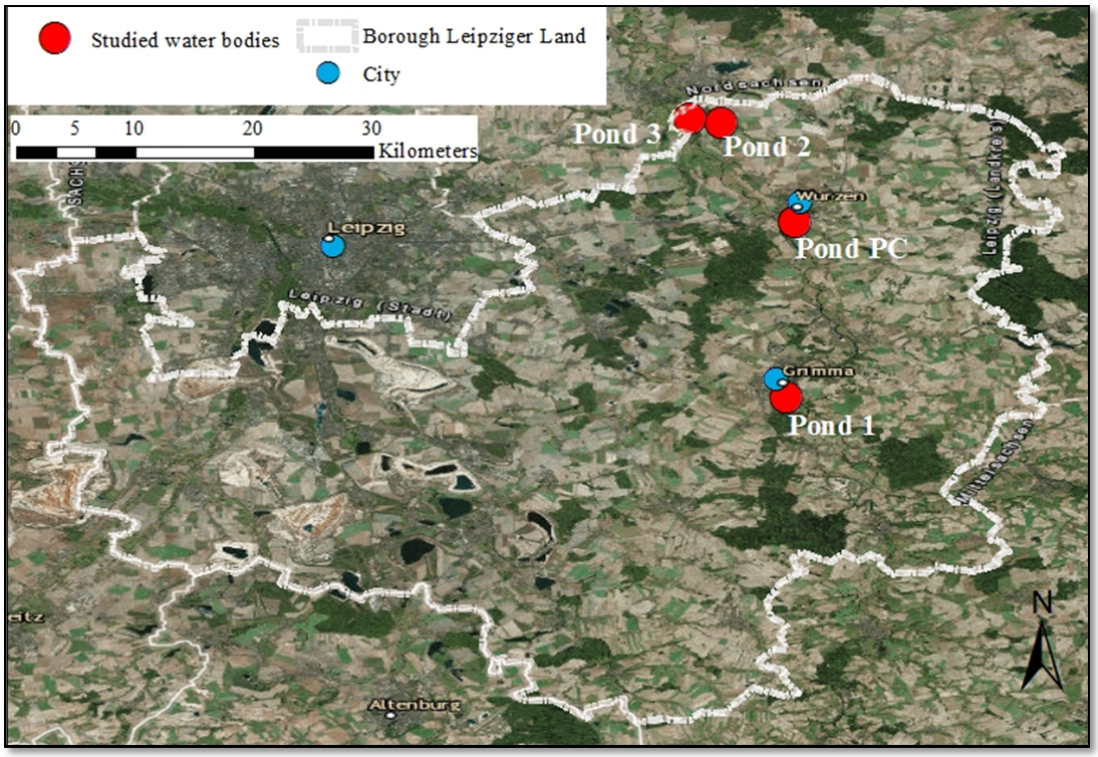


Figure 1: Study area in the borough "Leipziger Land" in the south-west of Saxony (1: Aueteich Grimma; 2: Drei Steine; 3: Kribbelwasser; PC: Wachtelberg).

Table 1: Specific measurements of the selected ponds for the current study

Water body	Pond 1	Pond 2	Pond 3	Pond PC
Size (ha) After drying out	~ 2 ~ 1	~ 0.5 ~ 0.5	~ 0.8 ~ 0.4	~ 0.0012 ~ 0.0006
Shallow area (%) Depth (m) Plant cover	80 0.2 – 0.5 Mainly reed cover	100 0.3 – 0.5 More than half reed cover	90 0.1 – 0.3 Mainly reed cover or grassed silt area	40 0.1 – 0.2 Grassed
Deep zone (%) Depth (m) Plant cover	20 ~ 3 Some reed cover	0 - -	10 1 Some reed cover	60 1 No plant cover

Target Species

The great crested newt (*Triturus cristatus*) is an already widely studied amphibian for eDNA studies (Biggs et al., 2015; Rees, Bishop, et al., 2014). With its 11-20 cm body length it is the largest out of four newt species existing in Germany, the only one protected under the Habitats Directive and one out of four or five crested newt species occurring in Europe (Arntzen et al., 2007; Arntzen & Wallis, 1999; Steinfartz et al., 2007). Specimen of *T. cristatus* have an individual belly pattern and show a clear sexual dimorphism in the breeding phenotype (Figure 2) (Glandt, 2016; Grosse & Seyring, 2015). It is a semiaquatic creature and mostly occurs in sun-exposed ponds, usually < 1 ha, with moderate vegetation and without or low level of fish occurrence (Glandt, 2016; Grosse & Seyring, 2015). Newts are ectotherm, meaning that their activity is highly dependent on ambient temperature (Glandt, 2016). Its immigration to the ponds begins mostly in February-March depending on weather factors (males 1-2 week earlier than females) and the emigration happens mostly in June-July with some individuals even hibernating in the pond (Glandt, 2016; Grosse & Seyring, 2015; Henle & Veith,). The reproduction phase (March to June) starts with a complex courtship behaviour and secretion of aromatic substances, the male then deposes a spermatophore on the ground from which the female picks up the sperm (Glandt, 2016; Grosse & Seyring, 2015). Mostly in April the female separately deposits 200-300 eggs on submersed vegetation and after about two weeks the larvae hatch (Grosse & Seyring, 2015). I assume that all these actions increase the eDNA concentration in the water and influence the probability of trapping success.

Figure 2: Belly pattern image of two bottle trapped *T. cristatus* individuals of the current study; male (left), female (right)

Trapping

Four trap plots with 5 bottle traps each were set in each of the three ponds (Figure 3) (Griffiths, 1985). They were placed in 1-2 m distance to the shore line with the single traps 0.5-2 m apart. Together with skilled staff, the trapping areas were selected in different sections in 30 to 160 m distance, based on its expected value as crested newt habitat, comprising features such as hiding spots and assumed emerging submerse vegetation for oviposition (Glandt, 2016; Grosse & Seyring, 2015). The exact trap locations were maintained until drying out forced their shifting, especially in the last two occasions. The funnel openings were placed on the pond ground, and also halfway to the surface if the water depth allowed for it, with a remaining air pocket providing an adequate amount of oxygen. Every second week the traps were set for three successive nights (cf. trapping occasion) and controlled regularly. The trapping design was inspired by the recommendations for the Habitats Directive monitoring in Germany (Kronshage et al., 2014). To prevent the transmission and spread of threatening diseases such as *Batrachochytrium dendrobatidis* and *B. salamandrivorans* I used disposable gloves, disinfectant and separate equipment sets for each studied pond, which dried up between each occasion (Glandt, 2016; Kronshage et al., 2014). After an introduction by experienced staff the trap setting was performed by students.

An amphibian-fence with pitfall traps, installed north-west of pond 1 the 1st of March for about seven weeks, allowed investigating start and intensity of *T. cristatus*-migration. The first individuals were already recorded the 1st of March and directly transferred to the pond. About two weeks later the bottle trapping started and continued until mid-June, resulting in 7 trapping periods (Table 2).

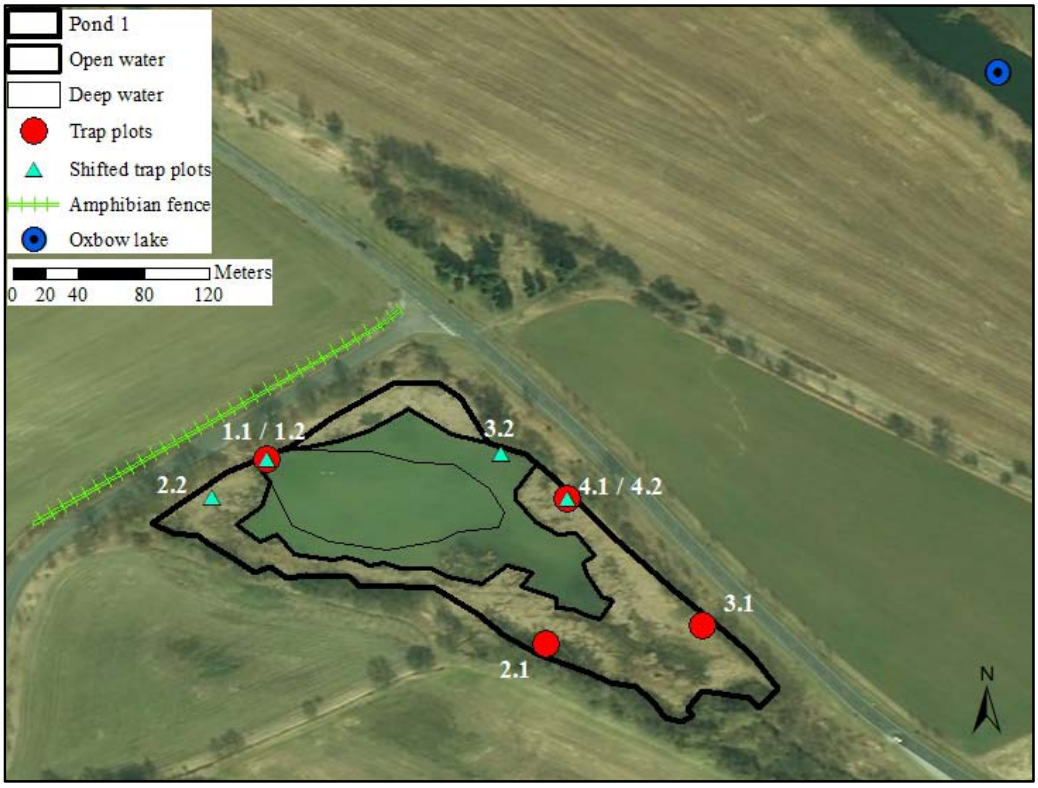


Figure 3: Locations of trap plots, amphibian fence and oxbow lake at pond 1. Two plots were totally shifted in the occasions 6 and 7 due to severe drying out.

Water Sampling

Collecting three 15 ml samples has established itself as a standard since the species detection rates of fewer samples showed significant reductions (Dejean et al., 2012; Ficetola et al., 2008; Rees, Maddison, et al., 2014; Thomsen et al., 2012). Therefore three 15 ml water samples were collected at arm's length from the pond's shore at each of the four trap plots and another three, each randomly taken at one of them (Figure 4). In the positive control pond (PC) only three samples were collected at three different places. The sampling started two weeks before the first bottle trapping was performed (Table 2).

The water sample locations were shifted in line with the trap plot shifting and in the last occasion even away from the shore. To prevent sample contamination from whirled up target eDNA preserved in the ground, sampling occurred directly before entering the pond for setting the traps (Biggs et al., 2015). The samples were taken with disposable Pasteur pipettes to reduce outside contamination of the sample tubes and were directly pipetted to a premixed ice-cold precipitation solution consisting of 1.5 ml sodium acetate 3M and 33 ml absolute ethanol (Ficetola et al., 2008; Valiere & Taberlet, 2000). The sample tubes were immediately placed on a mix of ice and dry ice to simulate the -20°C of the freezer also under field conditions. In the lab they were incubated at least over night at -20°C until DNA extraction. For detecting possible (cross-)contaminations while field sampling and DNA extraction resulting in potential fault positives, precipitation solutions with 15 ml of autoclaved purified water (precipitation negative control (NC)) were taken to the field, not opened and otherwise treated identically like the other samples (Figure 4) (Darling & Mahon, 2011; Rees, Maddison, et al., 2014; Valiere & Taberlet, 2000).

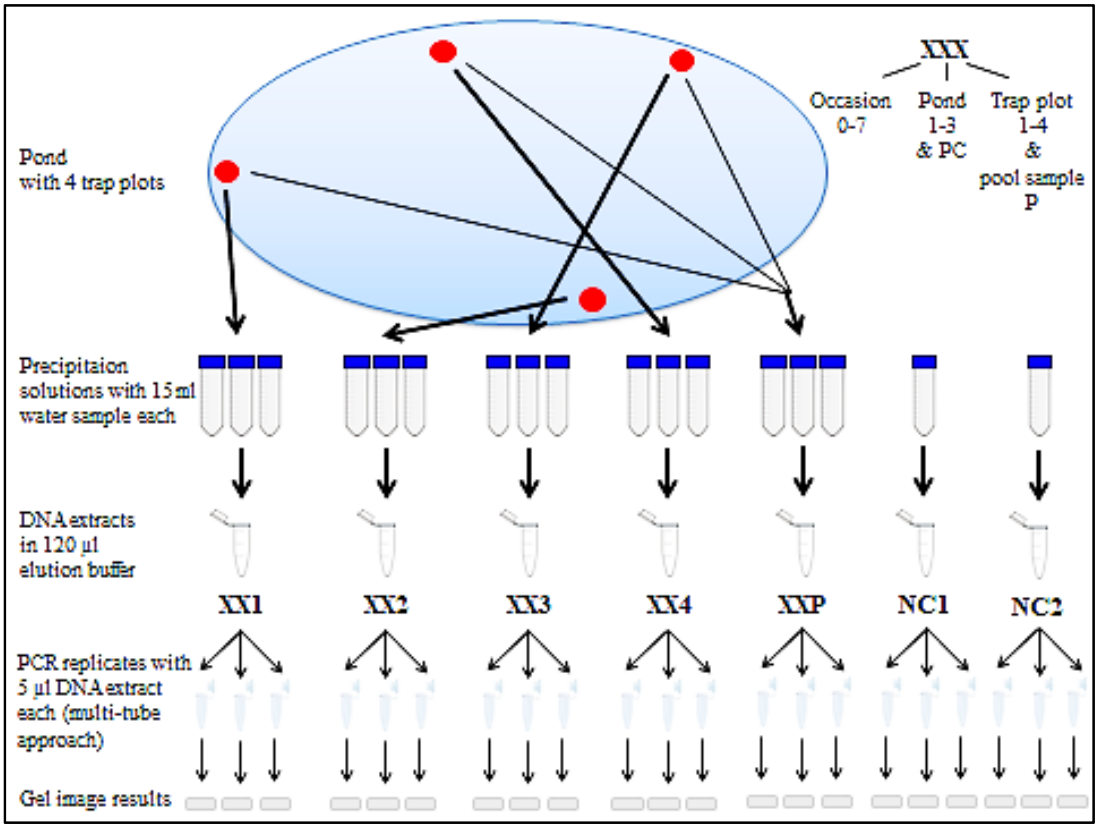


Figure 4: eDNA study design including all steps from water sampling until the final result on the gel image.

Table 2: Chronological table of water sampling (S) and bottle trapping (T) during the study period.

	Pond 1	Pond 2	Pond 3	Pond PC
Occasion 0	S	-	-	S
Occasion 1	S & T	S & T	S & T	S
Occasion 2	S & T	S & T	S & T	S
Occasion 3	S & T	S & T	S & T	S
Occasion 4	S & T	S & T	S & T	S
Occasion 5	S & T	-	S & T	S
Occasion 6	S & T	-	S & T	S
Occasion 7	S & T	-	-	S

Population Analysis

A picture of each crested newt was taken and the individuals were sexed. The unique belly patterns allow for mark-recapture models to estimate the population size (Halliday, 2006).

Primer Design & Testing

Species specific primers were designed including at least three base pair mismatches between species sequences to increase target specificity (Rees, Maddison, et al., 2014). Therefore I aligned sequences of the mitochondrial cytochrome b (cytb) segment of *T. cristatus* and the two closely related newt species of the study area (*Lissotriton vulgaris* and *Mesotriton alpestris*) from the database GenBank (http://www.ncbi.nlm.nih.gov/genbank/) using the multiple sequence alignment tool Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Burgener & Hubner, 1998). The primers were then tested *in silico* for their specificity using the NCBI primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). They only matched with the sequences of three further crested newt species (*T. carnifex, T. karelinii, T. dobrogicus*) available on GenBank, which do not occur in the study area. Primer specificity and efficiency were subsequently tested *in vitro* by using DNA of three individuals of each of *L. vulgaris* and *M. alpestris* extracted from tap water spiked with one individual for 24 h. None of these amplifications showed a positive result. Three replicate dilution series to test for the primers sensitivity were conducted with 0.1 ng to 0.0001 ng target DNA gained from tissue samples of three different individuals. Tests with additionally adding non-target DNA to the dilution series did not show effects on the primers performance (Darling & Mahon, 2011). I also tested the primers *in situ* on pond water with confirmed occurrence of *T. cristatus* as well as with samples from artificial pools imitating natural ponds with ensured presence or absence of the target and closely related species, both collected last year late in the season. For further tests the two most promising primer pairs TC12 and CfEr were selected out of several designed primer combinations, one amplifying less than 150 bp as recommended for eDNA and one relatively long around 300 bp to enhance specificity (see below) (Davison et al., 2016; Valentini et al., 2009). Both were species specific, able to detect *T. cristatus* in pond water and in the artificial ponds when the target species was present, but showed different detection limits of 0.1 ng for CfEr and up to 0.01 ng for TC12 based on the lab results.

TC12 forward 5'-GCTAACGGAGCCTCGCTAT-3',
reverse 5'-CCAGAAATAGTAAGACTACGCC-3' (118 bp amplicon, Tm 54 °C)
CfEr forward 5´-CGTAGTCTTACTATTTCTGGTC-3´,
reverse 3´-AGAGGAGATGCCTGTTGGATTG-5´ (291 bp amplicon, Tm 59.5°C)

DNA Extraction

To receive a DNA pellet for a classical DNA extraction, the falcon tubes with the mixture of precipitation solution and water sample were centrifuged (4700g, 60 min, 4 °C) and the supernatant discarded. I included a washing step by adding 20 ml 70% absolute ethanol to the pellet, followed by 5 sec vortexing, centrifugation (4700g, 60 min, 4 °C) and discarding the supernatant. The centrifuge only allowed processing of 12 falcon tubes, which is why only 3x3 or 2x3 tubes and one precipitation NC tube could be processed at a time. This resulted in two NCs per pond which were treated identically to the pond samples (Figure 4). The pellet was then gently air-dried for 10 min at room temperature before the DNA was extracted using the Spin-Column protocol for animal tissues of the DNeasy Blood & Tissue Kit (Qiagen) with some adjustments after trouble shooting. Each of the three related samples were merged to a final sample during the extraction (Figure 4). Then the DNA was eluted in two steps using each time 60 µl elution buffer containing EDTA (known to inhibit DNases (Junowicz & Spencer, 1973) resulting in a total volume of 120 µl. Remaining DNases were subsequently denatured by heating the DNA extract at 70 °C for 10 min. Finally the extract was stored at -20 °C. The DNA was quantified and its purity measured using a NanoDrop spectrometer.

PCR & Gel Images

The PCR was conducted in a total volume of 25 µl with 12.5 µl DreamTaq Hot Start Green PCR Master Mix (2X) (Thermo Scientific), 1 µl of each primer (10 pmol), 5µg BSA and 5 µl DNA extract, inspired by the study of Ficetola et al. (2008), using classical thermal cycling (Table 3) (Ficetola et al., 2008). For all PCRs positive (5 µl of 1 ng/µl high quality DNA from tissue) and negative controls (nuclease free water) were included (Darling & Mahon, 2011; Rees, Maddison, et al., 2014). The PCR amplification of each of the 16 final samples and the 6 precipitation NC samples per sampling occasion was conducted in three replicate tubes (Figure 4). This multi-tube approach with at least three replicates and at least one positive for the sample to be scored positive is recommended as standard practice (Rees, Maddison, et al., 2014; Taberlet et al., 1996). It increases the probability of getting a reliable result (Rees, Maddison, et al., 2014; Taberlet et al., 1996). Here the stochastic sampling or pipetting error has to be taken into account since I only took 5 µl for each replicate from a total of 120 µl template DNA (present case) with potentially very low proportions of target DNA.

The PCR products were visualized using gel electrophoresis on a 2.5% agarose gel (100 ml) where the DNA was stained with 5 µl RotiSafe. 20bp and 100 bp ladders were used as standards. The PCR amplification and gel electrophoresis was conducted separately for each primer.

Table 3: Thermal cycling for conducting the PCR.

	Temperature (C°)	Time (mm:ss)	Cycles
Denaturation	95	3:00	1
Denaturation Annealing Elongation	95 Tm 72	0:30 0:30 0:30	40
Elongation	72	5:00	1

Sequencing

For each primer about 50 % of the PCR products showing a positive result for *T. cristatus* were selected for sequencing to check for specific performance. The PCR product was used for a reamplification when it showed several unspecific or weakly visible bands. The

targeted band was cut out and the DNA purified using the Wizard SV Gel and PCR Clean-Up System (Promega Corporation, USA) when it still showed unspecific bands. Finally 10-15 µl of the PCR product or purified DNA was sent to the Seqlab-Microsynth for Sanger sequencing (https://srvweb.microsynth.ch/PlateSequencing). I also included one product with high quality DNA of the target being used for each of the primers as a control sequence. The resulting sequences were then edited and interpreted by using SeqTrace (http://seqtrace.googlecode.com/) and BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) to receive a base sequence which was then uploaded to the standard nucleotide BLAST of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) to analyse the amplification success and specificity of the primers. For applying the BLAST function I used default settings with „Nucleotide collection (nr/nt)" as database and optimizing for highly similar sequences (megablast), using a minimum confidence score of 30 and the Bayesian consensus algorithm.

Analyses

The statistical analyses were performed by using JMP Pro12. First I checked for a correlation between the total DNA amount in the sample (ng/µl) measured by NanoDrop Microvolume Spectrophotometer and the number of positive PCR results of the three replicates of this sample (0-3 bands), showing the success of detecting the target. The data of the whole study period (occasion 0-7) were included. I also looked at the proportion of success to confirm *T. cristatus* between bottle trapping and the eDNA method in the seven trapping occasions (Table 2). Here I focussed on the ability to confirm the target species rather than the intensity of confirmation.

For the quantitative correlation I summed up the numbers of trapped newts per trap plot and occasion and compared them to the number of bands (0-3) of the related sample. For the pool sample I summed up the total number of trapped newts per occasion. The trapping data of the amphibian fence and the weather data, both provided by local authorities, were used for linear and multiple regression (Pearson, 1908) tests. The agrometeorological weather station was located in Wurzen, close to the pond PC and measured different weather factors every hour. The factors air temperature measured in 0.2 m height above ground, ground temperature in 0.05 m depth and the precipitation in mm were used. I measured the water temperature at each trapping occasion at the location of trap plot 1 in pond 1.

Results

Most of the results were received from pond 1, while pond 2 had to be left out due to reasons explained below and 3 did not produce interpretable results. 191 *T. cristatus* individuals had been transferred from the amphibian fence to pond 1, while the bottle trapping resulted only in 15 catches. The eDNA method showed better results than the trapping in confirming the presence of newts. Here, the primer CfEr showed to be more reliable than TC12. However, the data clearly indicated an influence of the sampling location on the eDNA results, which is also partly the case for weather factors and / or sample timing within the season. In the following, the underlying results are presented in detail.

Trapping

Until occasion 1 already 51 and until occasion 2, 155 *T. cristatus* individuals with a sex ratio of about 30 % more females were transferred from the pitfall traps to pond 1 (Figure 3, Table 2, Table 4 and Graph 2). The last individuals were caught on the 13th of April, nine days before the pitfall traps were removed, resulting in a total number of 191 transferred great crested newts (90 males, 101 females).

The bottle trapping resulted in no captures in both pond 2 and 3. In pond 1 I only caught *T. cristatus* in the beginning and end of the study period and only in the trap plots 3 and 4 with no recaptures (Table 4). However, this dataset was too small for running population analysis. In the positive control pond the great crested newt could be confirmed by observation.

In pond 1 and 3 the amphibians *Lissotriton vulgaris*, *Pelophylax kl. esculentus* and *Bufo bufo* were confirmed from bycatches, while *Bombina bombina* and *Pelobates fuscus* were seen additionally in pond 3.

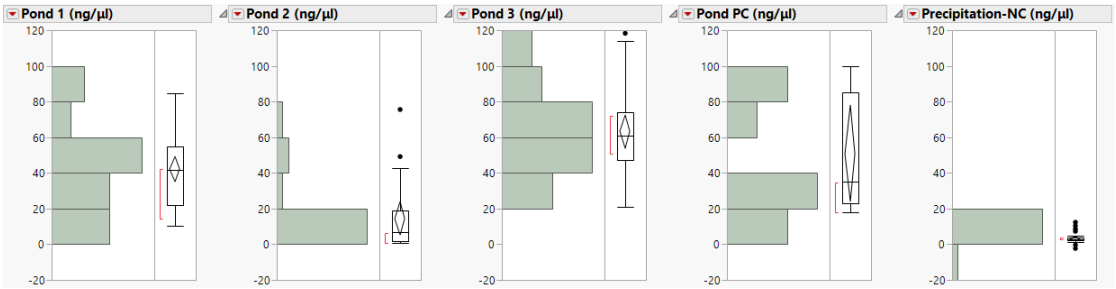
No target species were detected in pond 2. Here, the water analysis showed an extreme acidity of pH values of 3.1 to 3.2 in multiple samples, while the other two ponds showed values of about 6.2. Consequently, pond 2 was removed from the study after occasion 4 (Table 2). Pond 3 was omitted after occasion 6 due to lack of trapping success.

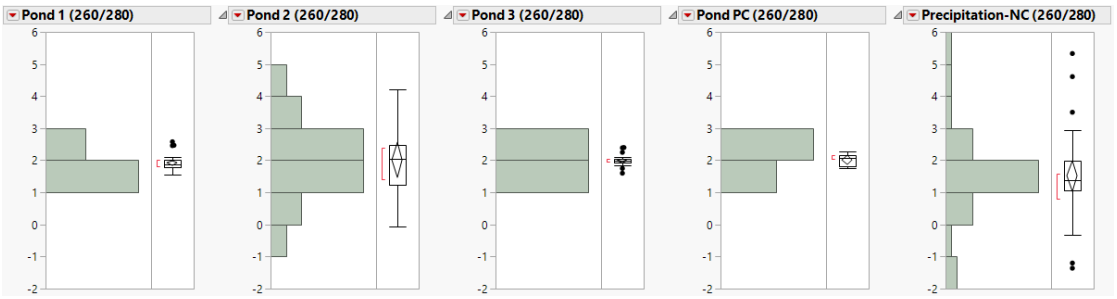
Table 4: Number of individuals of *T. cristatus* caught in the bottle traps in pond 1 over the total study period. In brackets: males.females.larvae

Pond 1	Trap plot 1	Trap plot 2	Trap plot 3	Trap plot 4	Total
Occasion 1	0	0	0	2 (2.0.0)	2 (2.0.0)
Occasion 2	0	0	1 (0.1.0)	9 (7.2.0)	10 (7.3.0)
Occasion 3	0	0	0	0	0
Occasion 4	0	0	0	0	0
Occasion 5	0	0	0	0	0
Occasion 6	0	0	1 (0.1.0)	1 (0.1.0)	2 (0.2.0)
Occasion 7	0	0	0	1 (0.0.1)	1 (0.0.1)

DNA Extraction

Not only the trapping results but also the DNA concentration and its purity showed differences between the ponds. While pond 1, 2, PC and the negative controls showed expected results with almost no irregularities in DNA concentration (ng/µl) and purity (260/280), this was not the case for pond 2 (Graph 1). The precipitation step showed conspicuous features as well. A jelly-like precipitation product, probably caused by the acidic properties, clogged the spin column membrane, and therefore limited the DNA extraction step.





Graph 1: Histograms showing the distribution of the DNA concentrations (ng/µl) and DNA purities (260/280) for each of the sampled ponds and the precipitation negative controls (NC) with differing numbers of samples (pond 1: occasion 0-7; pond 2: occasion 1-4; pond 3: occasion 1-6; pond PC: occasion 0-7; precipitation NC: occasion 0-7). View extreme values of pond 2 and precipitation NC were excluded for greater clarity of the graphs.

PCR & Gel Images

Primer TC12 could confirm *T. cristatus* 14 times and failed 26 times in pond 1, could not detect it in pond 3 and from the second occasion always with three bands in the positive control pond (Table 5). Whereas primer CfEr got 19 positive and 21 negative results in pond 1, it was also once positive in pond 3 in occasion 6 and reacted like primer TC12 in the positive control pond, but showed only one band in occasion 5 (Table 5). The positive result in pond 3 occurred when the trap plots had been shifted to the deep water. However, few positive results had to be removed from the interpretation, when the precipitation negative controls showed contamination also after repeating the PCR. For primer TC12 this only affected one positive band in pond 1, but six bands in pond 3, all in occasion 6, and another one in occasion 1 and 4. For primer CfEr only the bands for the pond PC in occasion 2 have to be treated with caution due to negative control contamination.

Table 5: Number of bands showing T. cristatus on the gel images for pond 1 and the positive control (PC) pond with both primers (TC12 & CfEr) over the whole study period. Pond 1 was divided in five samples, including the trap plots 1-4 and one pooled sample from three of the four trap plots. The different shades highlight the number of bands from 1-3, while the black highlighted bands have to be treated with caution due to contamination in the related precipitation negative controls.

Primer TC12

Pond 1	Trap plot 1	Trap plot 2	Trap plot 3	Trap plot 4	Pool	Pond PC
Occasion 0	**1**	**3**	0	0	0	0
Occasion 1	0	0	0	0	0	0
Occasion 2	0	0	**2**	0	**2**	**3**
Occasion 3	0	0	0	**1**	0	**3**
Occasion 4	**3**	0	0	0	**2**	**3**
Occasion 5	**3**	**1**	0	**1**	0	**3**
Occasion 6	**1**	0	0	0	**3**	**3**
Occasion 7	0	0	**3**	**3**	**2**	**3**

Primer CfEr

Pond 1	Trap plot 1	Trap plot 2	Trap plot 3	Trap plot 4	Pool	Pond PC
Occasion 0	0	0	0	0	0	0
Occasion 1	0	0	0	0	0	0
Occasion 2	0	0	**2**	**3**	**1**	**3**
Occasion 3	0	0	0	0	0	**3**
Occasion 4	**1**	0	**1**	**1**	**3**	**3**
Occasion 5	**3**	0	0	**1**	**1**	**1**
Occasion 6	**1**	**1**	0	**1**	**3**	**3**
Occasion 7	**2**	**2**	**3**	**1**	**2**	**3**

TC12 often produced unspecific secondary bands that, however, could only be seen for the samples of pond 1 (Figure 5). The secondary bands got weaker the stronger the target bands became. Primer CfEr was able to confirm the target more often and also performed totally target specific. Using the Hot Start Green PCR Master Mix instead of the common Green PCR Master Mix reduced the unspecific bands for TC12 and totally excluded them for primer CfEr where they also occurred before.

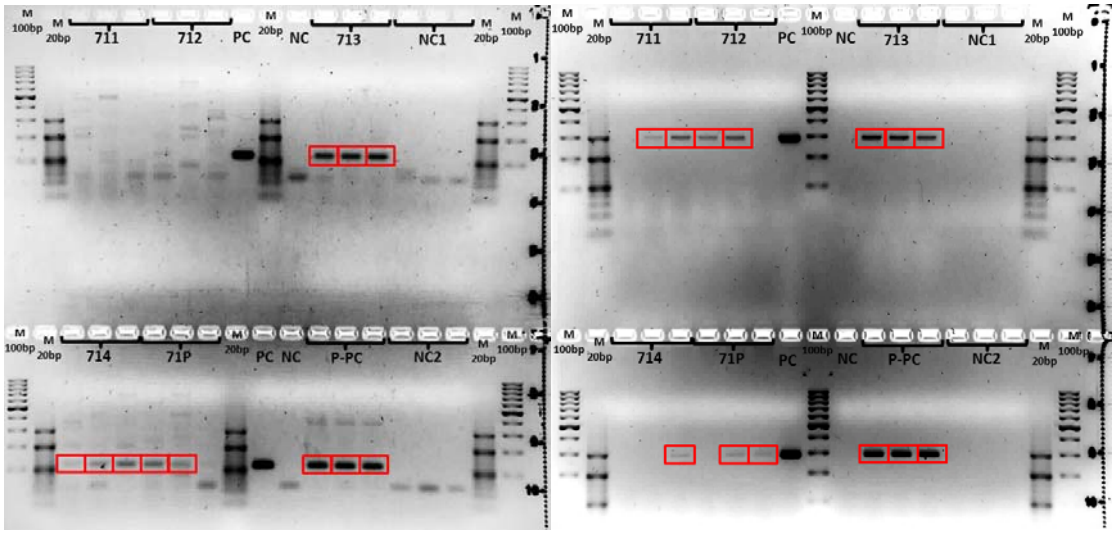


Figure 5: PCR results for sampling in pond 1 and pond PC at occasion 7 for both tested primers (TC12 left; CfEr right), showing the high sensitivity of both primers late in the season, the specific performance of CfEr and the partly unspecific performance seen in TC12. The red frames highlight the bands rated as positive for *T. cristatus*. Sample designation: Figure 4.

Sequencing

The primer TC12 resulted in a total of 60 bands and CfEr in 46 bands that might have been interpreted as belonging to *T. cristatus*, for all ponds including the contaminations, and about half of each resulted from pond 1. For each primer, 30 PCR products were sent in for sequencing, 17 from TC12 and 16 from CfEr from pond 1. Only 21 out of the 30 from TC12 and all from CfEr could be 100 % confirmed as *T. cristatus*, each including two with only 98 %. All remaining samples could not be sequenced at all or did not result in a high enough similarity with any other sequence existing in GenBank.

In TC12 mostly around 215 out of 315 matching sequences confirmed *T. cristatus*, while the other sequences included *Triturus carnifex* (one sequence to 100 %), *Triturus dobrogicus* and *Triturus karelinii* to less than 100 %. The samples of CfEr got mostly around 280 hits, 170 of these *T. cristatus*, and the rest including less than 100 % match with mostly *T. carnifex*, but also *T. karelinii*, *T. dobrogicus* and *T. macedonicus*. Additionally sometimes few matching sequences came from *Hynoblus nebulosus*, *Ambystoma macrodactylum* and *Bratrachoseps attenuatus*. These species are all amphibians, but none of them occurs close to the project area (Arntzen et al., 2007; Arntzen & Wallis, 1999).

DNA vs. Bands

I could not find any correlation between the total DNA amount and the amount of positive bands for both primers, TC12 (r^2=0.000953; p=0.7443) and CfEr (r^2=0.000378; p=0.8372). Due to bands in the precipitation negative controls, these were included in the analyses, while pond 2 had been excluded, as described before. This is likely explained by the fact that the water sample also includes DNA of other species existing in the pond and thus the extracted DNA does not represent the amount of target DNA. Also the primers were not inhibited by too much non-target DNA, since I could not find a negative correlation either.

Bottle Trapping vs. eDNA Method

Comparing the success of confirming the great crested newt in pond 1 between bottle trapping and eDNA water sampling, there was a difference between the methods but also within the eDNA method, depending on the primer used (Figure 6). In general the eDNA method was able to confirm *T. cristatus* in seven out of eight sampling occasions with at least one of the two tested primers, while the trapping only caught newts in four of the seven bottle trapping occasions (Graph 4).

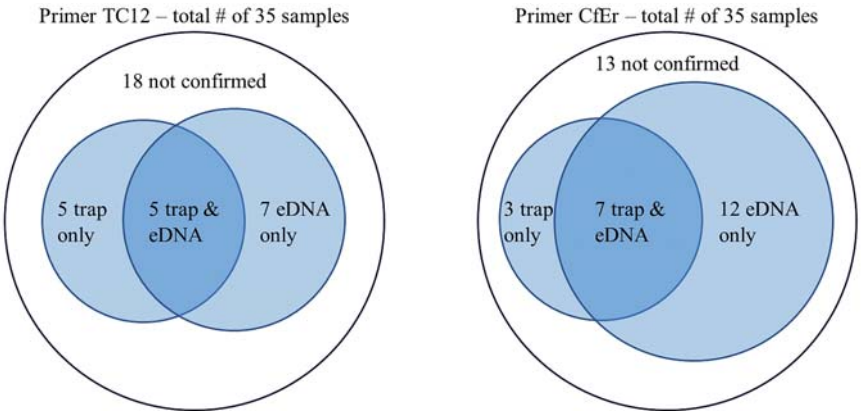


Figure 6: The two circular charts show the proportions of how often either one or both of the used methods, including traditional bottle trapping and emerging water sampling (eDNA), were able to confirm *T. cristatus* in pond 1 (divided in four trapping / sampling areas) over the entire trapping period, including the pooled samples, resulting in a total of 35 samples.

Looking at the bottle trapping results and comparing them with the results seen on the gel in occasion 2, there might be a correlation (Figure 7). Here no individual got caught in the trap plots 1 and 2 and also no bands were seen. One individual was trapped in plot 3, where two light bands confirmed the presence of *T. cristatus*. Plot 4 clearly showed three bands, where seven individuals were found in the traps. For all the other occasions there were too few newts caught to test for a correlation with eDNA success at different locations.

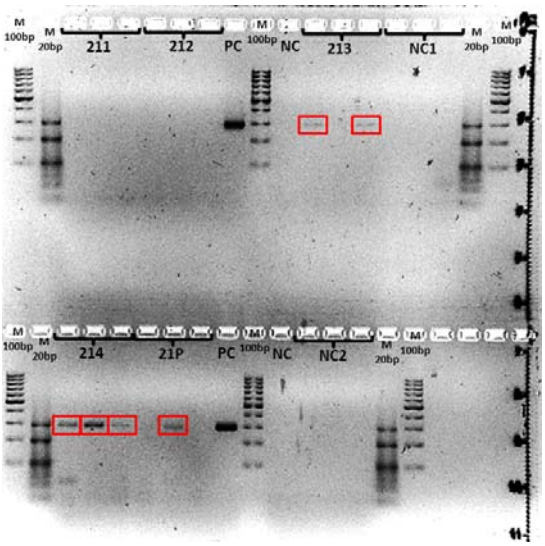


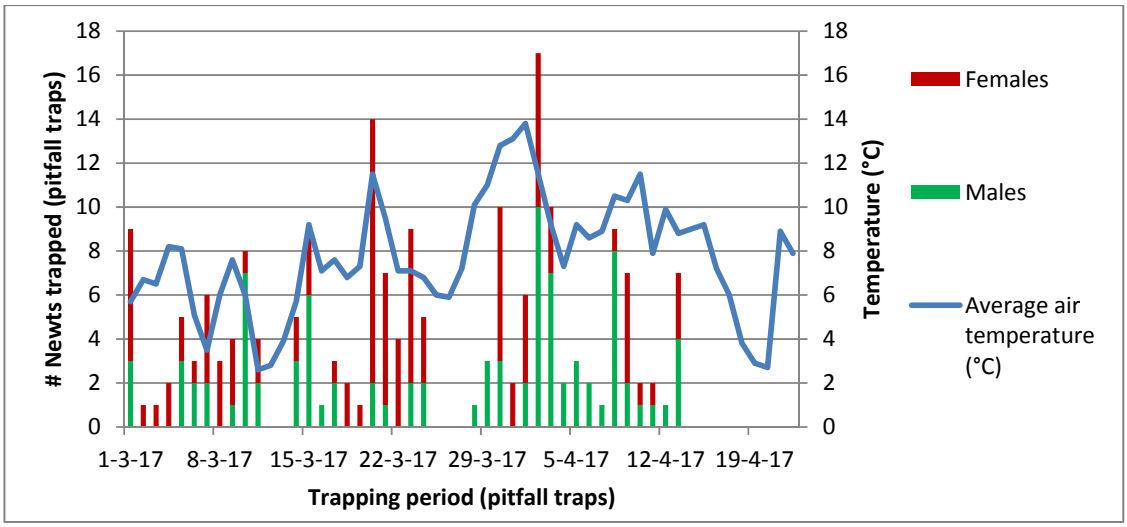
Figure 7: PCR results for sampling in pond 1 at occasion 2 for the very specific performing primer CfEr, showing its capability to confirm *T. cristatus* depending on the sampling location. The red frames highlight the bands rated as positive for *T. cristatus*.

Sample Location vs. Bands

The results of the trap plots clearly differ within one occasion for both primers, regarding the general ability and the intensity of confirming the target (Table 5). The traditional pool sample (Figure 4) failed sometimes for primer TC12 even when trap plot samples were positive. Here, primer CfEr could always detect the target, when it was also detected in a trap plot sample. Nevertheless, it is important to point out that sometimes the trap plot samples confirmed *T. cristatus* with more bands and thus higher certainty than the pool samples.
The results differ as well between sample locations as between occasions suggesting that also the timing of the sampling regarding weather factors and season matters.

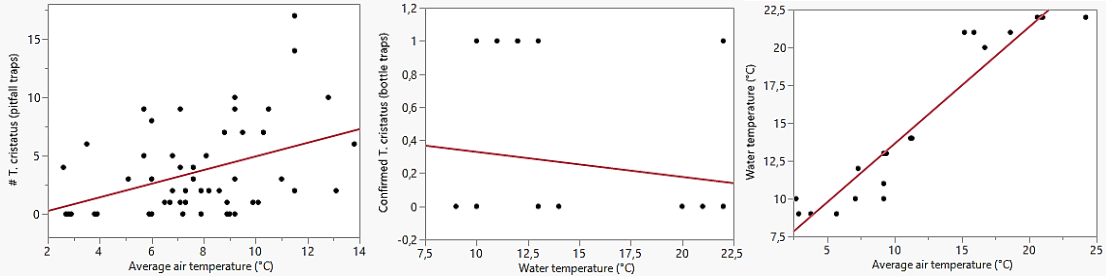
Seasonal / Weather Factors vs. Trapping Success

My findings support the conjecture that biological reasons like differing activity depending on weather conditions and season cause variations in detection probability for trapping amphibians (Graph 2) (Kronshage et al., 2014). Using multiple regression with the number of individuals trapped in the pitfall traps at the amphibian fence as the dependent variable and average, maximum and minimum air temperature, ground and water temperature and precipitation as independent variables, the average air temperature showed to be the significant predictor for this trapping success (r^2=0.157593; p=0.0032; Graph 2 and Graph 3).



Graph 2: Trapping success of *T. cristatus* of the pitfall traps along the amphibian fence located in the north-west of pond 1 and its correlation to the average air temperature over the first 7-8 weeks of the study period.

The bottle trapping confirmed the presence of *T. cristatus* in ~30 % of the sampling days in pond 1. Here, I used the binary data of the bottle trapping success (confirmed or not confirmed) to run the same multiple regression analysis as for the pitfall trapping. The remaining variables were average air temperature (r^2=0.000654; p=0.9148) and water temperature (r^2=0.033257; p=0.4416), which are strongly correlated (r^2=0.907619; p<0.0001) (Graph 3). The values indicate that my bottle trapping success is not significantly correlated with any included weather factor.

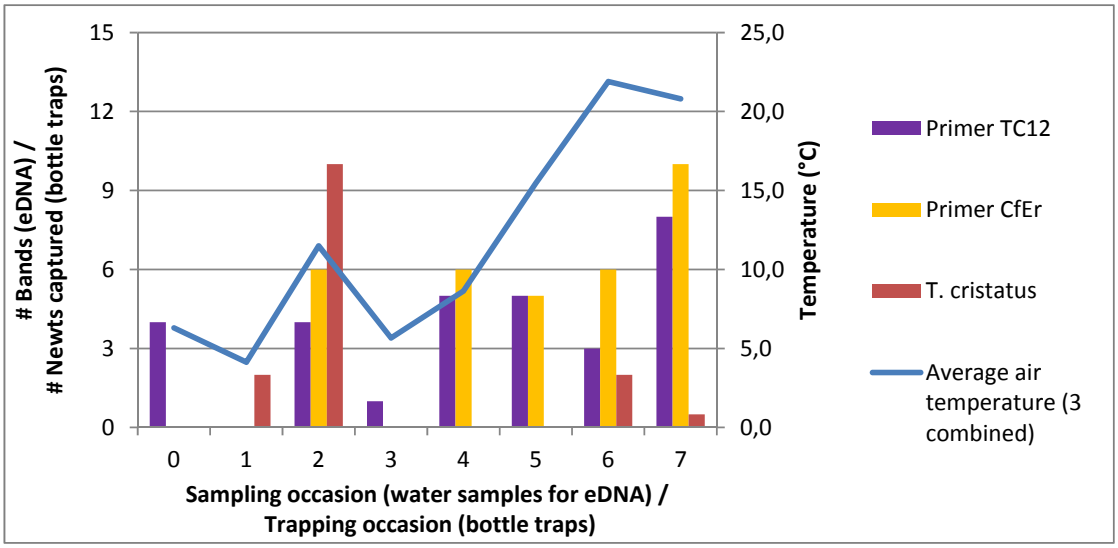


Graph 3: Linear regression between (left) average air temperature and number of *T. cristatus* specimens trapped in the pitfall traps along the amphibian fence located in the north-west of pond 1; (middle) water temperature of pond 1 and confirmation frequency of *T. cristatus*; (right) average air temperature and water temperature in pond 1.

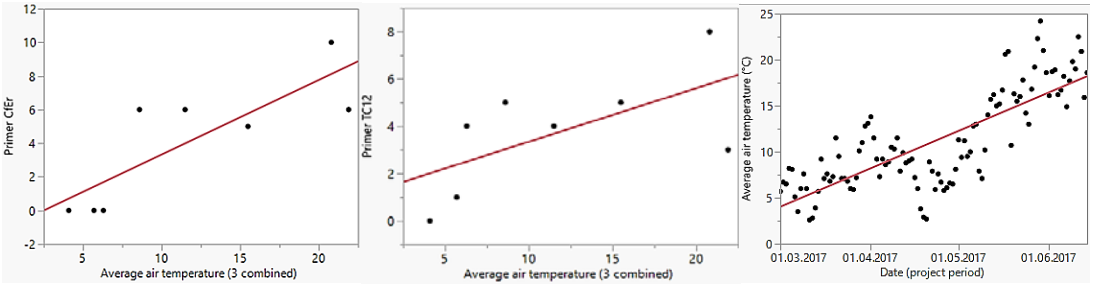
Seasonal / Weather Factors vs. eDNA Success

Based on the knowledge that weather, especially temperature, influences the intensity of newt migration, I assume that it also influences their movement within the pond and by this increases the amount of target DNA or its probability to be caught in one of the water samples, I finally assume that also the eDNA-method success is influenced by temperature (Glandt, 2016). Therefore I also tested for a correlation between the weather factors and success of confirming *T. cristatus* by using eDNA in pond 1. Here the average air temperature of the three days before the sampling was averaged. This was motivated by the assumption that not the weather and thus the movement of the newts on the sampling day but the movement prior to the sampling might have influenced the eDNA detecting success.

Further I used the amount of bands per occasion, including all received positive bands (30 for TC12; 33 for CfEr) out of the 120 possible from the eight sampling occasions (Figure 4). Hence, the average air temperature explains almost 68% of the variation in the results for the primer CfEr significantly (r^2=0.678325; p=0.0120) and only 39 % of the results for primer TC12, but without significance (r^2=0.392595; p=0.0964). These correlations might, however, rather be explained by the season than the actual temperature, since the average air temperature is significantly correlated with the time of the season within the project period (r^2=0.62191; p<0.0001). (Graph 4 and Graph 5)



Graph 4: Frequency and success of tested methods, including bottle trapping (number of individuals of *T. cristatus*) and eDNA method using primers TC12 and CfEr (number of bands on the gel image) ability to confirm *T. cristatus* in pond 1, compared to average air temperature three days prior to trapping / sampling.



Graph 5: Linear regression between average air temperature three days prior to sampling and number of bands on the gel images using primer CfEr (left) and TC12 (middle) over the complete study period in pond 1; (right) linear regression between time of season within the project period and average air temperature.

The period of using pitfall trapping and bottle trapping only overlapped in the beginning of the project period and resulted in a very limited data set. This was insufficient for running analyses looking on the relationship between numbers of newts transferred to the pond and the success of bottle traps and water sampling.

Discussion

Target Species

The habitat use of a population within a pond depending on daytime, season and local habitats is not totally understood yet. There are differences seen between males and females using rather shallow areas or deep water zones (Kronshage et al., 2014) but also other factors might cause core areas of higher and lower activity. Causatively this could lead to differences in detection probability using eDNA depending on sampling location, requiring further investigation.

Trapping

Both methods, trapping and eDNA sampling, showed differences in success depending on location and season, however, also my results suggest that using eDNA is more sensitive and therefore to be preferred in monitoring. Yet, some of the low trapping success might have been improved, if not for the specific study design, as described in the methods section. I could not always pick the most suitable spot when trapping and sampling since maintaining the comparability between e.g. the two tested methods and the occasions was rated more important for the study. This applies also for sticking to the regular sampling cycle regardless of sometimes unsuitable weather conditions.
The number and distribution of *T. cristatus* individuals might have been affected by transferring migrating newts from the fence into pond 1 possibly preventing or delaying further migration to the nearby oxbow lake. This might have caused the highest numbers in the bottle traps closest to the oxbow lake (Figure 3). Nevertheless, this does not influence the message of the results of this study.

Water Sampling

The water sampling depends a lot on probability to catch the often rare target DNA, since we have insufficient knowledge about what is going on below the water surface. However, my findings support my hypothesis, that eDNA is not evenly distributed within a pond. To balance some of these uncertainties, staff should be engaged for the sampling that is able to evaluate the different areas within a pond for the likelihood of being used by the target, also depending on the time of the season. Another important factor is the differing eDNA-concentration depending on population and pond size, so that it should be considered if taking always a fixed amount of samples is sufficient. As samples were immediately precipitated and transported around - 20 °C, DNA preservation is assumed high.

Primer Design

There is probably no such thing existing as the “perfect primer”, as the design always includes trade-offs (Valentini et al., 2009). Here, I especially had to take into consideration the primers sensitivity being opposite to its specificity. The longer the primers amplicon, the higher its probability to perform species specific, as I also saw in my results. However, here one faces restrictions due to the nature of eDNA samples. Different studies showed that DNA in the environment is exposed to degradation processes and thus eDNA methods are only able to confirm a target a certain number of weeks or even days until the remaining fragments are too small for primers to amplify successfully, depending on the amplicon length of a primer (Dejean et al., 2011; Hajibabaei et al., 2006; Valentini et al., 2009). Shorter primers show therefore a higher sensitivity in eDNA studies, what can be crucial for small populations, but as also in my case, can lead to unspecific (secondary) bands. This can limit the ability to interpret the confirmation success of the target. Notwithstanding the limitations, the designed primers showed to be very successful in the current study,

especially when combining the results of the two. I could confirm the target already very early in the season and the sequencing results did not show non-target amplifications for the expected amplicon size.

DNA extraction

Already basic water chemistry can affect the extraction success, as seen for the highly acid water samples of pond 2. Water samples in general have to be taken carefully to exclude suspended sediments and plant remains as far as possible, which were also seen to hamper the extraction steps. I also recommend including a negative control for each sampling and extraction step, since I could not determine the origin of the occurring contaminations by using a combined one (Rees, Maddison, et al., 2014). However, I assume that it happened in the student lab where high amount of target DNA was handled and it was not possible to assure the detailed requirements of cleanliness as demanded by other studies (Beja-Pereira et al., 2009; Deiner et al., 2015). In the field all possible measures to prevent cross-contaminations were taken. The contaminations did, however, only have a minor effect on the overall study results, since only few bands had to be excluded from the analyses. The trouble shooting of the study design to achieve the best results is crucial, as also seen in the current study. Adding a second washing step and heating in the end of the extraction enhanced the results, while it did not matter if the precipitation solution was premixed before adding the water sample. For every detail, from the amount of samples, to number of replicates, the attempt is made to standardize the process (Rees, Maddison, et al., 2014). However, one essential design element for being able to compare different studies was mostly left out in discussions so far: the used amount of elution buffer. It decides on the dilution factor of the (target) DNA and therefore fundamentally influences the probability of catching a target DNA segment when pipetting the replicates and of receiving a positive result for the sample. Therefore, reducing this volume might increase detection success and result reliability. However, the currently used 120 µl were the minimum reasonable amount, when taking into account that 50 µl is the minimum possible for the used micro-spin column and that the second elution step increases the DNA yield significantly according to the manufacturer manual.

PCR & Gel Images

The PCR design is another crucial factor for the probability of success using this method. First of all, the decisive factor to get any results in the gel image was adding the adjuvant BSA to the PCR-solution, after getting no results with the test-samples before using this additive (Beja-Pereira et al., 2009). Although the selected study design follows the suggested standards of other studies, e.g. regarding the amount of PCR replicates, it seems not unobjectionable, since I still had to face the difficulty of false negatives which are known for small target populations or sample collection errors (Biggs et al., 2015; Ma et al., 2016; Rees, Maddison, et al., 2014). However, it also occurred when another sample from the same occasion was positive. This shows again that the current standards need to be reassessed.

A possibility would be to enhance the effect of the multi-tube approach by increasing the number of replicates per sample in the PCR. It might even be reasonable to increase this number until the total eluted DNA is used, thus completely eliminating the pipetting error. Thereby even smallest amounts of DNA can be visualised. The current pipetting randomness could be an additional explanation for differing results between samples, as referred to in ‘Water Sampling’. However, using more replicates, there is also an increased risk of errors like false positives which needs to be taken into account and thus there has to be an agreement if one band is still enough to rate the sample as positive (Beja-Pereira et

al., 2009). Generally I rate false negatives more disastrous than false positives for studies working with protected species. False positives would be controlled for by verifying the positive result using comprehensive traditional monitoring methods, while false negatives cannot be detected (Darling & Mahon, 2011).
Regarding primer TC12, unspecific bands were another issue which was only seen for samples of pond 1. This might indicate an influence of water specifics on the performance of this primer. However, it makes a clear interpretation of the bands more difficult and might also cause false negatives, which is why measures should be taken to assure for a distinct separation of the bands and therefore a more reliable interpretation (Rees, Maddison, et al., 2014).

DNA vs. Bands

The possibility that the success of the PCR would be determined or correlated with the total DNA amount in the sample was low. Only the concentration of target DNA within the total sample would assumingly have an effect on the PCR success, but this cannot be measured with a spectrophotometer. This explains why it is not possible to standardise the amount but the volume of DNA used in the PCR.

Bottle Trapping vs. eDNA Method

For the actual application in monitoring the general ability to confirm presence or absence is crucial. Since primer CfEr was able to confirm *T. cristatus* in ~ 54 %, TC12 in ~ 34 % and trapping in ~ 29 % of the cases, the eDNA method again shows its higher sensitivity (Dejean et al., 2012; Smart et al., 2015). However, comparing the two primers directly, their results only partly overlapped so that a combination of two primers delivers even better results and might therefore be the method of choice (Beja-Pereira et al., 2009; Evans et al., 2016). Taking into account that this can also increase the risk of false positives, a total combination of methods is most reliable when using comprehensive trapping to confirm the eDNA results in the positive rated ponds.
Proving my assumption that the probability of capturing target DNA and therefore of a successful amplification is correlated with target activity centres interpreted from location-dependent quantitative trapping success was impossible due to lack of sufficient data in bottle trapping (Smart et al., 2015). My results show, however, that likely this is the case, but further investigation needs to be done. Nonetheless, I saw a clear difference of success between different trapping / sampling locations.

Sample Location vs. Bands

Another hypothesis in this study was that it does matter where the three water samples are taken and that it might not be enough to pool three samples taken at random locations in a pond to receive a reliable result, as seen in many papers (Ficetola et al., 2008; Sigsgaard et al., 2015; Thomsen et al., 2012). If this assumption would be true, then all trap plots as also the pool sample would need to show the same result. Looking at the related tables, this is clearly not the case. One explanation might be the expected change in preferences and concentration of habitation within one pond during the season due to habitat suitability for foraging and reproduction. It also needs to be taken into consideration that the concept of uniform mixing of water in a pond might rather be true for bigger water bodies with mostly free water area, since in reality plant cover has a high probability to hamper the mixing as also drying out during a season could (partly) cut off different deeper parts from each other. In general, the greater the eDNA success of confirming the target in one occasion, the higher the probability to still get a positive result in a repetition or other circumstances. This is desirable in monitoring which is mostly influenced by natural conditions. This is

why the concept of randomly taking three 15 ml water samples needs to be reconsidered. Yet, the differences in the results might also originate from the randomness in pipetting the eluted DNA. The timing within the season was seen to interfere with the reliability of the results as well. Here, I suspect that the occasions 0 and 1 were still too early, since also the positive control pond did not yet show a sign of the target species.

Seasonal / Weather Factors vs. Trapping Success

The calculated results in correlation between air temperature and migration behaviour and therefore trapping success of the pitfall traps were to be expected. This highlights the dependence of the target species on thermal energy for their activity in general (Glandt, 2016). At the same time, my data were not able to support the hypothesis of correlation between bottle trapping success resembling target movement within the pond and the interdependent factors of time within the season and air temperature but also other weather factors. However, it is important to point out that the data basis of bottle trapping was probably too small to make reliable statements and that bottle trapping success resembles the newts' movement within a pond is only an assumption. However, Kronshage et al. (2014) refer to reduced amphibian activity and affected trapping success due to low temperatures in the early season (Kronshage et al., 2014).

Seasonal / Weather Factors vs. eDNA Success

My two tested primers showed different results when looking for a dependence upon air temperature when collecting the water samples. Firstly it is important to keep in mind that my findings could either be caused by the temperature itself or by time of the season including different actions like mating, oviposition and hatching since they were positively correlated. Since CfEr showed a strong and significant correlation which I could not see in TC12, the reason might lie more specific in the nature of my primers. CfEr has a rather long amplicon and relies upon longer, less degraded DNA segments in the water, which are therefore probably more recently introduced (Dejean et al., 2011). This might be an explanation why I see the correlation to the data three days prior to the sampling. TC12 amplifies a rather short segment and is therefore likely to detect also DNA segments which are in a later degradation state and therefore also introduced before the used data. This would result in no direct correlation.

Based on my results I cannot agree that eDNA sampling is independent of weather conditions (Thomsen & Willerslev, 2015). If the sampling design is shifted towards the main period of *T. cristatus*, with high enough temperatures and the peak of the newt population in its aquatic phase (including hatchlings), both primers were always successful, while the trapping success was unclear. If the samples shall be taken already early in the season, the primer TC12 might be suitable, but it should be combined with sequencing due to the unspecific bands. Otherwise Primer CfEr seems more reliable.

Derivations and Conclusions

My results indicate, that the currently used methodology of eDNA is not always reliable, especially since there is yet no sufficient understanding of target DNA-quantities needed for successful detection and in particular how this varies temporarily and spatially in the sampling body. I saw clear differences in the results between different sample locations within one occasion but also in the influence that seasoning and weather have on eDNA sampling as known for traditional trapping. This should induce rethinking of the eDNA

implementation methodology. I suggest different approaches, which often call for further investigation and standardisation, to overcome the encountered challenges:

1) Deciding on the number of samples taken or merged depending on pond volume, influencing the target DNA concentration.
2) Generally increasing the number of samples taken to either more than three 15 ml samples or taking e.g. 2-3 times 3x 15 ml at different locations.
3) Premixing several water samples from all along the shore of a pond and only then taking the 15 ml subsamples and adding them to the precipitation solution, as already conducted in some studies (Biggs et al., 2015). Here tests for a sufficient number of subsamples are still necessary.
4) Continuing to restrict the (random) sampling to the main season to reach a sufficient target DNA concentration in the pond. However, I did not look into the possible extension during the juvenile time (Rees, Maddison, et al., 2014).
5) Continuing to use trained or experienced staff that is able to conduct more target-oriented sampling. This might allow for expanding the time frame.
6) Enhancing the multi-tube approach effect by increasing the PCR replicate number per sample, using the total eluted DNA to completely eliminate pipetting error, or reducing the volume of elution buffer and so the DNA dilution factor per sample.
7) Generally trying to reduce the DNA loss while processing the samples to increase the overall performance, by for example replacing common by low-retention plastic tubes (Beja-Pereira et al., 2009).

In conclusion, using environmental DNA as a time- and cost-effective, non-invasive detection tool in monitoring of aquatic species, remains promising and worth further research. As Rees et al. (2014) already pointed out it should generally be seen as an additional preselecting tool prior to full ecological surveys (Rees, Maddison, et al., 2014). However, implementing one or more of the listed approaches would achieve more reliable results. This makes the eDNA method a practicable and reliable tool in monitoring, where the limited resources can be redirected and therefore be used reasonably.

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Pictures

Source: S. Malt BfUL / FB55

Water sampling

Pond 1 Trap plot 1

Pond 1 Trap plot 2

Pond 1 Trap plot 3

Pond 1 Trap plot 4

Pond 2 Precipitation at plants

Pond 3 Shallow water level situation

Pond 3 Deep water

Pond PC

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