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Environmental DNA sampling for African clawed frog in Flanders, Wallonia and France in 2020

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ENVIRONMENTAL DNA SAMPLING FOR AFRICAN CLAWED FROG IN FLANDERS, WALLONIA AND FRANCE IN 2020

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Abstract

In 2018 a population of African clawed frogs (*Xenopus laevis*) was discovered in a single pond in La Chapelle-d'Armentières, France, located 2,5 km south of Komen-Waasten, Wallonia, Belgium and 5,3 km southeast of Heuvelland, Flanders, Belgium. To assess the potential presence of this species in Belgium, 74 samples of water bodies in Flanders, 23 in Wallonia and 9 in France were analysed for presence of *X. laevis* DNA in April 2020. Results indicate that *X. laevis* is not present in the prospected sites in Flanders and Wallonia at this time, whereas a strong positive signal was detected in the pond harbouring the source population in France. If the species manages to invade Belgium in the future, successful eradication will prove to be difficult and costly. Swift action in France, including, but not limited to, draining of occupied water bodies, combined with fencing and extensive environmental DNA (eDNA) screening of additional water bodies within dispersal distance is urgently needed to prevent such invasion. Furthermore, monitoring of eradication success via eDNA is recommended for several years after eradication measures have ceased.

Résumé

En 2018 une population de Xénope lisse a été découverte dans une seule mare à La Chapelle-d'Armentières en France, située 2,5 km au sud de Komen-Waasten en Wallonie, Belgique et 5,3 km au sud-est de Heuvelland, Flandre, Belgique. Pour évaluer la présence potentielle de cette espèce en Belgique, 74 échantillons de plans d'eau en Flandre, 23 en Wallonie et 9 en France ont été analysés pour la présence d'ADN de *X. laevis* en avril 2020. Les résultats indiquent que *X. laevis* n'est pas présent dans les sites prospectés en Flandre et Wallonie à cette époque, alors qu'un fort signal positif a été détecté dans l'étang abritant la population source en France. Si l'espèce parvient à envahir la Belgique à l'avenir, une éradication complète sera difficile et coûteuse. Une action rapide en France, y compris, mais sans s'y limiter, le drainage des masses d'eau occupées, combinée à des clôtures et à un dépistage étendu de l'ADN environnemental (ADNe) de masses d'eau supplémentaires à distance de dispersion est nécessaire de toute urgence pour empêcher une invasion. De plus, le suivi du succès de l'éradication via l'ADNe est recommandé pendant plusieurs années après l'arrêt des mesures d'éradication.

Samenvatting

In 2018 werd een populatie Afrikaanse klauwkikkers (*Xenopus laevis*) ontdekt in één poel in La Chapelle-d'Armentières in Frankrijk, 2,5 km ten zuiden van Komen-Waasten, Wallonië en 5,3 km ten zuidoosten van Heuvelland, Vlaanderen. Om het potentiële voorkomen van deze soort in België na te gaan, zijn 74 waterlichamen in Vlaanderen, 23 in Wallonië en 9 in Frankrijk geanalyseerd voor *X. laevis* DNA in april 2020. De resultaten tonen aan dat *X. laevis* momenteel niet voorkomt in de onderzochte locaties in Vlaanderen en Wallonië, maar een sterk positief signaal werd gedetecteerd in de poel waar de bronpopulatie zich bevindt in Frankrijk. Als deze soort zich in de toekomst in België kan vestigen, zal succesvolle eliminatie moeilijk en kostelijk zijn. Snelle actie in Frankrijk, door onder meer het leegpompen en omheinen van besmette wateren en een doorgedreven environmental DNA (eDNA) onderzoek van bijkomende waterpartijen binnen dispersie-afstand is dringend nodig om deze invasie tegen te gaan. Daarnaast is ook de monitoring van beheersingrepen gedurende enkele jaren via eDNA aangeraden.

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Recommendations for management and/or policy

African clawed frogs are thought to be absent from the Flemish areas bordering the French population at the time of sampling (spring 2020). Additional comprehensive eDNA sampling in Wallonia and France is advised, as only a subset of water bodies were sampled. The results from our in depth sampling campaign indicate that the species is still in the early stages of invasion. Thus, the time to act in France is now, considering the financial and ecological consequences of further delay. Conservation importance of the pond in which the reproducing population is located is negligible. The pond is in the early stages of succession, submersed vegetation is largely absent, and the presence of both fish (*Pungitius pungitius*) and African clawed frogs have reduced the suitability of the habitat for both invertebrates and the few native amphibian species (*Pelophylax sp., Lissotriton vulgaris, Ichthyosaura alpestris*, *Bufo bufo* and *Rana temporaria*) co-occuring in the pond. The pond is well-suited for complete drainage, with steep slopes and deeper areas. Combined with a drift fence completely surrounding the pond, to ensure no animals escape, a quick and thorough draining of the pond is the best option to increase efficacy of the mitigation measure and minimize workload and financial investment. Optimally, drainage occurs before the African clawed frogs become more active in spring, in this way potential emigration risks are further reduced and the impact on native amphibians is reduced. After complete removal of all life stages of *X. laevis*, if necessary through repeated draining of the pond, a thorough eDNA campaign during the next spring including stagnant, temporary, and running water bodies within an area of at least 5 km surrounding the pond should be paramount, to ensure no unnoticed populations persist in the wider surroundings. If detected, these locations should be dealt with accordingly. After all infected sites are cleared of the species, eDNA sampling should be repeated periodically for 5 years to ensure this invasive species does not regain a foothold.

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Introduction

The African clawed frog *Xenopus laevis*, native to Sub-Saharan Africa, was introduced to natural systems around the globe. Introductions originate from escapes or releases linked to the pet trade and from laboratories that use animals as model organisms in developmental and cellular biology (Measey et al. 2012). There are several physiological and demographic traits that can explain the success of the species as an invasive species. *Xenopus laevis* has a largely aquatic ecology, can adapt to a wide range of (anthropogenic) habitats, tolerate temperature fluctuations, salinity and pollution. It is able to burrow itself in the substrate, thereby persisting unfavourable conditions such as drought periods (Sousa et al. 2018). Apart from aquatic migration, the species is able to disperse over land (Measey 2016). Furthermore, this species has large clutch sizes (up to 17.000 ova/female) and can reproduce several times per year and can attain an age of at least 15 years.

In a global review of alien amphibian impacts *Xenopus laevis* was ranked second (after cane toad *Rhinella marina*) among the seven amphibian species part of the '100 of the world's worst' invasive species, based on its environmental and socio-economic impacts (Measey et al. 2016). *Xenopus laevis* is a generalist aquatic carnivore, predating on invertebrates, amphibians, fish and smaller vertebrates. Invertebrates and native amphibian species have been shown to decline when co-occurring with *X. laevis* (see Scalera et al. 2019 and references therein). Furthermore, *X. laevis* is a known vector for amphibian diseases. A risk assessment has been performed for the European Union (Scalera et al. 2019), and based on the results the species is currently (January 2021) under consideration for regulation under the European Union IAS Regulation (1143/2014).

In Europe, the species is presently established in France, Portugal and Italy (Sicily) and has been recorded in six additional countries. In Belgium, three observations are documented, but no established populations are currently known. In France, African clawed frogs were introduced in the 1980ies, in the Two-Sèvres region, following the closure of a breeding centre and 30 years later occupy an area of over 2000 square kilometres (Louppe et al. 2017). The French populations are under management in the framework of the EU co-funded Life project CROAA (Control stRategies Of Alien invasive Amphibians, 2016-2022) LIFE15 NAT/FR/000864 (www.life-croaa.eu/).

In September 2018 a population was reported in La Chapelle-d'Armentières (département du Nord, région Hauts-de-France, Lille), in a 0.25 ha permanent pond (figure 1) owned and managed by the municipality, at about 2,5 km from the border with Belgium (Labadesse & Quevillart 2020).

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Figure 1: Infected pond in La Chapelle-d'Armentières, France. November 2020 (Loïc van Doorn).

Important, protected wetlands areas in the vicinity include the nature reserve of Ploegsteert (100ha), a marsh area with old clay pits. Also relevant is the Valley of the Lys which could potentially serve as a dispersal route into Belgium. In La Chapelle-d'Armentières, the species is reproducing as adults and larvae of different cohorts have been found (figure 2). Observations of the species in 2006 and in 2016 in Komen-Waasten on the Walloon side of the border were reported on the citizen science portal www.observations.be.

In response to this newly discovered population in France, a working group of relevant French and Belgian actors was founded in 2019 to discuss potential mitigation actions. Submerged fyke trapping was performed between September and November 2020. Four isolated water bodies within 1km were visually surveyed for the species and eDNA samples were taken from these locations.

Considering the invasive character of the species and its potential impact on native amphibians, nearby protected wetland areas, dispersal corridors via the river valley at just a few kilometres from the site and the presence of a large metapopulation of the protected great crested newt (*Triturus cristatus*) just across the border in Flanders, in April 2020, INBO decided to perform a landscape-scale eDNA sampling in a buffer area around the known population in France and the casual records in Wallonia. This short report describes the results of this initial survey, that can serve as a baseline for future surveillance initiatives.

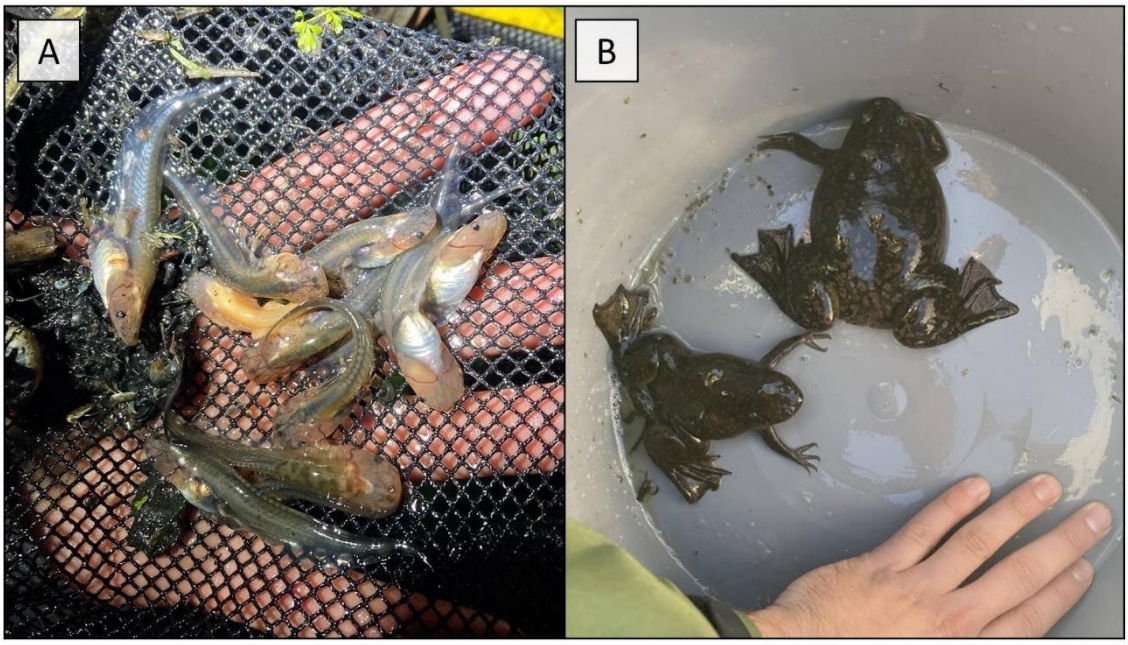

Figure 2: African clawed frogs removed from a pond in La Chapelle-d'Armentières, France. A) Subset of larvae caught in October 2020. Note the unique morphology, different stages of development, and the albinistic individual. Larvae are pelagic filter feeders and school together in deep parts of the pond. B) Adult male (left) and female (top) caught with submerged fykes in November 2020. Note the pronounced sexual size dimorphism in this species (Loïc van Doorn).

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Materials and methods

Field sampling

Environmental DNA (eDNA) sampling was performed on the 21st and 25th of April 2020. In total 74 water bodies (ponds, drainage ditches, canals and lakes) were sampled in Flanders, 23 in Wallonia and 9 in France, mostly lakes (figure 3, appendix table 1). Samples were taken in a standardized approach. In most cases, subsamples from several water bodies located close to each other (clusters) were pooled to obtain a single integrated and homogenous sample. For Flanders, this amounts to 23 pooled samples, for Wallonia 6, and for France 5. The water bodies were thoroughly sampled with subsamples of 0,5L scooped from the complete surface area just below the water surface, using a telescopic sterile pole with a sterile bag attached at the end (figure 4). A strict hygienic protocol was followed to exclude sample contamination and to eliminate the potential spread of pathogens.

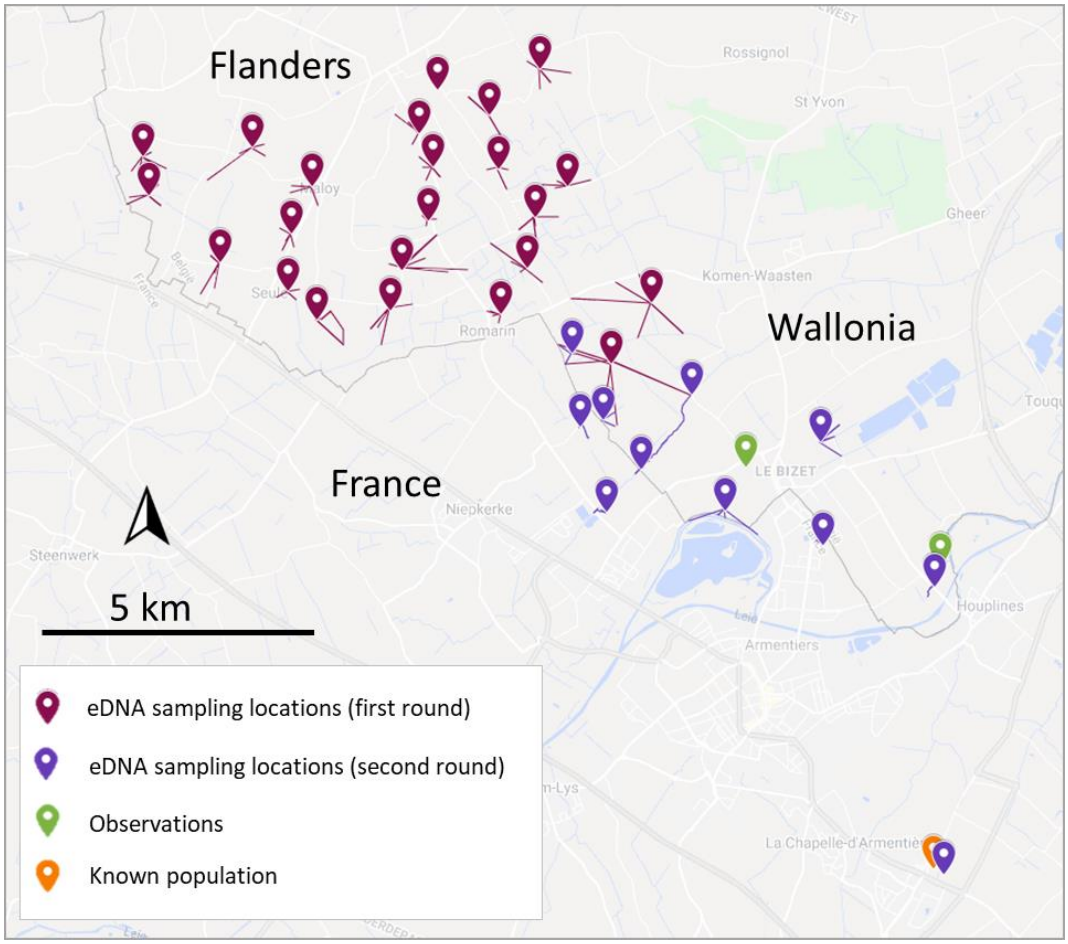

Figure 3. eDNA sampling locations in spring 2020. To decrease workload while maintaining a high landscape coverage, several water bodies in close proximity were pooled in one sample. Different lines that are connected with these pooled sample locations point on the water bodies in which subsamples are taken. Samples taken on the 21st of April in black, on the 25th in blue. Reported observations of *X. laevis* are presented in green, the known population in La Chapelle-d'Armentières in orange (see appendix table 1 for precise locations).

For each pooled sample, new sterile equipment was used, and 2% Virkon S (Antec - DuPont, Suffolk, UK) was used to decontaminate all reusable field material in between sampling locations. The collected samples were filtered in the field using enclosed Sterlitech filters (50 mm diameter syringe disk filter with an integrated 5 µm glass fiber prefilter and a 0.8 µm PES membrane) and a peristaltic pump, allowing a larger amount of water to be filtered. After filtration, the remaining water inside capsules was expelled by forcing air through the capsule. In a next step each filter was capped at both ends, and stored at −21°C in anticipation of further analyses in the laboratory. During field sampling field blancs were included in the workflow (i.e., filtering following the same procedures but with clean, uncontaminated source water).

Figure 4. A subset of sampling locations during the 2020 survey. Note the use of the telescopic pole with an attached 0.5L bag allowing for better coverage of the water body surface. All equipment is sterilised between clusters/pooled locations.

Laboratory analyses

Prior to PCR, all eDNA samples were stored and processed in a PCR-free building at INBO, dedicated to low copy number template extractions, with controlled DNA-free high-efficiency particulate air (HEPA)-filtered compartments with positive pressure to prevent eDNA sample contamination. On each filter an internal positive control (IPC) was added in the first step of the extraction together with the lysis buffer, in order to test for potential inhibition and thus to avoid false negative detections (see figure 5 and 6) to evaluate extraction efficiency of each sample separately. This IPC is a plasmid with a 149 bp insert sequence from Dengue virus type 2 (GenBank M29095.1) and can be quantified with a primers/probes assay with droplet digital PCR (ddPCR). This assay is compatible to run in duplex with the primer/probe assay for the target species.

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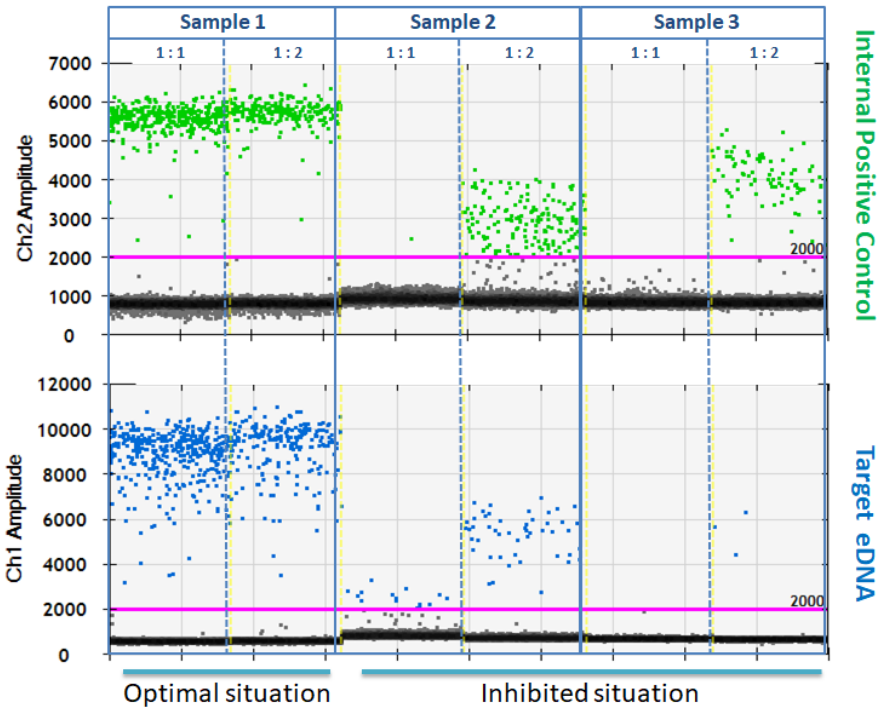

Figure 5. ddPCR output of a subset of field samples, showing optimal amplification of both the Internal Positive Control and Target eDNA in sample 1, whereas sample 2 and 3 illustrate complete and substantial inhibition in the 1:1 and 1:2 dilution respectively.

In a next step, each of the filters was recapped and placed in a rotating incubator overnight at 56 °C. The DNA was extracted from the filters using Qiagen's DNeasy Blood & Tissue Kit according to the manufacturer's instructions. DNA extracts were additionally purified with the DNeasy PowerClean Cleanup Kit (Qiagen) according to the guidelines provided by the manufacturer, and were eluted in 100 µL of TE. Comparing the concentration of IPC initially added to each filter and finally quantified by ddPCR after extraction or cleaning up, allows to standardize variation in target eDNA concentrations attributable to sample-specific differences in DNA extraction or amplification efficiency and hence to increase the comparability of eDNA samples in space and time.

During the lab workflow several technical blancs and positive reference samples were included, to test for either potential contamination during handling procedures in the lab, or potential failure of successful amplification of reference material, due to, for instance, inhibition (see Fig. 5).

Finally, the obtained DNA extracts were analysed via ddPCR using the primer/probe assay for the positive internal control in combination with a second primer/probe assay developed for detection of Xenopus laevis DNA. The latter amplifies a 83-bp fragment (primers included) on the 12S region, and those primers amplify 12 (sub)species (*Tragulus javanicus*, *X. tropicalis*, *X. andrei, X. boumbaensis, X. cf. boumbaensis* (BJE-2007), X. cf. *fraseri* (BJE-2004), *X. gilli, X. laevis, X. largeni, X. longipes, X. petersii* and *X. victorianus*). It is expected that only *X. laevis* currently occurs in France (Secondi et al. 2016).

Results

Each of the field- and lab-blancs (see Fig. 6, PCR neg. controls) did not show any amplification of *X. laevis* DNA, whereas all positive reference samples (see Fig. 6, PCR pos. control) showed optimal amplification without any sign of pcr failure or certain levels of inhibition.

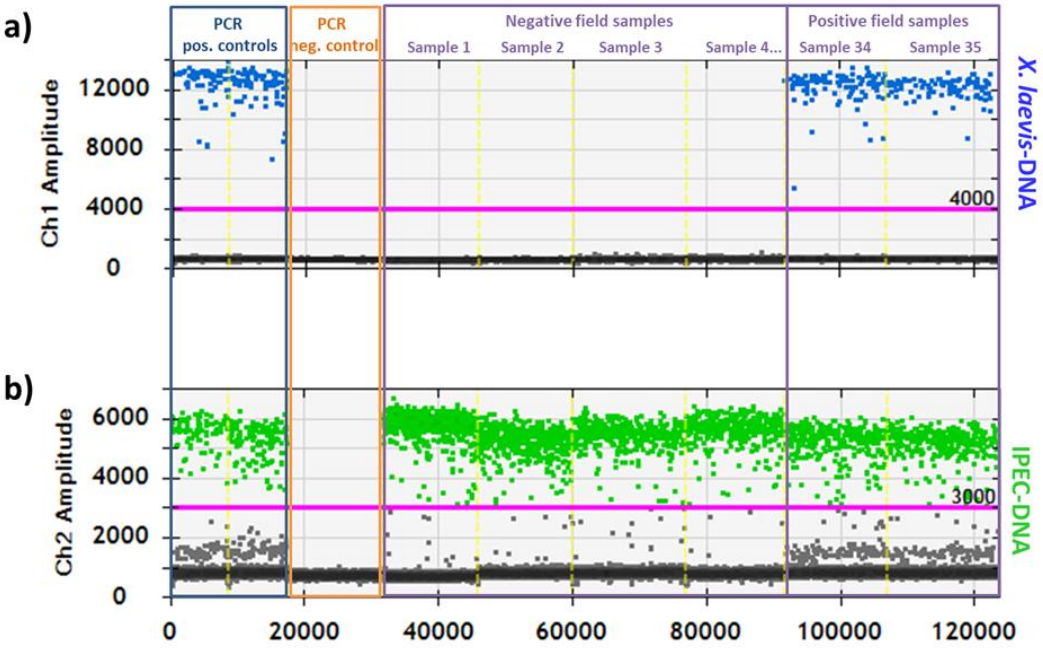

Figure 6. ddPCR output of a positive and negative control sample included at each run, four negative field samples (sample 1 - 4), and two positive samples (sample 34 and 35) taken at the infected pond in La Chapelle-d'Armentières, France.

None of the field samples taken in Flemish or the Walloon part of the study area showed any positive sign of *X. laevis* DNA, although the positive internal control amplified well and without any sign of inhibition (see figure 6, sample 1 - 4 as an example). Only the two samples taken in the source population (sample 34 and 35, figure 6, samples FRC4W1 and FRC5W1, appendix table 1) at the infected pond in La Chapelle-d'Armentières, showed relatively high eDNA concentrations of *X. laevis* in both samples taken (on average 7.51 ± 0.85 copies / µL DNA extract).

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Appendix

Table 1: Merged table containing samples and metadata for the 2020 campaign and observations of *Xenopus laevis* in the area. Cluster indicates the pooling of the samples.

Name	Type	Cluster	Location	Waterbody	Latitude	Longitude	Description
C1W1	Sample	F1	Flanders	Pond	50,73645142	2,78719246	eDNA sample 2020
FC1W2	Sample	F1	Flanders	Pond	50,73651201	2,78857909	eDNA sample 2020
FC1W3	Sample	F1	Flanders	Pond	50,73697698	2,79070558	eDNA sample 2020
FC1W4	Sample	F1	Flanders	Pond	50,73659756	2,79247679	eDNA sample 2020
FC1W5	Sample	F1	Flanders	Pond	50,73826039	2,78757358	eDNA sample 2020
FC2W1	Sample	F2	Flanders	Pond	50,7338762	2,78697347	eDNA sample 2020
FC2W2	Sample	F2	Flanders	Pond	50,73307131	2,79167945	eDNA sample 2020
FC2W3	Sample	F2	Flanders	Pond	50,73510565	2,79111173	eDNA sample 2020
FC2W4	Sample	F2	Flanders	Pond	50,73463696	2,78765907	eDNA sample 2020
FC3W1	Sample	F3	Flanders	Pond	50,73554127	2,79844268	eDNA sample 2020
FC3W2	Sample	F3	Flanders	Pond	50,73789629	2,80723297	eDNA sample 2020
FC3W3	Sample	F3	Flanders	Pond	50,73889925	2,8071185	eDNA sample 2020
FC3W4	Sample	F3	Flanders	Pond	50,74134904	2,80588204	eDNA sample 2020
FC3W5	Sample	F3	Flanders	Ditch	50,7379158	2,80337645	eDNA sample 2020
FC4W1	Sample	F4	Flanders	Pond	50,74172535	2,82661563	eDNA sample 2020
FC4W2	Sample	F4	Flanders	Pond	50,73891729	2,829243	eDNA sample 2020
FC4W3	Sample	F4	Flanders	Pond	50,74115047	2,83110585	eDNA sample 2020
FC4W4	Sample	F4	Flanders	Pond	50,74100452	2,82866296	eDNA sample 2020
FC5W1	Sample	F5	Flanders	Pond	50,74394251	2,82937622	eDNA sample 2020
FC5W2	Sample	F5	Flanders	Pond	50,74315383	2,83311416	eDNA sample 2020
FC5W3	Sample	F5	Flanders	Pond	50,74498151	2,83361797	eDNA sample 2020
FC5W4	Sample	F5	Flanders	Pond	50,74464639	2,8322309	eDNA sample 2020
FC6W1	Sample	F6	Flanders	Pond	50,74334636	2,83761155	eDNA sample 2020
FC6W2	Sample	F6	Flanders	Pond	50,7386103	2,84339086	eDNA sample 2020
FC7W1	Sample	F7	Flanders	Pond	50,74662725	2,84634389	eDNA sample 2020
FC7W2	Sample	F7	Flanders	Pond	50,74413906	2,84823761	eDNA sample 2020
FC7W3	Sample	F7	Wallonia	Pond	50,74482408	2,84974479	eDNA sample 2020
FC7W4	Sample	F7	Wallonia	Pond	50,7456761	2,85284101	eDNA sample 2020
FC8W1	Sample	F8	Wallonia	Ditch	50,73464418	2,84321475	eDNA sample 2020
FC8W2	Sample	F8	Flanders	Pond	50,73656194	2,84338307	eDNA sample 2020
FC9W1	Sample	F9	Flanders	Pond	50,73772465	2,83004112	eDNA sample 2020
FC9W2	Sample	F9	Flanders	Pond	50,73676804	2,83073136	eDNA sample 2020

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FC9W3	Sample	F9	Flanders	Pond	50,73557284	2,8313436	eDNA sample 2020
FC9W4	Sample	F9	Flanders	Pond	50,73589717	2,83324623	eDNA sample 2020
FC10W1	Sample	F10	Flanders	Pond	50,73494684	2,81114197	eDNA sample 2020
FC10W2	Sample	F10	Flanders	Pond	50,73434178	2,81094394	eDNA sample 2020
FC10W3	Sample	F10	Flanders	Pond	50,7331594	2,81506484	eDNA sample 2020
FC11W1	Sample	F11	Flanders	Pond	50,73100527	2,80944487	eDNA sample 2020
FC11W2	Sample	F11	Flanders	Pond	50,72974121	2,81010035	eDNA sample 2020
FC11W3	Sample	F11	Flanders	Pond	50,72892957	2,80953651	eDNA sample 2020
FC11W4	Sample	F11	Flanders	Pond	50,7292242	2,8115783	eDNA sample 2020
FC12W1	Sample	F12	Flanders	Pond	50,72834621	2,79913595	eDNA sample 2020
FC12W2	Sample	F12	Flanders	Pond	50,72480134	2,79774551	eDNA sample 2020
FC12W3	Sample	F12	Flanders	Pond	50,72673513	2,79960961	eDNA sample 2020
FC12W4	Sample	F12	Flanders	Pond	50,72527155	2,79969767	eDNA sample 2020
FC13W1	Sample	F13	Flanders	Pond	50,72609217	2,80983768	eDNA sample 2020
FC13W2	Sample	F13	Flanders	Pond	50,72446753	2,80882638	eDNA sample 2020
FC13W3	Sample	F13	Flanders	Pond	50,72523764	2,81225577	eDNA sample 2020
FC14W1	Sample	F14	Flanders	Pond	50,7231623	2,81692762	eDNA sample 2020
FC14W2	Sample	F14	Flanders	Pond	50,72236437	2,81559138	eDNA sample 2020
FC14W3	Sample	F14	Flanders	Pond	50,72004213	2,8190294	eDNA sample 2020
FC14W4	Sample	F14	Flanders	Pond	50,72139848	2,81887514	eDNA sample 2020
FC15W1	Sample	F15	Flanders	Pond	50,72147877	2,82294502	eDNA sample 2020
FC15W2	Sample	F15	Flanders	Ditch	50,71997532	2,82429093	eDNA sample 2020
FC15W3	Sample	F15	Flanders	Ditch	50,72361924	2,82917721	eDNA sample 2020
FC16W1	Sample	F16	Flanders	Pond	50,72808338	2,83087644	eDNA sample 2020
FC16W2	Sample	F16	Flanders	Pond	50,72577489	2,82875659	eDNA sample 2020
FC16W3	Sample	F16	Flanders	Pond	50,72687115	2,83456355	eDNA sample 2020
FC16W4	Sample	F16	Flanders	Pond	50,72706074	2,83746945	eDNA sample 2020
FC17W1	Sample	F17	Flanders	Pond	50,73228495	2,83069437	eDNA sample 2020
FC17W2	Sample	F17	Flanders	Pond	50,73129682	2,83121281	eDNA sample 2020
FC17W3	Sample	F17	Flanders	Pond	50,7319158	2,83280342	eDNA sample 2020
FC18W1	Sample	F18	Flanders	Pond	50,72782217	2,84395363	eDNA sample 2020
FC18W2	Sample	F18	Flanders	Pond	50,72667893	2,8454267	eDNA sample 2020
FC18W3	Sample	F18	Flanders	Pond	50,72589911	2,84860519	eDNA sample 2020
FC19W1	Sample	F19	Flanders	Ditch	50,72323063	2,84036349	eDNA sample 2020
FC19W2	Sample	F19	Flanders	Pond	50,72272061	2,84112441	eDNA sample 2020
FC19W3	Sample	F19	Flanders	Pond	50,72211344	2,84197396	eDNA sample 2020
FC20W1	Sample	F20	Flanders	Pond	50,72058585	2,8532142	eDNA sample 2020

FC20W2	Sample	F20	Flanders	Pond	50,71934923	2,85362635	eDNA sample 2020
FC20W3	Sample	F20	Flanders	Ditch	50,71810488	2,85195348	eDNA sample 2020
FC20W4	Sample	F20	Flanders	Pond	50,719092	2,853748	eDNA sample 2020
FC21W1	Sample	F21	Flanders	Ditch	50,71301886	2,86961024	eDNA sample 2020
FC22W1	Sample	F22	Flanders	Ditch	50,70832108	2,86321682	eDNA sample 2020
FC23W1	Sample	F23	Flanders	Pond	50,71237271	2,85955669	eDNA sample 2020
FC23W2	Sample	F23	Flanders	Ditch	50,713309	2,858538	eDNA sample 2020
WC1W1	Sample	W1	Wallonia	Ditch	50,73659014	2,85123576	eDNA sample 2020
WC1W2	Sample	W1	Wallonia	Pond	50,73547616	2,85071247	eDNA sample 2020
WC1W3	Sample	W1	Wallonia	Ditch	50,73507758	2,84855499	eDNA sample 2020
WC1W4	Sample	W1	Wallonia	Pond	50,73484501	2,85031908	eDNA sample 2020
WC1W5	Sample	W1	Wallonia	Pond	50,73556684	2,855811	eDNA sample 2020
WC1W6	Sample	W1	Wallonia	Pond	50,73581391	2,85291998	eDNA sample 2020
WC2W1	Sample	W2	Flanders	Pond	50,73097417	2,84510673	eDNA sample 2020
WC2W2	Sample	W2	Wallonia	Ditch	50,72950197	2,84777957	eDNA sample 2020
WC2W3	Sample	W2	Wallonia	Pond	50,73211524	2,85098553	eDNA sample 2020
WC2W4	Sample	W2	Wallonia	Pond	50,731172	2,847548	eDNA sample 2020
WC3W1	Sample	W3	Wallonia	Ditch	50,72664158	2,86310478	eDNA sample 2020
WC3W2	Sample	W3	Wallonia	Ditch	50,72437055	2,8528524	eDNA sample 2020
WC3W3	Sample	W3	Wallonia	Ditch	50,72067519	2,86259816	eDNA sample 2020
WC3W4	Sample	W3	Wallonia	Pond	50,725811	2,859583	eDNA sample 2020
WC3W5	Sample	W3	Wallonia	Ditch	50,72097802	2,86936162	eDNA sample 2020
WC4W1	Sample	W4	Wallonia	River	50,703989	2,874443	eDNA sample 2020
WC5W1	Sample	W5	Wallonia	Ditch	50,713068	2,890848	eDNA sample 2020
WC5W2	Sample	W5	Wallonia	Pond	50,712467	2,892805	eDNA sample 2020
WC5W3	Sample	W5	Wallonia	Ditch	50,711336	2,892272	eDNA sample 2020
WC5W4	Sample	W5	Wallonia	Ditch	50,709509	2,893378	eDNA sample 2020
WC6W1	Sample	W6	Wallonia	River	50,698368	2,90749	eDNA sample 2020
FRC1W1	Sample	FR1	France	Ditch	50,712591	2,854114	eDNA sample 2020
FRC2W1	Sample	FR2	France	Ditch	50,704234	2,856457	eDNA sample 2020
FRC2W2	Sample	FR2	France	Pond	50,704303	2,857162	eDNA sample 2020
FRC2W3	Sample	FR2	France	Pond	50,70466	2,858465	eDNA sample 2020
FRC3W1	Sample	FR3	France	Pond	50,702273	2,889605	eDNA sample 2020
FRC3W2	Sample	FR3	France	Pond	50,701576	2,889443	eDNA sample 2020
FRC3W3	Sample	FR3	France	Pond	50,701746	2,89097	eDNA sample 2020
FRC4W1	Sample	FR4	France	Pond	50,670734	2,906984	eDNA sample 2020
FRC5W1	Sample	FR5	France	Pond	50,670178	2,906932	eDNA sample 2020

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ObsB2006	Observation	NA	Wallonia	Pond	50,70841291	2,87868765	Observation Wallonia 2006
ObsB2016	Observation	NA	Wallonia	River	50,69896398	2,90776707	Observation Wallonia 2016
ObsF2018	Population	NA	France	Pond	50,67045	2,906893	Known since 2018
ObsF2019	Observation	NA	France	Unknown	50,66929585	2,94707687	Observation France 2019