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The invasive amphibian fungus *Batrachochytrium dendrobatidis (Bd)* in Vienna: Biomonitoring using environmental DNA

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# Contents

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# Abstract

There are many reasons for the worldwide amphibian decline since the 1950s such as loss of natural habitats, climate change or environmental pollution. One of the most devastating causes is the pathogenic, aquatic zoosporic fungus *Batrachochytrium dendrobatidis (Bd)*, which is responsible for the global disease chytridiomycosis in amphibians. In Austria infected specimens were detected in all nine provinces. For example, in Vienna the fungus was detected in five of seven sampled sites in the year 2011. There was still no mass mortality provoked by *Bd* in Vienna, but the quantity of *Bd* positive tested animals is alarming. Further observation of the fungus distribution is necessary. This study examined the applicability of the Environmental DNA method for a monitoring of *Bd* and investigated the actual infection situation in Vienna.

Water samples were collected from ten water bodies in Vienna over a period of two months. DNA of the water samples was extracted, purified and analyzed by Real Time PCR methods. Additionally, skin swabs were taken from amphibians around or in the tested ponds and *Bd* positive specimens from the Vienna Zoo were included as positive control.

All the skin swabs and all water samples were tested *Bd* negative.

Fortunately, there are a few fungus-limiting factors such as, for example, a temperature over 25°C, or the amphibian skin microbiome, or the fact that some planktonic predators feed on *Bd*. The load with the chytrid-fungus on amphibian species varies from time to time, so it is very important to survey its distribution regularly.

# Zusammenfassung

Es gibt viele Gründe für den weltweiten Rückgang der Amphibien seit den 1950er Jahren, wie den Verlust natürlicher Lebensräume, den Klimawandel oder Umweltverschmutzung. Eine der verheerendsten Ursachen ist der pathogene, aquatische Pilz *Batrachochytrium dendrobatidis* (*Bd)*, der für die weltweite Krankheit Chytridiomykose bei Amphibien verantwortlich ist. In Österreich wurden infizierte Exemplare in allen neun Bundesländern nachgewiesen. Zum Beispiel wurde der Pilz im Jahr 2011 in fünf von sieben Probenstellen in Wien festgestellt. Eine Massensterblichkeit, die durch *Bd* in Wien verursacht wurde, gab es noch nicht, jedoch ist die Anzahl der positiv getesteten Tiere alarmierend. Eine weitere Beobachtung der Pilzverbreitung ist notwendig. Diese Studie überprüfte die Anwendbarkeit der Umwelt-DNA-Methode (eDNA) für ein Monitoring von *Bd* und gab einen aktuellen Überblick über die Infektionssituation in Wien.

Wasserproben wurden über einen Zeitraum von zwei Monaten aus zehn Gewässern in Wien entnommen. Die DNA der Wasserproben wurde extrahiert, gereinigt und mittels Real-Time-PCR-Methode analysiert. Zusätzlich wurden Hautabstriche von Amphibien aus der Umgebung der getesteten Teiche entnommen, und *Bd*-positive Exemplare aus dem Tiergarten Schönbrunn dienten als Positivkontrolle.
Alle Hautabstriche und Wasserproben wurden *Bd*-negativ getestet.
Es gibt einige pilzlimitierende Faktoren, wie zum Beispiel eine Temperatur über 25°C, das Amphibien-Hautmikrobiom, oder die Tatsache, dass einige Arten von Plankton *Bd* fressen. Die Belastung durch den Chytrid-Pilz auf Amphibienarten variiert von Zeit zu Zeit, weshalb es sehr wichtig ist, regelmäßig einen Überblick über seine Ausbreitung zu haben.

# 1 Introduction

The chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) is one of the most significant pathogens in amphibian history. Its taxonomic classification places it within the kingdom Fungi, phylum Chytridiomycota, order Rhizophydiales and class Chytridiomycetes (Longcore et al., 1999).

Most of the Chytridiomycota inhabit moist soil or fresh water and they are saprotrophic or parasitize algae and plants. Within the genus Batrachochytrium, *Bd* is phylogenetically closely related to *Batrachochytrium salamandrivorans* (*Bsal*), another highly pathogenic chytrid fungus that primarily infects urodeles (Martel et al., 2013). Another interesting relative is *Ichthyochytrium vulgare* which was described as parasite of fresh-water fishes (Plehn, 1920).

There is substantial genetic diversity within *Bd* itself. Several distinct lineages have been described, including the highly virulent *BdGPL* (Global Panzootic Lineage), which has been linked to mass mortalities and declines of amphibian species worldwide (O`Hanlon et al., 2018). Other lineages such as *BdCAPE*, *BdBRAZIL* and *BdAsia-1* are more geographically restricted and exhibit lower levels of virulence (Byrne et al., 2019).

## 1.1 Biology and life cycle of *Batrachochytrium dendrobatidis*

The peculiarity of *Bd* lies in its exclusively epidermal infection and its asexual two-phase life cycle consisting of motile zoospores and sessile sporangia. The infectious form is the 3–5 µm large, uniflagellate zoospore which shows chemotaxis to attractants such as sugars, proteins or amino acids (Moss et al., 2008). For example, *Bd* zoospores react positive on skin mucus from *Xenopus laevis*, which contains free sugars such as alpha-D-mannose, alpha-D-galactose, alpha-L-fucose and N-acetylglucosamine (Van Rooij et al., 2015). Once the zoospore reaches its host it absorbs its flagellum, forms a cell wall and encysts on the amphibian surface. Then it begins to penetrate the outer layers of the skin (*Stratum corneum*, *Stratum granulosum*) by a germ tube. Inside the host epidermis it develops into a sessile sporangium. At the same time, rhizoids, root-like runners grow, that anchor the sporangium in the tissue and absorb nutrients. After maturation, the sporangium forms a discharge tube through which the mature zoospores are released into the surrounding medium (water or a film of water on the skin) and can infect other amphibians (Longcore et al., 1999; Berger et al., 2005; Rosenblum et al., 2008).

The entire life cycle from the initial zoospore through encystation, sporangia maturation to the release of new zoospores takes about 4 to 5 days at temperatures around 17–25°C (Piotrowski et al., 2004). Unlike many other chytrid fungi, *Bd* does not produce typical resting or persistent stages (zygospores or chlamydospores). However, long-lasting sporangia with delayed spore release have been observed in vitro under stress conditions, which may allow

for some ecological persistence (Johnson and Speare, 2003). The zoospore has a limited lifespan of less than 72 hours, depending on environmental factors such as temperature and humidity (Piotrowski et al., 2004). The life cycle is thus an example of the adaptation of a microorganism to a specific host organism and an aquatic environment. Its simple structure, and the high reproductive rate, makes *Bd* a highly infectious and ecologically successful pathogen that has caused significant damage to amphibian populations worldwide (Fisher et al., 2009).

### 1.2 Chytridiomycosis

*Batrachochytrium dendrobatidis* only infects keratinized cells, which in adult amphibians are found throughout the outer skin but in tadpoles only in the mouth area. This infection causes hyperkeratosis, epidermal hyperplasia and cell apoptosis. The skin of amphibians is a central site for osmoregulation, respiration and water uptake. Functional impairment due to *Bd* infection leads to a reduced water uptake and excretion, a reduced electrolyte transport (especially Na+ an K+), hyponatremia and hypokalemia. This electrolyte imbalance can impair cardiac function and is the major cause of death in animal models. Ventricular fibrillation and cardiac arrest occur in the end stage. Typical disease courses last 7–21 days from infection to death in highly susceptible species. Many animals show no symptoms as long as the infection load remains low (Berger et al., 2005; Voyles et al., 2009).

### 1.3 Limiting factors for *Batrachochytrium dendrobatidis*

The skin of amphibians is not only an important interface with the environment but also harbours a complex microbial community, the so-called skin microbiome. This community consists of bacterial, viral and fungal symbionts that interact closely with the host and play a significant role in protection against pathogenic microorganisms such as *Bd* (McKenzie et al., 2011). This skin microbiome is species-specific but also influenced by environmental factors, developmental stage, habitat and season (Kueneman et al., 2013). Dominant bacterial genera include *Janthinobacterium*, *Pseudomonas*, *Acinetobacter*, *Chryseobacterium*. Some of these microorganisms produce secondary metabolites with antifungal effects. The bacterium *Janthinobacterium lividum* produces Violacein with proven activity against *Bd* (Brucker et al., 2008). There is also a negative chemotaxis of *Bd*, provoked by antifungal metabolites. For example, 2,4-Diacetylphloroglucinol (DAPG) is produced by certain strains of the genus *Pseudomonas*, has fungicidal properties and can alter *Bd* zoospore attachment behaviour. Another antifungal molecule is Indole-3-Carboxaldehyde (I3C) which is produced by *Janthinobacterium lividum* and affects the motility and settlement preferences of *Bd* zoospores (Brucker et al., 2008; Loudon et al., 2014).

Protective bacteria could be transferred to other amphibian species to increase resistance to *Bd* (Bletz et al., 2013). But not all amphibian species own effective bacteria against *Bd*, and *Bd* itself can develop antimicrobial strategies to evade such bacteria (Woodhams et al., 2007).
In addition to the skin microbiome, there are several abiotic factors that can limit the infection and spread of *Bd*. Behavioural thermoregulation (behavioural fever) is an adaptive behaviour in which amphibians deliberately seek out warmer microhabitats to increase their body temperature which inhibits the growth or directly kills *Bd* (Woodhams et al., 2003; Richards-Zawacki, 2009; Rowley and Alford, 2013).
An in vitro study of Johnson et al. (2003) showed that *Bd* is sensitive to UVB radiation, even low doses significantly reduced zoospore viability. Higher UVB radiation exposure in the habitat correlates with lower *Bd* prevalence in frog populations (Ortiz-Santaliestra et al., 2011). There is a narrow pH optimum for *Bd* growth between 6 and 6,5 (Piotrowski et al., 2004). The fungus is also sensitive to osmosis; a high salinity leads to cell lysis or inhibition of spore movement (Stockwell et al., 2012). Amphibians in saline or brackish habitats show lower *Bd* prevalence (Clulow et al., 2017).
Amphibians possess numerous antimicrobial peptides such as for example Dermaseptins, Brevinins and Temporins, synthesized in the granular glands of the skin and secreted upon stress or pathogen contact. These peptides are part of the innate immune system and can directly inhibit *Bd* cells, particularly zoospores (Rollins-Smith et al., 2005).
Savage and Zamudio (2011) provide strong evidence that genetic variation in the immune system plays a central role in natural resistance to *Bd*. Their findings support the idea that conservation programs should promote genetic diversity, particularly at the MHC locus, to strengthen the resistance of amphibians to *Bd*.
Buck et al. (2014) provide insights into the importance of zooplankton as natural predators of *Bd*. Zooplankton could represent a potential tool for biological control of *Bd* (Schmeller et al., 2014).

### 1.4 Origin and distribution

The first scientific description of chytridiomycosis as disease in amphibians was published by Berger et al. (1998). One year later the first official mycological description of the fungus by Longcore et al. (1999) was published. In the meantime, the fungus is documented worldwide. About 700 of 1300 tested amphibian species are infected. There was a decline of 501 species, 90 of them are extinct (Scheele et al., 2019).
The origin and spread of *Bd* remained unclear for a long time. Previous studies suggested that the fungus originated in tropical regions of Africa (Weldon et al., 2004; Vredenburg et al.,

2013). Since there was also a large mass extinction of amphibian species in Australia it was argued that this was caused by *Bd* as a novel pathogen (Skerratt et al., 2007). O`Hanlon et al. (2018) showed by genetic analysis of more than 200 *Bd* isolates from different regions worldwide that the fungus has an Asian population of origin, from which it spread to the rest of the world. They also build on previous work suggesting that human activities, particularly the Korea-war (1950-1953), the international trade in amphibians, and the release of animals from breeding programs, may have played a key role in the rapid spread of *Bd*.
Some studies highlight the African clawed frog (*Xenopus laevis*) as a vector for the spread of *Bd*, as it was used for pregnancy tests or for biomedical studies from the 1930s to 1960s and therefore was internationally highly traded (Weldon et al., 2004; Schloegel et al., 2006). Clawed frogs are often asymptomatic carriers, so-called reservoir hosts of *Bd*.
The American bullfrog (*Rana catesbeiana*) has also been found to be a reservoir host for *Bd*, facilitating the transmission of the fungus to other amphibian species in the wild and influencing the distribution of the fungus in Europe and North America (Garner et al., 2006).
Another key finding was that *Bd* probably arrived in Mallorca through the reintroduction project of the midwife toad (*Alytes obstetricans*) in 1991 (Walker et al., 2008).
Sun et al. (2025) reinforce the theory that *Bd* originated in the tropical amphibian communities of Asia, especially South China, where the high genetic diversity and ecological specialization of *Bd* suggest that the fungus has been established there for a long time and has adapted to different environmental conditions.

### 1.5 Situation in Austria

In Austria, *Bd* is widespread. One of the central surveys on this topic was published by Sztatecsny and Hödl (2011). Particularly high infection rates were observed in eastern and southeastern Austria, as well as in Vorarlberg. Commercially used waters and floodplain waters showed high detection rates (Sztatecsny and Hödl, 2011).
Another study on the geographical distribution of *Bd* in Austria proved the highest occurrence of the fungus to date in the European Alps at 1630 metres above sea level. The study included nine amphibian species, with yellow-bellied toad (*Bombina bombina*) being particularly affected. Notably many infected individuals did not show clinical symptoms of chytridiomycosis (Sztatecsny and Glaser, 2011).
The occurrence of *Bd* has also been documented in Vienna. In 2011 five of seven sample sites along the Danube and Western Vienna were tested positive on *Bd* (Sztatecsny and Glaser, 2011).

### 1.6 Detection methods

One of the first methods to detect the fungus was cultivation of *Bd*, which involves culturing amphibian skin samples on culture media to observe the growth of the fungus. But cultivation is a time-consuming method, as *Bd* growth under laboratory conditions can take days to weeks. It is also susceptible to contamination and not all *Bd* strains grow under laboratory conditions, which can lead to false negative results (Piotrowski et al., 2004).

Another traditional method is microscopic examination of skin samples.

Polymerase Chain Reaction (PCR) technology, developed by Kary Mullis in 1983, revolutionized the diagnosis of *Bd*. It allows specific *Bd* DNA (from an amphibian skin swab) sequences to be amplified to detect the presence of the fungus even in small amounts of DNA, what has significantly improved the sensitivity and accuracy of diagnosis compared to culture and microscopic examination (Annis et al., 2004).

However, all these methods are invasive, as they require catching and skin swabbing, which is stressful for the animals. The environmental DNA (eDNA) method has emerged in recent years as one of the most promising techniques for the detection of *Bd*. Environmental DNA refers to the genetic material released by organisms into their environment, for example, through skin secretion, urine, feces or dead cells. This DNA remains in the environment after its release and can be extracted from water samples. For the detection of *Bd*, eDNA is analyzed for the presence of specific *Bd* DNA sequences using PCR. Due to its high sensitivity, this method is particularly effective for detecting *Bd* in waters where amphibians live, without the need to take direct skin samples from the animals (Kirshtein et al., 2007; Kamoroff and Goldberg, 2017).

A disadvantage of eDNA is that it can rapidly degrade or be affected by environmental factors such as temperature, pH, UV radiation, which reduce DNA stability and can lead to false negatives, especially in contaminated or changing surroundings (Ficetola et al., 2014).

Quantitative PCR (qPCR) is a more advanced form of PCR that allows to quantify not only the presence of *Bd* but also the amount of *Bd* DNA in a sample. In qPCR, DNA is amplified (replicated) in a series of cycles with the amount of target DNA doubling in each PCR cycle. Unlike traditional PCR, where the result is only visualized by gel electrophoresis, qPCR measures the amount of amplified DNA product in real time during each PCR cycle. This procedure is done by using a fluorescent dye or probe that binds to the amplified DNA product. The intensity of the fluorescent signal increase with each cycle and is monitored throughout the process. The amount of amplified DNA is compared to a reference standard, which allows DNA concentration in the sample to be calculated. The point at which the

fluorescence reaches the specified threshold is recorded (Cycle threshold value). A lower Ct value indicates a higher initial amount of DNA, as fewer cycles are required to generate measurable fluorescence. This method allows for accurate quantification of eDNA and provides information about the *Bd* infection load in the environment. The eDNA concentration can be tracked in relation to environment conditions or the presence of *Bd* in different samples and over different time periods. A high concentration of *Bd* DNA indicates a high infection load in a water body, possibly indicating an outbreak of chytridiomycosis (Kamoroff and Goldberg, 2017).

Quantitative PCR is very sensitive and can be contaminated by other DNA (from humans, equipment, environment). This circumstance poses a major problem as even tiny amounts of exogenous DNA can lead to false positive results (Kirshtein et al., 2007).

Next-generation sequencing (NGS) goes a step further by allowing the analysis of the entire DNA spectrum of a sample. With NGS it is possible to investigate a sample besides for *Bd* also for other microorganisms and even the microbiome of amphibians and their environment (Valentini et al., 2015).

### 1.7 Aim of this study

This project consisted of two parallel investigations using the same methods. This study set out to provide an overview of the actual occurrence and distribution of *Bd* in Western Vienna, by testing 10 water bodies and 50 of their inhabiting amphibians while the parallel investigation in the East of Vienna was carried out by Katharina Vesely. The applicability of the eDNA detection method for a monitoring of *Bd* was examined by comparing results of skin swabs and tested water samples.

# 2 Material and Methods

For this work ten Viennese water bodies were chosen. The field work consisted of taking swabs from five amphibians per waterbody and water samples which were collected three times per pond. Water samples from an aquarium, which contained *Bd*-positive tested newts from the Zoo Vienna were collected too. Swabbing and water sampling was started in April 2017 and was finished in June 2017. Water samples were filtrated and frozen until the laboratory work which included the DNA extraction, DNA purification, measuring of the DNA concentration with Nanodrop and finally the sample-analysing by a quantitative PCR according to Sztatecsny and Hödl (2011) and add-on bacterial DNA cloning. All laboratory work steps were performed in collaboration with Katharina Vesely (Vesely, 2019) in the laboratory spaces at the Department Biomedical Engineering of the University of Applied Sciences Vienna. To avoid contamination of the samples, the extraction, purification, qPCR and bacterial cloning were performed in different laboratory spaces.

## 2.1 Sampling sites

The following ten sampling sites were tested (Fig. 1, Table 1).

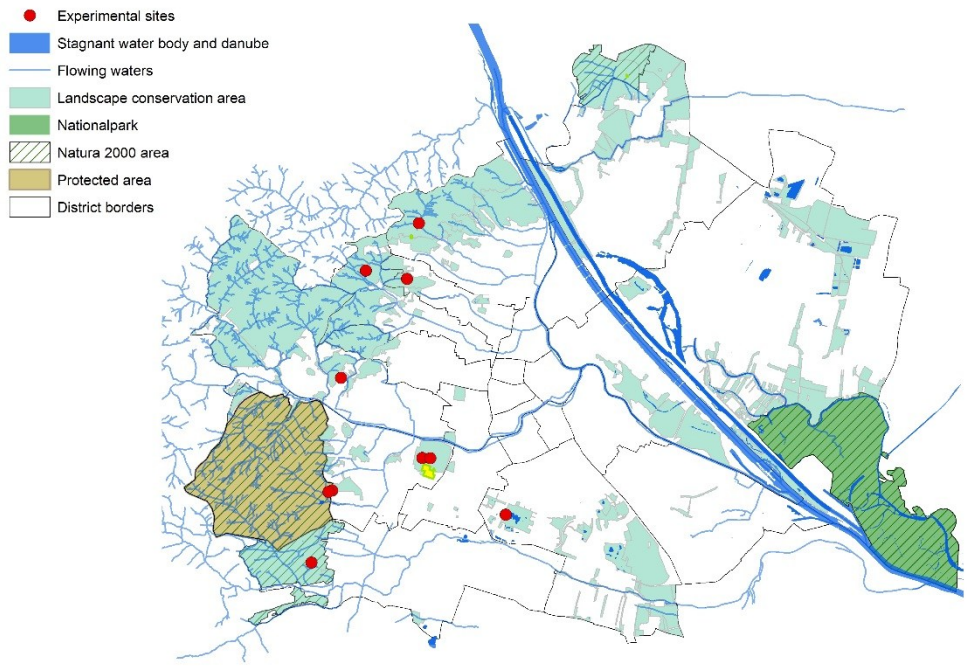


Figure 1: Map of Vienna and the ten red marked sampling sites. Afritschteich and Wachsstöcklteich are located close together at the protected area (marked in light brown).

Table 1: Names and GPS Data of the sampling sites. Red marked ponds were tested *Bd* positive in 2011.

| Place Name | WGS84 O | WGS84 N |
|---|---|---|
| Wienerberg | 16,35420 | 48,16100 |
| Silbersee | 16,26324 | 48,20908 |
| Pappelteich | 16,24719 | 48,14544 |
| Afritschteich | 16,25770 | 48,17009 |
| Schwarzenbergpark | 16,275799/ 16,268902 | 48,245749/ 48,245478 |
| Bombentrichter 1/ Paraplouie Teich | | |
| Sieveringer Wehr | 16,30296 | 48,26197 |
| Pötzleinsdorfer Schlosspark West | 16,29699 | 48,24293 |
| Tiergarten Alpenteich | 16,30482 | 48,18176 |
| Nashornteich | 16,30885 | 48,18167 |
| Wachsstöcklteich | 16,25690 | 48,17012 |

**Wienerbergteich**:

Since the Roman empire, the area Wienerberg was used as clay pit for brick fabrication. From 1960 until 1983 it served as dumpsite. Nowadays, the Wienerberg with the pond is a recreation area (Fig. 2). The ponds size is about 16,1 ha. The high stock of reed provides many nesting opportunities for birds and many other animals. Little bittern (*Ixobrychus minutus*), European pond terrapin (*Emys orbicularis orbicularis*), the endangered large cooper (*Lycaena dispar*), numerous populations of waterfrogs (*Pelophylax sp*.) and many fishes can be found.

Due to algae and greasy film, as a consequence of sunscreen, the filtration of this water worked very slow.

Figure 2: Wienerbergteich.

**Silbersee**:

This pond with a size of approximately 300 $m^2$, is located in the Wienerwald near a settlement zone. It is an idyllic water body surrounded by a broadleaf forest with a few places of reed and deadwood (Fig. 3). Growth of algae increased in the warmer period. Common toads (*Bufo bufo*) (both, adults and tadpoles), agile frogs (*Rana dalmatina*), grass snakes (*Natrix natrix*), grey herons (*Ardea cinerea*) were found.

Figure 3: Silbersee.

**Pappelteich**:

This artificial lentic water was first documented in 1872. It is surrounded by a few poplars and stands free near a children playground inside the recreation area Maurer Forest, which belongs to the Wienerwald (Fig. 4). It includes common stonewort and water lilies but also algae, which are increasing in warmer months and makes a filtration more difficult. Pappelteich is a very important habitat for many amphibians such as common toads, yellow-bellied toads (*Bombina variegata*), alpine newts (*Ichthyosaura alpestris*), common newts (*Lissotriton vulgaris*), and agile frogs (*Rana dalmatina*). Further, goldfish and grass snakes could be found. Because this pond was only fed by rain and slope water, the increasing aridity by climate change, endangered this habitat. In 2018 it nearly dried out (meinbezirk.at 23.10.2018). To counteract this, a permanent water pipe was laid in 2022 to supply the pond with fresh water (Magistratsabteilung 45 Wien, 2022).

Figure 4: Pappelteich.

**Afritschteich**:

This biotope with a size of approximately 30 $m^2$, originated from a former swimming pool of a childrens´s home (Josef-Afritsch-Heim) (Fig. 5). It is situated next to the gardens of a settlement zone at the edge of Wienerwald. To avoid injuries and dogs to go into the water, the pond is surrounded by a wire-fence. At this water body, water frogs, crested newts (*Triturus carnifex*), grass snakes, common newts, common toads, many water snails and leeches were found. The water body contains reed, water lilies and is surrounded by bushes and broadleaf trees.

Figure 5: Afritschteich.

**Schwarzenbergpark (Bombentrichter 1/Parapluieteich)**:

Bombentrichter 1 is a waterfilled bomb crater in the broadleaf forest of Schwarzenbergpark with a size of approximately 8 $m^2$ (Fig. 6). Schwarzenbergpark is the first landscape garden of Austria. The surrounding of the small pond is, despite a nearby trail, natural, with a lot of bear leek around. This water body contained many larvae of fire salamander (*Salamandra salamandra salamandra*), but unfortunately the water level got lower rapidly. At the last water sampling round in June, it was dried out completely. For this reason, the third water sample was taken from Parapluieteich, which is located also in the Schwarzenbergpark. It is a fishpond near a trail and a street, surrounded by a mixed forest and it contains fishes, common newts, alpine newts and many tadpoles of common toads, agile frogs and common frogs.

Figure 6: Bombentrichter.

**Sieveringer Wehr**:

Sieveringer Wehr is a small dam in the stream Erbsenbach in the Wienerwald (Fig. 7). It protects the Viennese district Sievering from high water, which appeared frequently in Viennese history. A small pond with a size of 20-30 $m^2$ is fed by the stream and offers a habitat for many animals. Especially larvae of the fire salamander were found, but also common newts, crested newts, agile frogs, yellow-bellied toads, water frogs, larvae of dragonflies and many more. The pond is located near a broadleaf forest in a typical fire salamander area.

Figure 7: Sieveringer Wehr.

**Pötzleinsdorfer Schlosspark**:

This park, near the mansion Pötzleinsdorf was built in 1801 by K. Rosenthal, who also established the ponds. The area was destroyed during the second world war and was reopened in 1949 (Stadt Wien Homepage). The water body, with a size of approximately 200 $m^2$ near a trail, is surrounded by broadleaf trees and contained reed and deadwood (Fig. 8). Many animals such as mallards (*Anas platyrhynhos*), deer (*Capreolus capreolus*), and grass snake were found. There were many leeches, common toad tadpoles and a few agile frogs and common newts.

Figure 8: Pötzleinsdorfer Schlosspark.

**Alpenteich**:

This pond is located near a visitor pathway beside the rainforest house of the Zoo Vienna (Fig. 9). It is an artificial water body of about 15 $m^2$ with clear and filtrated water and contains many native fish species such as endangered Danube salmon (*Hucho hucho*), common barbel (*Barbus barbus*), bream (*Abramis brama*), common chub (*Squalius cephalus*), pike (*Esox lucius*), European perch (*Perca fluviatilis*), common nase (*Chondrostoma nasus*), wels catfish (*Silurus glanis*), sterlet (*Acipenser ruthenus*), gudgeon (*Gobio gobio*), roach (*Rutilus rutilus*), wild carp (*Cyprinus carpio*). At visiting hours no amphibians were found at or in this pond, but at night many amphibians such as the endangered green toads (*Bufotes viridis*), common toads, common newts, and water frogs can be seen throughout the zoo area.

Figure 9: Alpenteich in the Zoo Vienna.

**Nashornteich**:

The water body of about 30 $m^2$ is located near a zoo entrance and is part of the great one-horned rhinoceros (*Rhonoceros unicornis*) enclosure (Fig. 10). It contains filtrated water but also more suspended solids than the Alpenteich. At this pond the same amphibian species were found as in the rest of the zoo area, especially in the Alpenteich.

Figure 10: Nashornteich in the Zoo Vienna.

**Wachsstöcklteich**:

This artificial pond of about 20 $m^2$ is located a few meters beside Afritschteich. It is densely covered with duckweeds and contains reed (Fig. 11). The place is surrounded by a broadleaf forest (Hörndlwald). Amphibians seemed to prefer Afritschteich, none could be found at Wachsstöcklteich. For this reason, skin swabs were taken from the Afritschteich inhabiting amphibians (in Table 10 these skin swabs were assigned to Wachsstöcklteich). Only water snails and great diving beetles (*Dytiscus marginalis*) were discovered at Wachsstöcklteich. Residents told about yellow-bellied sliders (*Trachemis scripta scripta*) which were released into Wachsstöcklteich.

Figure 11: Wachsstöcklteich.

**Aquarium *Ambystoma dumerilii***:

Water from the aquariums of the Mexican newts (Fig. 12) at the Zoo Vienna was used as positive control according to Kabrt (2016). These newts were tested *Bd* positive by Dr. J. Spergser (University of Veterinary Medicine Vienna) in 2015, and therefore were kept backstage in quarantine in 200 l aquariums at a water temperature of 21 °C. They were fed with lumbricids. Water samples were taken before the water change, which was done twice a week (information of Dr. Preininger).

Figure 12: *Ambystoma dumerilii* at the Zoo Vienna.

## 2.2 Water Sampling

First, gloves and overshoes or gumboots were put on, and the on-site situation was documented by measuring pH-value, oxygen content and water temperature by a multimeter and taking pictures of the pond. All Parameters were listed in water sampling protocols (see Appendix A). Additionally, a mapping form was filled in which includes a description of the vegetation and encountered amphibians, reptiles or other animals.

For water abstraction empty mineral water bottles were used.

From each pond three litres of water were sampled from three different sites, where most of the amphibians were found. Water sampling was done three times per pond, to compare the samples at different periods.

1. 26.4.2017-9.5.2017
2. 16.5.2017-20.5.2017
3. 9.6.2017-22.6.2017

In round three water bottles were fixed by a sliced bicycle tube on a metal rod. With this elongation it was possible to sample deeper water layers.

Table 2: Applied materials for water sampling

| **Equipment** |
|---|
| Water bottles 1,5 l, empty |
| Overshoes |
| Gloves |
| Multimeter and distilled water |
| Gumboots |
| Slides of bicycle tube |
| Metal rod |
| Sheets of water sampling protocol and mapping protocol |
| Paper towels |
| Camera (Samsung S5 mini) |

## 2.3 Skin swab sampling

Figure 13: Common newt (*Lissotriton vulgaris*) captured for taking a skin swab.

In addition to water sampling, skin swabs from five amphibians per waterbody were collected in the same period (end of April until end of June) (Table 3).

Catching amphibians in Austria is permitted only for research studies, and a notification from Wiener Umweltschutzabteilung Magistratsabteilung 22 is required (the notification number for this study: MA 22 – 984143-2015-5).

To get more reference values, swabs were taken over a range of days. Amphibians were caught by nets and wiped off by swabs twenty times on ventral side and fungus-exposed body areas like armpits and groins. Skin swabs were labelled with number of the waterbody, date, swab-number and stored at 23 °C in a dry and light-excluded place. For further evaluation by Hechinger according to Blooi et al. (2013), swabs were sent to the Landesbetrieb Hessisches Landeslabor in July 2017.

To prevent a spread of *Bd* in other waterbodies, all used gloves and overshoes were changed. Nets and gumboots were used only for one pond per day, cleaned and dried for two days before next amphibian catching and taking water samples.

Table 3: Applied materials for skin swab sampling.

| Equipment |
|---|
| Swabs |
| Nets |
| Overshoes |
| Gumboots |
| Gloves |
| Permanent marker |

## 2.4 Filtration

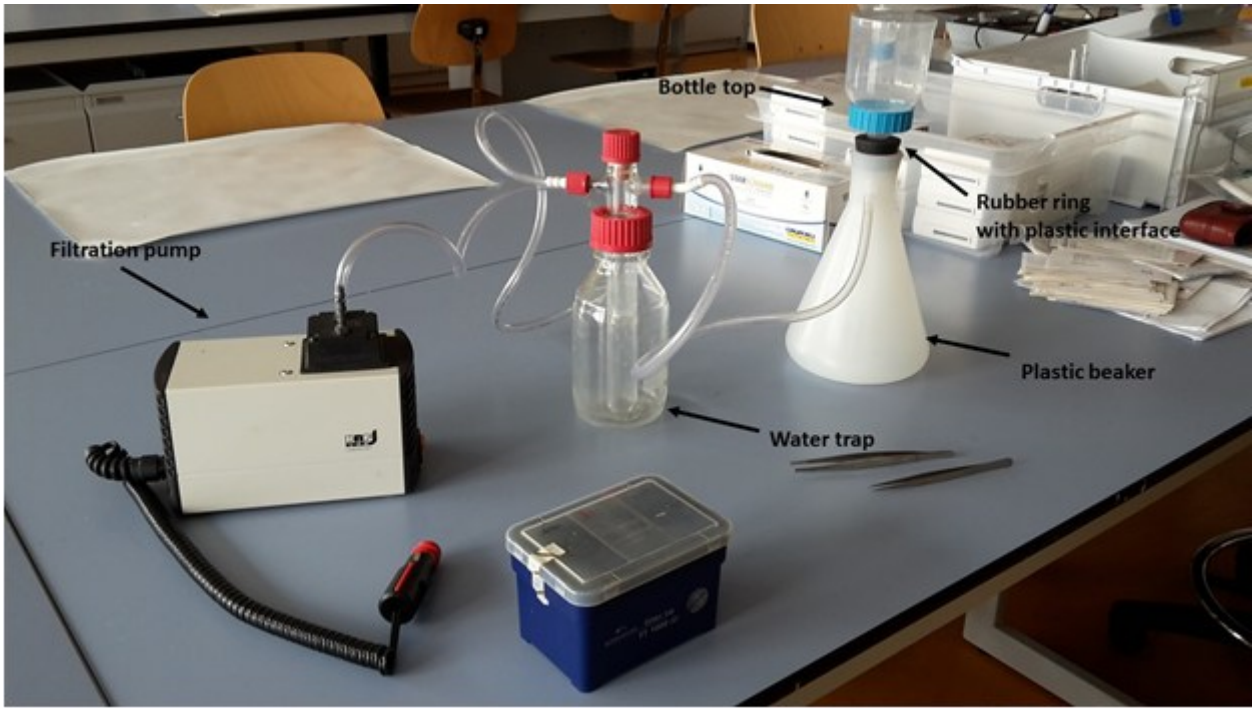


Figure 14: Equipment construction for filtration.

Water samples were filtrated subsequently on site. Samples were stored cool and filtrated within 6 hours. Filtration was performed by a portable vacuum pump (Laboport® 12V field filtration pump, KNF). A 12V DC adapter plugs into car outlets and enabled an on-site filtration. The pump was connected by a rubber tube to a water trap and further to a plasticbeaker (1000ml). This plastic beaker included a rubber ring for a plastic interface on which a bottle top could be insert. The lid and upper part of the bottle top was remoted to replace the pre-existing filter and set in a Millipore filter (0,22 µm pore size). Then the upper part was racked back. Subsequently 100-200 ml of a water sample were filled into the bottle top and were filtrated. The filtrate was collected in the plastic beaker and deflated if necessary. The water trap avoided a damage to the pump, if the beaker was not emptied in time (Fig. 14, Table 4).

It was considered to use at least two filters per pond, and filtration round, whereas 1,5 l were filtrated through each filter. For water bodies including a high range of algae or suspended solids, more filters were required (maximum eight filters were used at Wienerbergteich in filtration round one). As soon as no water flowed through a filter anymore, the filter was folded in the middle four times by sterilized forceps, put into a labelled 1,5ml Eppendorf tube and stored in a box with icepacks until it got freeze at -20 °C. Tubes were labelled as follows: for example: 1/1/A, the first number describes the sampling site, the second number describes the sampling round and the letter describes the number of filters.

Table 4: Applied materials for filtration.

| **Equipment** | |
|---|---|
| Plasticbeaker 1000 ml | Eppendorf tubes 1,5 ml (1/filter) |
| Sterivex-GP Sterile Vented Filter Unit (0,22µm) EMD Milipore | Nalgene™ Rapid-Flow™ Sterile Disposable Bottle Tops |
| filtration pump (Laboport® 12 V field filtration pump, KNF) | Water trap |
| Fridge (Liebherr) | Gloves |
| Forceps, Ethanol 20 %, Lighter | Rubber interface (between plasticbeaker and pump, and between plasticbeaker and water trap |
| Nylon stockings, rubber bands | Wastebox |
| Icebox with icepacks | Permanent marker |

### 2.5 DNA extraction

For the DNA extraction the Gentra Puregene Tissue Kit (Qiagen) was used (Table 5). Only the quantities of the reagents were modified upon consultation with Bettina Dekrout (University of Applied Sciences, Vienna). For each filter two sets of 2 ml tubes and one set of 1,5 ml tubes were prepared and labelled. One set of the 2 ml tubes was prefilled with 900 µl lysis buffer, the other with 810 µl Isopropanol (99%). Each single filter was cut with a scalpel blade on a culture dish, which was standing on a cooled metal plate in a styrofoam box with crushed ice. The cut filter pieces were added by forceps into the 2ml tubes with lysis buffer. These tubes were heated at thermomixer at 65 °C for 15 minutes. For each filter new culture dishes and gloves were used, and scalpel blades and forceps were sterilized by flaming. After heating the 2 ml tubes were centrifuged at 10000 x g for five minutes. The supernatant was transferred into the 1,5 ml tubes and 4,5 µl Proteinase K solution was added. The tubes were inverted 25 times to mix the solution and heated at thermomixer at 55 °C for 60 minutes. Afterwards, 4,5 µl RNase A solution was added and the tubes again were inverted 25 times and heated at 37 °C for 60 minutes, then immediately placed on ice for one minute. 300 µl protein precipitation solution was added; solutions were mixed on the vortex for 20 seconds

and centrifuged at 16000 x g for five minutes. A protein pellet was now visible in each tube. Supernatant was transferred into the 2 ml tubes filled with Isopropanol (leaving the protein pellet in the 1,5 ml tubes) and mixed up by inverting 50 times. After centrifugation at 16.000 x g for five minutes, a barely visible DNA pellet originated in each tube. Now the supernatant was discarded carefully, and the pellet was washed by adding 300 µl ethanol (70 %) and inverting the tubes several times. Then the solution was centrifuged at 16.000 x g for five minutes, the supernatant was discarded, and the now washed DNA pellet was air-dried by placing the opened 2ml tubes on a paper towel for ten minutes. Last extraction working step included adding 50 µl hydration solution into each tube and incubating them at 65 °C for 60 minutes to dissolve the DNA (tubes were mixed after 30 minutes by sniping carefully). Then the tubes were incubated overnight at 20 °C and 300 rpm (gently shaking) at thermomixer. On the next day samples (50 µl extracted DNA) were purified subsequently or stored at -20 °C.

Table 5: Applied materials for DNA extraction. Gentra Puregene tissue kit- reagents by Qiagen are marked with *.

| **Equipment** | **Reagents** |
|---|---|
| Eppendorf Thermomixer comfort | Isopropanol 99 % |
| Centrifuge (Thermo Scientific Hereaus Multifuge 8) | Ethanol 70 % |
| Vortex | Lysis buffer* |
| Styrofoam box filled with crushed ice | Proteinase K solution* |
| Metal plate | RNAse A solution* |
| Gloves | Protein precipitation solution* |
| Discovery Micropipettes and tips (1000 µl, 100 µl, 10 µl) | Hydration solution* |
| Paper towels | |
| Eppendorf tubes (1,5 ml, 2 ml) | |
| Scalpel blades | |
| Forceps | |
| Gas cartridge | |
| Wasteboxes | |
| Culture dishes (100x20 mm) Cyto one | |

### 2.6 DNA Purification

For the DNA clean-up the Quiagen Gel extraction kit was used. The kit included collection tubes, which were composed of two parts. The upper tube contains a membrane which collects DNA, the lower part picks up the discharge (Table 6).

Tubes containing 50 µl DNA extract were unfreezed and centrifuged, afterwards 150 µl QG-buffer was added to each tube and incubated at 50 °C for 10 minutes. 50 µl Isopropanol (99 %) was added, then the mixture was transferred into prepared and labelled 2 ml collection

tubes and centrifuged at 18.000 x g for 1 minute. Discharges were discarded, and the collection tubes were filled with 500 µl QG-buffer. Again, the tubes were centrifuged at 18.000 x g for 1 minute and the discharges deflated. Now the DNA in the violet parts of the collection tubes was washed by adding 750 µl PE-buffer. After 5 minutes the tubes were centrifuged at 18.000 x g for 1 minute and the discharges were deflated. To be sure that all of the PE-buffer was gone through the membrane, the tubes were centrifuged one more time. The lower part of the collection tubes could now be discarded, while the upper part was put into prepared and labelled 1,5 ml Eppendorf tubes. In the last working step, 30 µl EB-buffer was added to release the DNA from the membrane. After 4 minutes tubes were centrifuged at 18.000 x g for 1 minute. Collecting tubes were discarded and the 1,5 ml tubes containing 30 µl purified DNA were stored at -20 °C.

Table 6: Applied materials for DNA purification. Quiagen Gel extraction kit- components are marked with *.

| **Equipment** | **Reagents** |
|---|---|
| Eppendorf Thermomixer comfort | Isopropanol 99 % |
| Centrifuge (Thermo Scientific Heraeus Multifuge 8) | QG-buffer* |
| 1,5 ml Eppendorf tubes | PE-buffer* |
| 2ml collection tubes* | EB-buffer* |
| Gloves | |
| Discovery micropipettes and tips (1000 µl, 200 µl, 100 µl) | |
| Wasteboxes (one for pipette tips and one for liquid) | |

### 2.7 Measuring DNA Concentration (Quantification)

For DNA concentration measuring a NanoDrop® Spectrophotometer (Implen, Type Pearl) was used. The following setting was selected: nuclear acids, double strand DNA, lid factor 10, measurement unit ng/µl. 4 µl from each purified DNA sample were applied. The measurement was implemented three times per sample to configure a mean value. A zero adjustment was done by measure 4 µl Hydration solution (Gentra Puregene Tissue Kit (Qiagen)), before measuring the next sample.

### 2.8 Quantitative PCR

The qPCR was implemented according to Angelika Kabrt (2016), Sztatecsny and Hödl (2011) and consultation with Mag. Philipp Heher from the University of Applied Sciences Vienna.

First the samples, positive control (water samples from *Bd* positive tested *Ambystoma dumerilii* aquariums) and the internal positive control (IPC) had to be diluted with Milli-Q (Millipore corporation) water to a concentration of 12,5 ng/µl (Table 7).
Sztatecsny and Hödl (2011) used the following primer and probe sequences:

- Chytr-n-fwd: TTGATATAATACAGTGTGCCATATGTC
- Chytr-n-r: CAAGAGATCCGTTGTCAAAAGTT
- Probe: VIC-CGAGTCGAACAAA-MGB-NFQ
- IPC: 5'-TTG ATA TAA TAC AGT GTG CCA TAT GTC ACG AGT CGA ACA AAA TTT ATT TAT TTT TTC GAC AAA TTA ATT GGA AAT TGA ATA ATT TAA TTG AAA AAA ATT GAA AAT AAA TAT TAA AAA CAA CTT TTG ACA ACG GAT CTC TTG-3'

Primers and IPC were ordered from Mycrosynth, the probe from Thermofisher Scientifics.
The realtime qPCR means a duplication of DNA (denaturation, annealing, elongation) and allows a quantification of the product by measuring fluorescence. A probe (oligonucleotide) consisting of a quencher and a reporter-fluorescence colour is integrated in the DNA. When the taq-polymerase synthesizes a strand, it modifies the probe, whereby the reporter-fluorescence increases and gets measurable (Boyle et al., 2004).
For the qPCR 96 well plates were used. Reactions (29 samples and three controls per well plate) were run in triplicates at thermocycler (Stratagene mx3005p, Agilent Technologies). Reactions in each well consisted of 2 µl diluted DNA samples respectively positive control (total concentration of 25 ng/µl) and 18 µl mastermix, which was prepared for 100 wells and was compounded as follows:

- **Milli-Q-water**: 783 µl
- **TaqMan ™ Fast mix**: 1 ml
- **Primer forward**.: 6 µl
- **Primer reverse**.: 6 µl
- **Probe**: 5 µl

The total amount in each well was 20 µl. As negative control 2 µl Milli-Q-water were used instead of the DNA sample. As internal positive control a synthesized Bd-nucleotide sequence was availed. Because of the IPC purity its dilution factor was very high (1:100000000).
Well plates were covered up by a specific plastic foil to avoid contamination of the single wells. Before the reaction, the well plate was centrifuged at 1000 rpm for 3 seconds.

Then the well plate was set in the thermocycler and the following thermal profile (Fig. 15) according to Kabrt (2016) was entered:

- 5 minutes at 95 °C (segment 1, 1 cycle)
- 15 seconds at 95 °C (segment 2, 55 cycles)
- 30 seconds at 56 °C (segment 2, 55 cycles)
- 30 seconds at 60 °C (segment 2, 55 cycles)

After 2 hours the run was completed, and the results were displayed by MxPro QPCR Software.

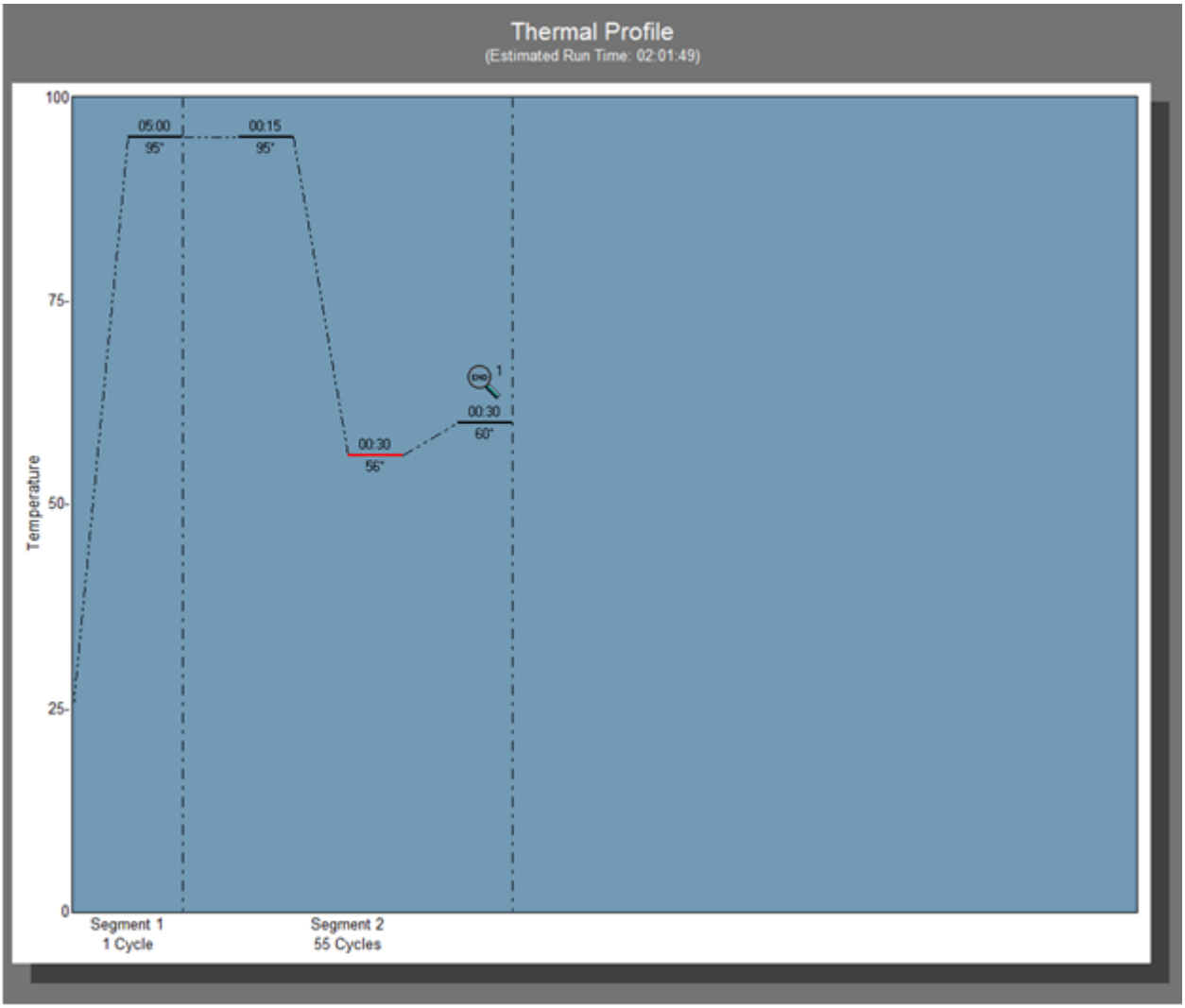

Figure 15: Thermal profile of the qPCR reactions.

Table 7: Applied materials for qPCR.

| Equipment | Reagents |
|---|---|
| Thermocycler mx3005p, Stratagene, Agilent Technologies | Milli-Q-water, Millipore |
| Discovery micropipettes and tips (1000 µl, 100 µl, 10 µl) | Primer fwd, Microsynth |
| Eppendorf Xplorer® (multichannel pipette) | Primer r, Microsynth |
| 96 well plates, Startlab | Probe, Thermofisher Scientific |
| Centrifuge Thermo Scientific Heraeus Multifuge 8 | TaqMan™ fast mix, Thermofisher Scientific |

### 2.8 Standard curve

To determine the number of *Bd* zoospores respectively the genome equivalent (GE) in the water samples, a standard curve was compiled. According to Kirshtein et.al (2007), one zoospore contains 169 copies. First, dilution series of the IPC were performed (1:10 intervals). Dilutions of 8.000, 800, 80, 8, 0,8 copies per µl were used in a qPCR. In triplicates, in each well were pipetted 2 µl of each dilution (total amount in the wells: 16.000, 1.600, 160, 16, 1,6 copies) and 18 µl of mastermix.

### 2.9 Cloning and Sequencing

To make sure, that the primers bound to the right sequence and it was *Bd* DNA indeed, the qPCR product of the positive control (*Ambystoma dumerilii* aquarium water) was manifolded by bacterial cloning (Table 8) and afterwards was send to Microsynth for sequencing. Bacterial cloning consists of A-tailing, ligation and transformation. It was necessary to work on ice.

For the A-tailing the following prescription was used:

- Taq-polymerase 10x reaction buffer: 1 µl
- $MgCl_2$: 0,6 µl
- dATP: 1 µl
- Taq-polymerase (5 units): 1 µl
- Enhancer (5x): 1 µl
- Cleaned up PCR product: 5,4 µl

The mixture was incubated for 30 minutes at 70 °C. In the meantime, 10 culture dishes were prepared for bacterial growth. Therefore, 6,28 g LB Broth with Agar (1,2 %) and 200 ml Aqua dest. were mixed and autoclaved for 90 minutes. The solution was cooled down to 60 °C and 400 µl ampicillin were added. Culture dishes were filled with the agar-gel and stored in a cling film at 4 °C.

Ligation was done after the Promega protocol as follows:

- 2x rapid ligation buffer: 5 µl
- pGEM-T easy Vector (50ng): 1 µl
- PCR product after A-tailing: 3 µl
- T4 DNA ligase (3 U/µl): 1 µl

For negative control the PCR product was excluded. The solution was incubated over night at 4 °C. For transformation 3 culture dishes and SOC-medium were prepared and warmed in the incubator. 40 µl IPTG and 80 µl x-Gal were pipetted on the culture dishes and greased by a glass stick. Then the culture dishes were dried in the incubator. 50 µl of bacterial suspension (NEB®5) were mixed with 2 µl ligation reaction and stored 30 minutes on ice. Then the solution was heat-shocked for 45 seconds at 42 °C in the thermomixer and put on ice immediately for 5 minutes. 950 µl SOC-medium were added and incubated for 90 minutes at 37 °C and 300 rpm. Respectively 100 µl of the solution was greased on the culture dishes and they were incubated for 22 hours at 37 °C ad 300 rpm. Then the culture dishes were closed-up with parafilm and stored for one day at room temperature until a blue-white colony was visible.

The white bacterial colonies were picked up by a pipette tip carefully without gel, added to a PCR tube with 50 µl Aqua dest. and stored at 4 °C in the fridge.

Now 10 glass tubes were filled with 4 ml LB-Broth consisting of 200 ml Aqua dest., 4 g LB-Broth and 400 µl ampicillin. Then 20 µl of the bacterial colony suspension was added to each glass tube and they were incubated over night at 37 °C and 300 rpm.

A colony PCR was implemented by the following working steps:

First a mastermix was prepared:

- Yield buffer: 25 µl
- $MgCl_2$: 15 µl
- Enhancer P: 50 µl
- dNTP: 5 µl
- Primer forward.: 2,5 µl
- Primer reverse.: 2,5 µl
- Taq-polymerase: 1,2 µl

The total volume was 101,25 µl, it was stocked up with 147,25 µl Aqua dest. to 248,5 µl. Each PCR tube was filled with 23,5 µl mastermix and 1,5 µl bacterial suspension from the glass tubes.

Thermal profile of the colony PCR:

- 5 minutes at 95 °C
- 45 seconds at 95 °C (28 cycles)
- 45 seconds at 56 °C (28 cycles)
- 1 minute at 72 °C (28 cycles)
- Hold at 4 °C

A gel electrophoresis was carried out to determine which of the ten samples should be sent to sequencing. Therefore, a 1,5 % gel was prepared by mixing 50 ml SB-buffer with 0,72 g gel powder (1,2 %, LB universal). It was heated in the microwave and after cooling down, 5 µl gel green were added. In each gel pocket were filled 20 µl colony PCR product and 4 µl loading dye. Two extra pockets were filled with 5 µl 500 bp (SM0643, Thermo Scientific) and the other with 5 µl 100 bp (N3231S, Thermo Scientific) marker.
Only 4 samples (number 3,4,7,10) showed usable results (C600 Azur Biosystems) (see Appendix A, Fig. A4) and were send after a miniprep to Mycrosynth for sequencing.

Table 8: Applied materials for bacterial cloning.

| **Equipment** | **Reagents** |
|---|---|
| Eppendorf Mastercycler (ep Gradient s) | NEB®5-alpha competent e.coli |
| C600 Azur Biosystems | Taq-Polymerase 10xreaction buffer |
| Culture dishes (Cyto one 100x20mm) | $MgCl_2$ |
| Discovery comfort Micropipettes | dATP |
| Greiner bio-one filter tips | Taq-Polymerase 5units |
| Glass stick | Enhancer |
| Eppendorf tubes (1,5 ml, 2 ml) | 2x rapid ligation buffer |
| PCR tubes (0,2 ml) | pGEM-T easy vector |
| Incubator | T4 DNA Ligase |
| Ice | Yield buffer |
| | dNTP |
| | Primer forw., primer r. |
| | SB buffer |
| | IPTG |
| | x-Gal |
| | LB-Broth |
| | Ampicillin |
| | LB universal |
| | SOC-medium |
| | 500 bp marker SM0643, 100 bp marker N3231S (Thermo Scientific) |

# 3 Results

## 3.1 Swabs

All swab samples (Table 9) were tested *Bd* negative. Detailed result tables with all sample sites, swab numbers and dates can be found in Appendix A (Table A1).

Table 9: Result of total species found at all ten sampling sites.

| Species | Number of Swabs | Results |
|---|---|---|
| *Bufo bufo* | 9 | Negative |
| *Bufotes viridis* | 1 | Negative |
| *Rana dalmatina* | 4 | Negative |
| *Pelophylax sp.* | 18 | Negative |
| *Salamandra salamandra salamandra* | 2 | Negative |
| *Lissotriton vulgaris* | 5 | Negative |
| *Ichthyosaura alpestris* | 5 | Negative |
| *Triturus carnifex* | 6 | Negative |
| **Total swab number** | 50 | |

## 3.2. QPCR and Standard curve

All water samples were tested *Bd* negative, which is in accordance with the skin swab results. Figure 16 to 19 show amplification plots of qPCRs. In each reaction (Fig. 16–19) the positive control (*Ambystoma dumerilii* samples) increased over the threshold (horizontal green line) after 22–27 cycles (Ct-value= 22–27). The Ct-value indicates after how many PCR cycles the fluorescence curve of a sample exceeds the threshold. Some field samples (Fig. 16,18,19) and the negative control pass the threshold after 35 cycles, what can be interpreted as negative result (see Fig. 21 standard curve). Detailed result figures including the measured DNA concentrations, dilutions and Ct-values can be found in Appendix A (Fig. A1–A3).

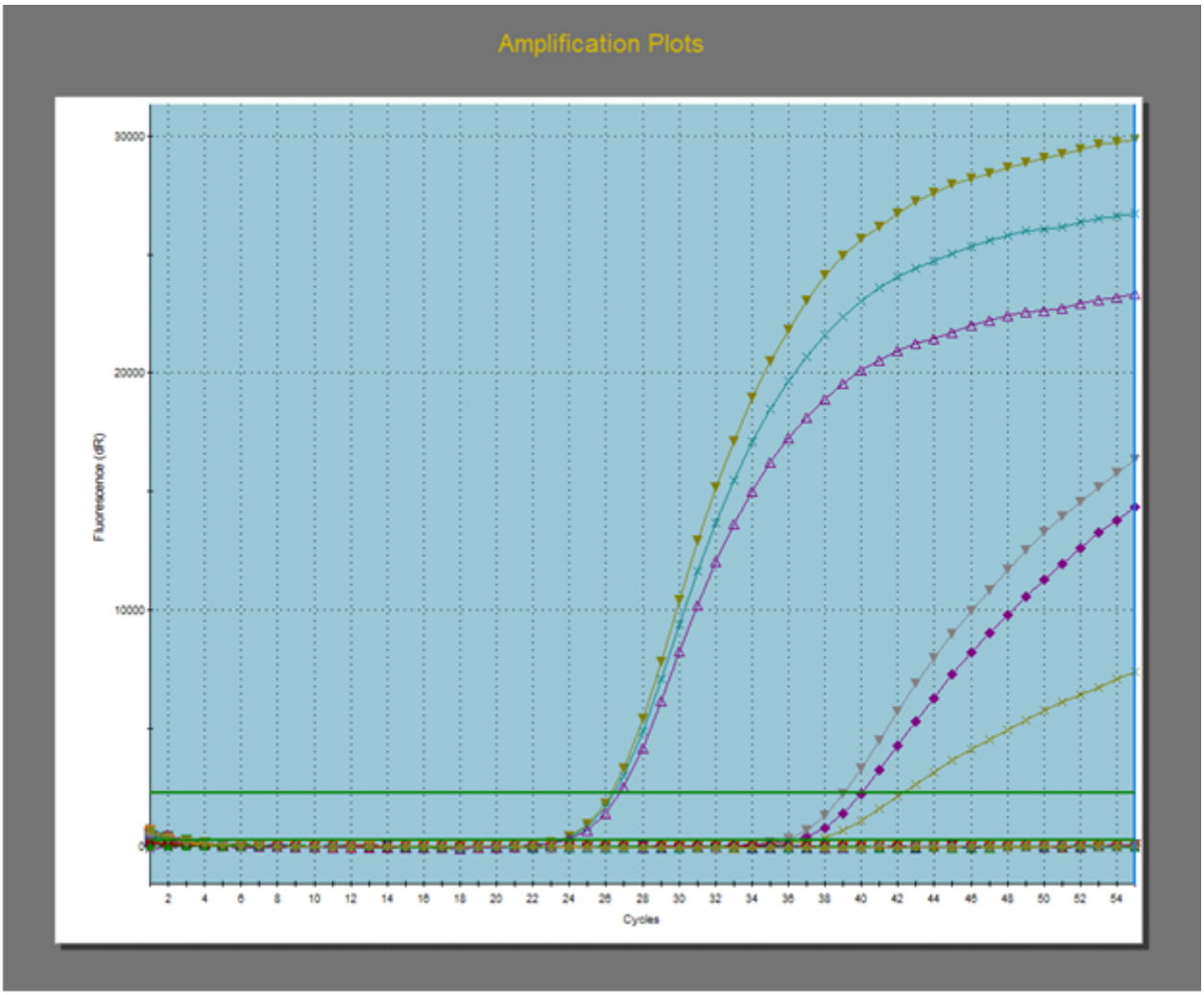


Figure 16: QPCR from 5.9.2017. Amplitudes describe DNA replications (each sample amplitude has another colour and symbol). The first three curves show the triplicate of the positive control (each of the three wells). Curves of positive control exceed the threshold after 25–27 cycles (Ct-value= 25–27). Lines under the threshold (= No Ct) are field samples and negative control. Later curve deflections (Ct-value >35) are insignificant (see standard curve).

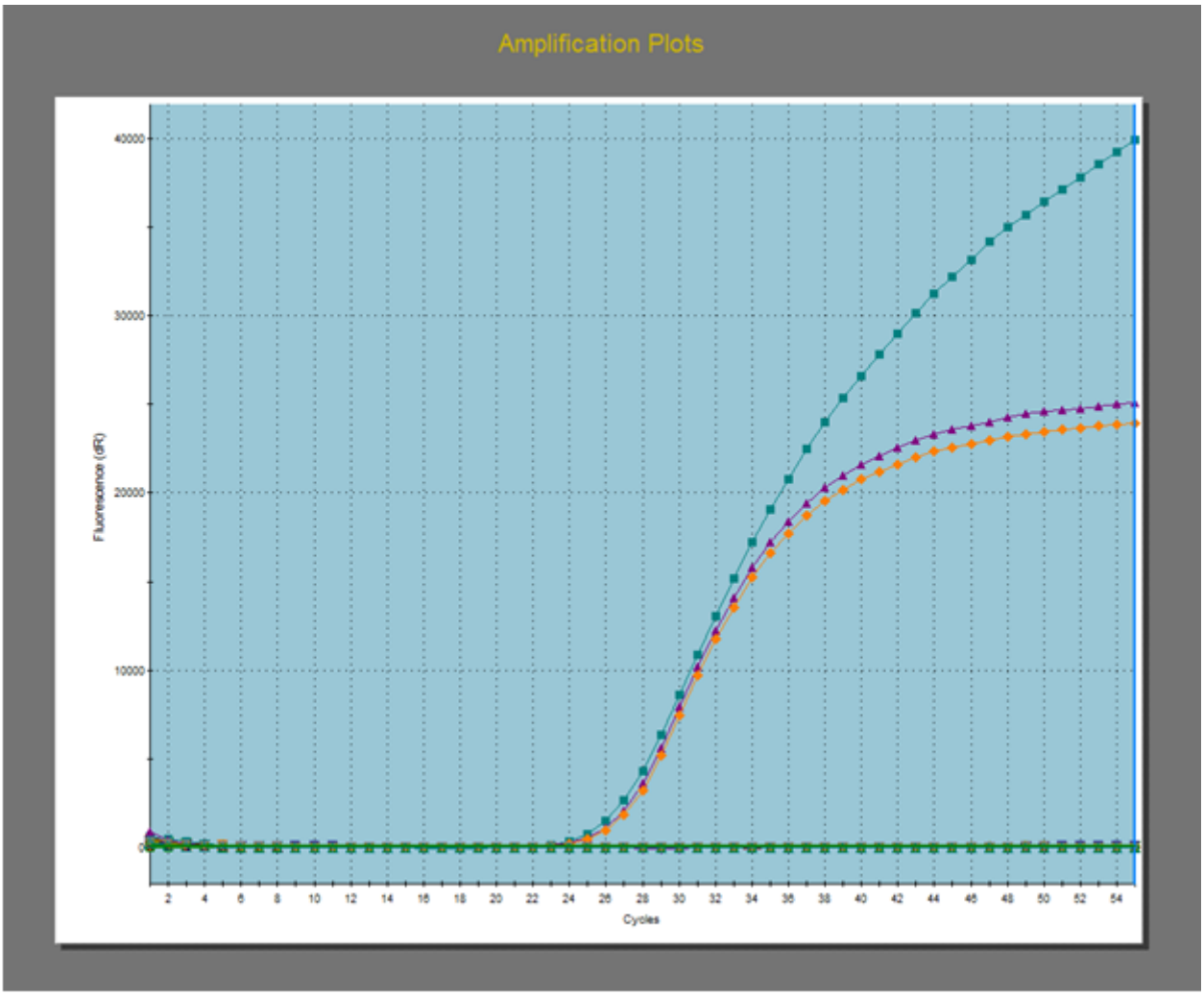

Figure 17: QPCR from 22.9.2017. Amplitudes of the positive control (three curves of the triplicate) pass the threshold after 24 cycles. All other samples remain under the threshold (each sample amplitude has another colour and symbol).

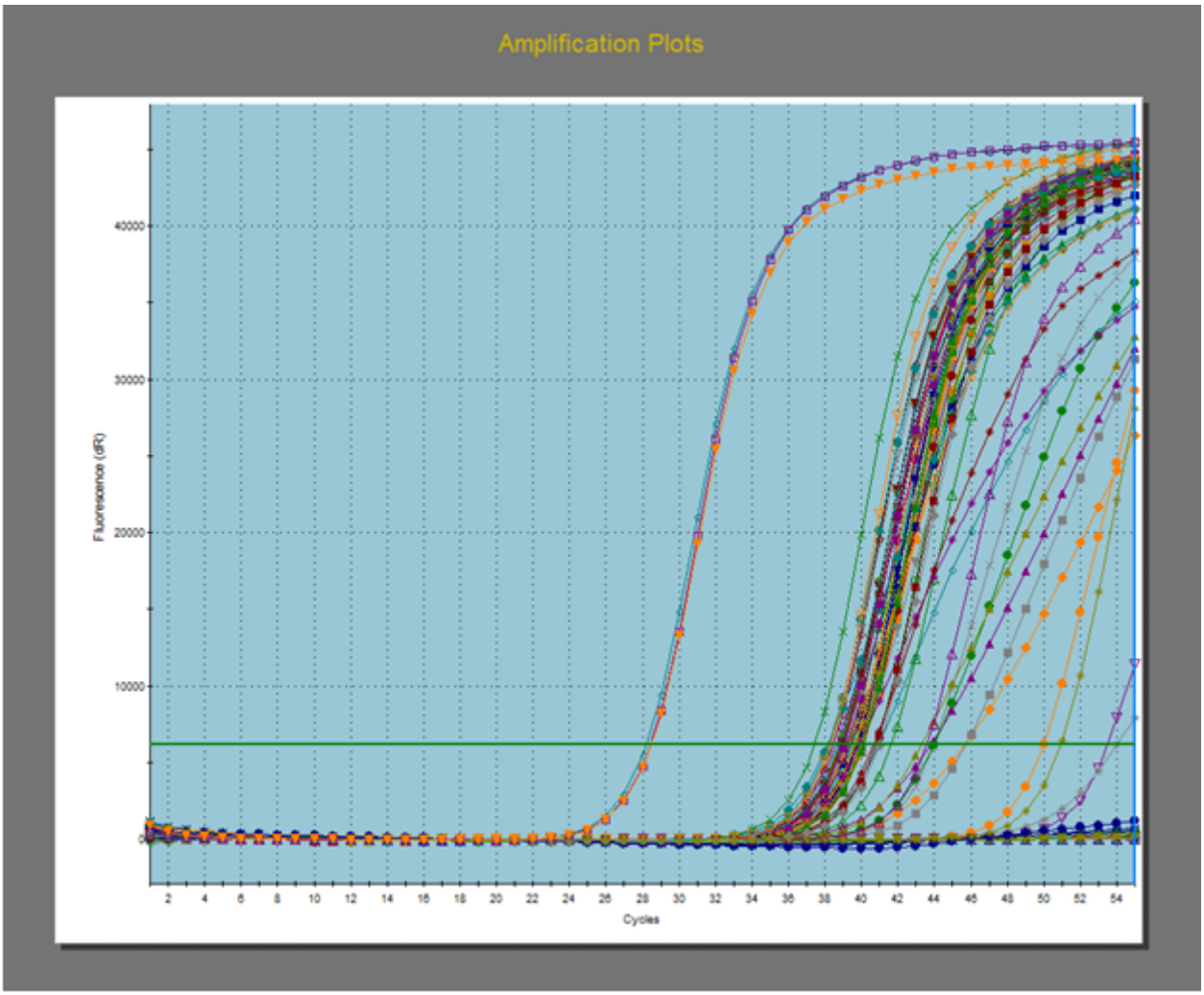

Figure 18: QPCR from 9.5.2018. Amplitudes of field samples increase after 36 cycles (each sample amplitude has another colour and symbol). Positive control passes the threshold after 26 cycles (first three curves of the triplicate).

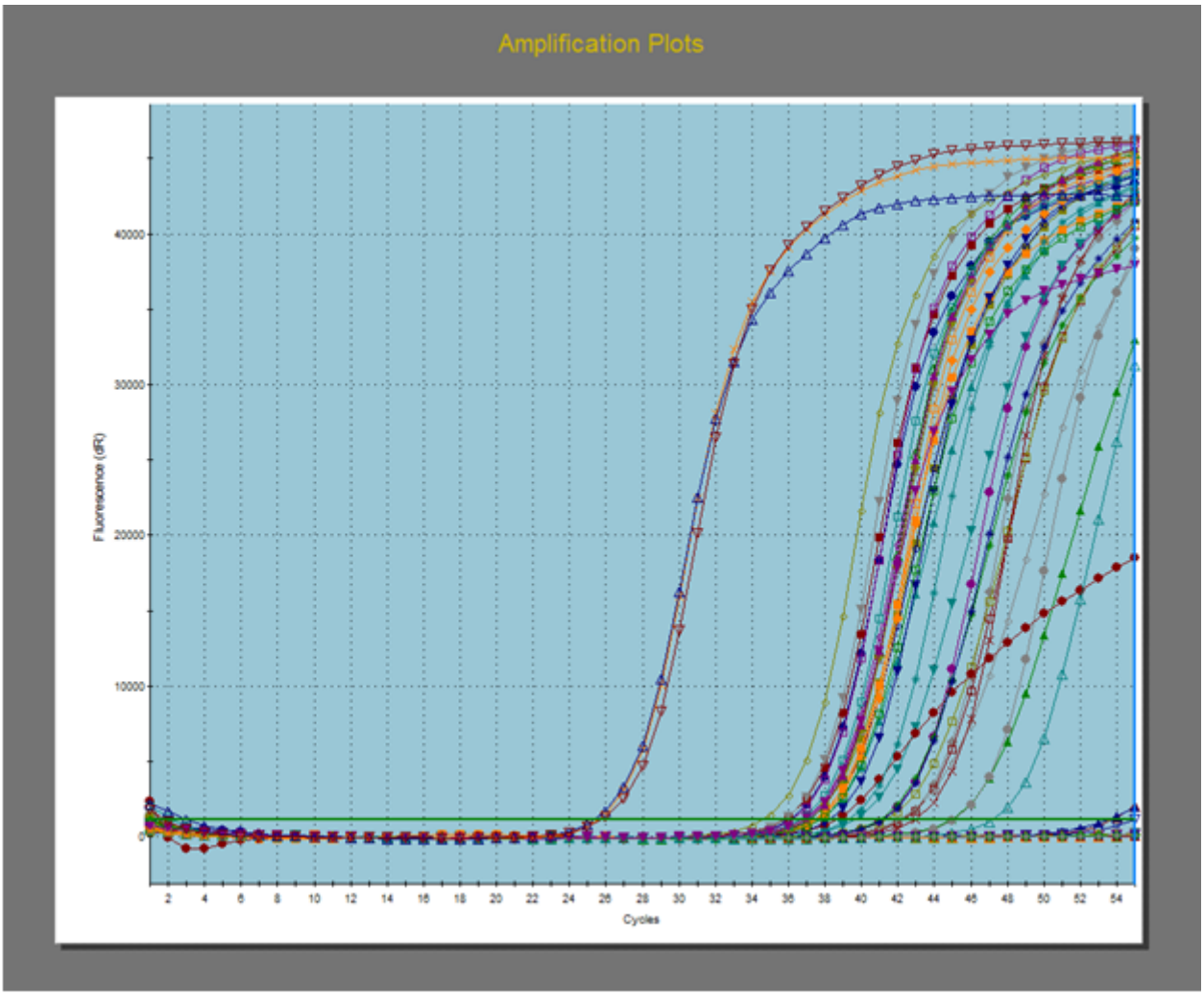

Figure 19: QPCR from 11.5.2018. Positive control passes the threshold after 24 cycles (first three curves of the triplicate). Field samples pass the threshold after 35 cycles (each sample amplitude has another colour and symbol).

**Standard curve**

The amplitudes of the dilution series (Fig. 20) increased at 26 cycles (≙ 16.000 copies), at 31 cycles (≙ 1600 copies), at 35 cycles (≙ 160 copies), at 38 cycles (≙ 16 copies) and at 40 cycles (≙ 1,6 copies). One *Bd* zoospore is one genome equivalent (GE), what corresponds to 169 copies. Therefore, a threshold exceedance at or after 35 cycles (≙ 160 copies and fewer) signifies that the sample is *Bd*-negative (Fig. 21).

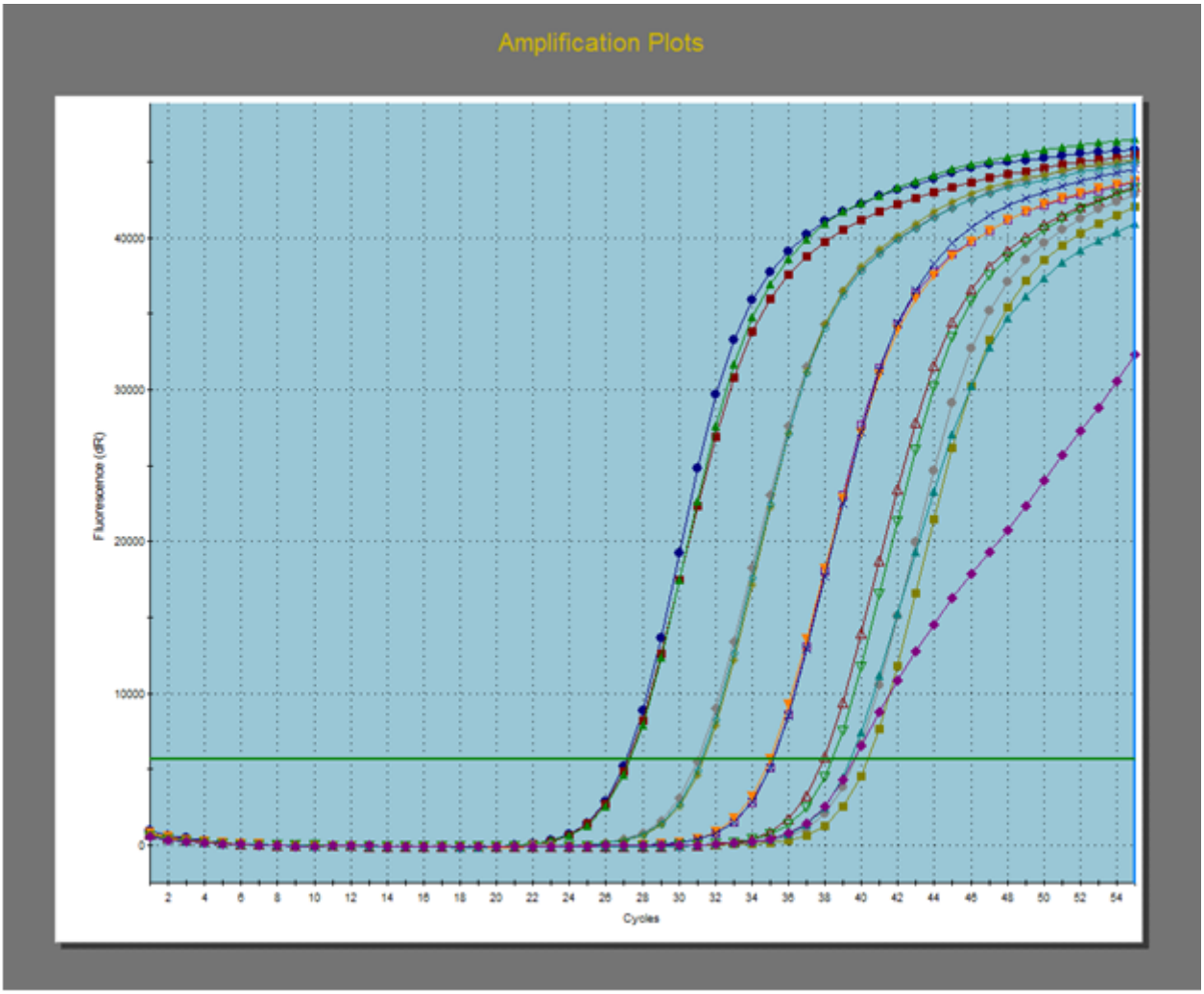


Figure 20: Amplitudes of dilution series for the standard curve. From left to right the dilution increases and with it the Ct-value; the genome equivalent decreases.

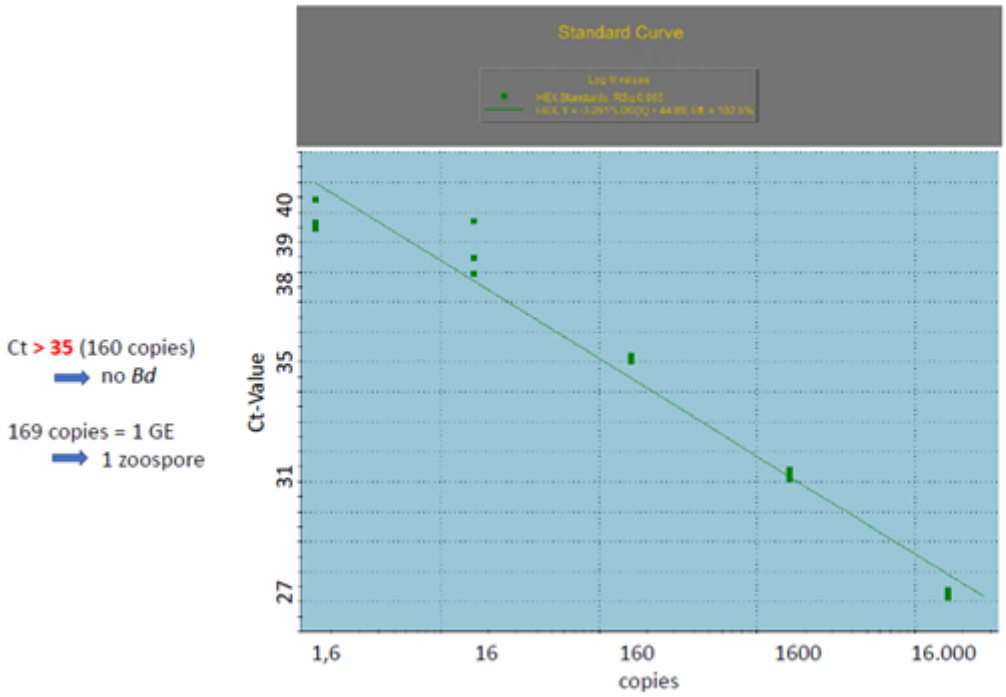


Figure 21: Standard curve. A threshold exceedance after >35 cycles ≙160 copies and signifies the sample is *Bd* negative. 169 copies ≙ one genome equivalent (GE) or 1 *Bd*-zoospore. 16.000 copies correspond to 100 GE, 1600 copies to 10 GE, 160, 16 and 1,6 comply with 0 GE.

### 3.3 Sequencing

Results of the sequencing were evaluated by the free ApE-software and then blasted in the NCBI database, where a match could be found (Fig. 22). Select sequence gene bank |KX115405.1|, *Batrachochytrium dendrobatidis* isolate L820609 internal transcribed spacer 1 and 5.8S ribosomal RNA gene, partial sequence. Primers indeed bound to the right sequence.

Ttagttgttggggtggatgggagttttattgatgtgtaaatgttgatggaatgacccattgtttttttcaaaaaaca
ccttgatataatacagtgtgccatatgtcacgagtcgaacaaaatttatttatttttcgacaaattaattggaaa
ttgaatgattttaatttaattgaaaaaaattgaaaataaatattaaaaacaactttgacaacggatctcttggct
ctcgcaacgatgaagaacgcagcgaaatgcgatacgtaatgtgaattgcaaacctttgtgaatcattaaatcttt
gaacgcacattgcactcgtaaaagagtatacatgtttgagaattataaaaatacattgtccgaattgactggaca
gatatgaaccatgtcaaaattgtttgacaatttgacaggttttaaaagtagtagtaaatgagtgatacaaaaagt
agtgggtctaaacaaccccgtccatcacaccataca

Figure 22: Green marked parts describe the primers (primer fwd. 27 bp, primer r. 23 bp), which were used by Sztatecsny and Hödl (2011). Blooi et al. (2013) used longer primer sequences (blue marked). *Bd* sequence is marked grey.

# 4 Discussion

In the present study, *Bd* was neither detected in the ten examined Viennese water bodies using eDNA, nor in the 50 amphibian skin swabs. The parallel study by Vesely (2019) in Eastern Vienna has obtained some positive results. Five of 95 water samples were positive for *Bd*, while only two of 50 animals were identified as infected. Despite that difference, predominantly parallels of the results of these two surveys are more important. Vesely had six negative tested sampling sites; for example, at the Ölhafen Lobau, where Sztatecsny and Glaser (2011) tested many fire-bellied toads *Bd*-positive, Vesely had only negative results. This fact speaks for the reliability of the negative results in this study.

Of course, negative detections do not necessarily indicate the actual absence of the pathogen. Rather, they could be due to temporarily low prevalence, local absence, sampling artifacts, or methodological limitations (Bletz et al., 2014). *Batrachochytrium dendrobatidis* depends strongly on environmental factors such as temperature, humidity, pH value, UV exposure, and anthropogenic influences (Piotrowski et al., 2004). Limitations also need to be considered regarding the detection methods. While the eDNA method is considered sensitive and non-invasive, its reliability can be limited by factors such as DNA degradation, dilution effects, or inhibitors in the water (Goldberg et al., 2016; Mosher et al., 2017). Particularly in shallow or flowing urban waters, eDNA can rapidly degrade or be removed by sedimentation. False negative results can also occur in skin swabs, for example, if the infection burden is below the detection limit or if the sample was not collected optimally (Shin et al.,2014).

Additionally, the presence of planktonic predators in water bodies in Vienna was investigated by Vesely in 2017. The finding that at least one of the zooplankton families that can potentially feed on free *Bd* zoospores was present in eight of ten ponds is significant. If plankton are sufficiently numerous and active, they may reduce zoospore concentrations in the water before they can be detected by eDNA filtration or skin swabs. Even if *Bd* was present, the free zoospore phase could be so suppressed that detection methods fail or only rarely detect positive samples (Vesely, 2019).

The fact that both eDNA and skin swab results were negative indicates a low or non-existent prevalence of *Bd* in these tested locations, but this should not be interpreted as a general all clear for Vienna. Although no infections were detectable at the time of the study, *Bd* remains a potential threat, especially given its global spread and its known role in causing massive amphibian declines (Scheele et al., 2019; Fisher and Garner, 2020). Regular and long-term monitoring is essential, especially in urban areas like Vienna, which are characterized by high human impact, fragmented habitats and climatic changes.

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**Magistratsabteilung 45** – Wiener Gewässer; „Sima/Bischof: Neue Wasserleitung versorgt Pappelteich in Wien-Liesing mit Frischwasser“, Presse-Aussendung der Stadt Wien, 08. Juni 2022

# Appendix A

Water sampling protocols, tables of samples, result skin swab, result gel electrophoresis (UV)

## Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Wienerberg Teich | Protocol number | 01 |
| Name | Dekrout, Vesely, Disacke | Start | 9:45 |
| Date | 2.5.2017 | End | 10:15 |
| Atmospheric conditions | cloudy | Air temperature in °C | 17 |
| Atmospheric pressure in hPa | 1010 | Weather previous day | Rain, wind |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Reed, close to nature, meadow at the shore | | |
| Species | tadpoles of common toad (Bufo bufo), water frogs, fishes, ducks, | | |
| Size in m² | 161000 | Colour | clear ☐ |
| Depth in m | 4-5 | | turbid ☐ |
| Water temperature °C | 13,5 | Odour | normal ☐ |
| Oxygen content mg/L | 10,9 | | must ☐ |
| pH-value | 8,1 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 1/1/A-H |

Notes

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Wienerberg Teich | Protocol number | 01 |
| Name | Bettina, Denise | Start | 17:00 |
| Date | 16.5.2017 | End | 17:15 |
| Atmospheric conditions | sun, few clouds | Air temperature in °C | 20 |
| Atmospheric pressure in hPa | 1020 | Weather previous day | sun |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | same | | |
| Species | many people, fishes, water frogs | | |
| Size in m² | 161000 | Colour | clear ☐ |
| Depth in m | 4-5 | | turbid ☐ |
| Water temperature °C | 20,7 | Odour | normal ☐ |
| Oxygen content mg/L | 10,37 | | must ☐ |
| pH-value | 8,17 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 1/2/A-C |

| Notes |
|---|
| |

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Wienerberg Teich | Protocol number | 01 |
| Name | Denise | Start | 16:20 |
| Date | 18.6.2017 | End | 17:35 |
| Atmospheric conditions | sun, few clouds, warm | Air temperature in °C | 23 |
| Atmospheric pressure in hPa | 1023 | Weather previous day | cloudy, rain |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | same | | |
| Species | Fishers, rat, water frogs, fishes, swamp chicken | | |
| Size in m² | 161000 | Colour | clear ☐ |
| Depth in m | 4-5 | | turbid ☐ |
| Water temperature °C | 24 | Odour | normal ☐ |
| Oxygen content mg/L | 8,76 | | must ☐ |
| pH-value | 8,3 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 1/3/A-C |

| Notes |
|---|
| |

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Silbersee | Protocol number | 02 |
| Name | Denise, Bettina | Start | 12:11 |
| Date | 3.5.2017 | End | 12:45 |
| Atmospheric conditions | cloudy | Air temperature in °C | 17 |
| Atmospheric pressure in hPa | 1019 | Weather previous day | cloudy |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Reed, natural pond, algae | | |
| Species | Mallards, tadpoles from common toads, leap frog | | |
| Size in m² | 300 | Colour | clear ☐ |
| Depth in m | 4 | | turbid ☐ |
| Water temperature °C | 14,1 | Odour | normal ☐ |
| Oxygen content mg/L | 7,73 | | must ☐ |
| pH-value | 7,03 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 2/1/A-C |

| Notes |
|---|
| |

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Silbersee | Protocol number | 02 |
| Name | Denise, Susi | Start | 10:50 |
| Date | 18.5.2017 | End | 11:15 |
| Atmospheric conditions | Sun, warm, dry | Air temperature in °C | 22 |
| Atmospheric pressure in hPa | 1026 | Weather previous day | Sun, warm, cloudy |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Reed, natural pond, algae, broadleaf forest arround, dogs swimming in water | | |
| Species | many tadpoles | | |
| Size in m² | 300 | Colour | clear ☐ |
| Depth in m | 4 | | turbid ☐ |
| Water temperature °C | 17,7 | Odour | normal ☐ |
| Oxygen content mg/L | 3,65 | | must ☐ |
| pH-value | 7,1 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 2/2/A-D |

| Notes |
|---|
| |

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Silbersee | Protocol number | 02 |
| Name | Denise | Start | 19:25 |
| Date | 22.6.2017 | End | 19:40 |
| Atmospheric conditions | Sun, hot | Air temperature in °C | 29,9 |
| Atmospheric pressure in hPa | 1011,4 | Weather previous day | Sun, hot |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Reed, natural pond, algae, broadleaf forest arround, dogs swimming in water | | |
| Species | many tadpoles | | |
| Size in m² | 300 | Colour | clear ☐ |
| Depth in m | 4 | | turbid ☐ |
| Water temperature °C | 25,9 | Odour | normal ☐ |
| Oxygen content mg/L | 7,37 | | must ☐ |
| pH-value | 8,2 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 2/3/A-B |

| Notes |
|---|
| |

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Pappelteich | Protocol number | 03 |
| Name | Denise | Start | 9:20 |
| Date | 26.4.2017 | End | 9:40 |
| Atmospheric conditions | Cloudy, cool | Air temperature in °C | 13 |
| Atmospheric pressure in hPa | 1019 | Weather previous day | Warm, sun |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Artificial pond, basin made of concrete | | |
| Species | Tadpoles of common toad, gras frog (sounds), goldfish | | |
| Size in m² | 40 | Colour | clear ☐ |
| Depth in m | 1-3 | | turbid ☐ |
| Water temperature °C | 13,2 | Odour | normal ☐ |
| Oxygen content mg/L | 7,73 | | must ☐ |
| pH-value | 7,88 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 3/1/A-D |

| Notes |
|---|
| |

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Pappelteich | Protocol number | 03 |
| Name | Denise, Bettina | Start | 9:50 |
| Date | 17.5.2017 | End | 10:24 |
| Atmospheric conditions | Sun, some clouds | Air temperature in °C | 23 |
| Atmospheric pressure in hPa | 1026 | Weather previous day | sun |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Artificial pond, basin made of concrete | | |
| Species | Alpine newts, common newts, water frogs | | |
| Size in m² | 40 | Colour | clear ☐ |
| Depth in m | 1-3 | | turbid ☐ |
| Water temperature °C | 18,3 | Odour | normal ☐ |
| Oxygen content mg/L | 14,14 | | must ☐ |
| pH-value | 7,92 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 3/2/A-D |

| Notes |
|---|
| |

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Pappelteich | Protocol number | 03 |
| Name | Denise | Start | 11:40 |
| Date | 18.6,2017 | End | 12:00 |
| Atmospheric conditions | Cloudy, warm, wind | Air temperature in °C | 22 |
| Atmospheric pressure in hPa | 1023 | Weather previous day | Rain, cloudy |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Artificial pond, basin made of concrete, more algae | | |
| Species | common newts, water frogs | | |
| Size in m² | 40 | Colour | clear ☐ |
| Depth in m | 1-3 | | turbid ☐ |
| Water temperature °C | 20,6 | Odour | normal ☐ |
| Oxygen content mg/L | 18 | | must ☐ |
| pH-value | 7,9 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 3/3/A-E |

| Notes |
|---|
| |

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Afritschteich | Protocol number | 04 |
| Name | Denise, Bettina | Start | 14:00 |
| Date | 2.5.2017 | End | 14:35 |
| Atmospheric conditions | Cloudy | Air temperature in °C | 16 |
| Atmospheric pressure in hPa | 1017 | Weather previous day | cloudy |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Artificial pond, swimming pool, mixed forest, near a settlement zone | | |
| Species | common newts, warty newt (larvae and adults), common toad, many watersnails | | |
| Size in m² | 30 | Colour | clear ☐ |
| Depth in m | 1-2 | | turbid ☐ |
| Water temperature °C | 16,1 | Odour | normal ☐ |
| Oxygen content mg/L | 9,4 | | must ☐ |
| pH-value | 7,33 | | different ☐ |

Fotodocumentation (including points of sampling)

## Samples

| Sample identification | |
|---|---|
| | 4/1/A-D |

Notes

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Afritschteich | Protocol number | 04 |
| Name | Denise, Bettina | Start | 13:45 |
| Date | 17.5.2017 | End | 15:35 |
| Atmospheric conditions | sun | Air temperature in °C | 22 |
| Atmospheric pressure in hPa | 1026 | Weather previous day | Sun, cloudy |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Artificial pond, swimming pool, mixed forest, near a settlement zone | | |
| Species | Water frogs, fishes, many watersnails, fishes, blindworm (dead) | | |
| Size in m² | 30 | Colour | clear ☐ |
| Depth in m | 1-2 | | turbid ☐ |
| Water temperature °C | 22,1 | Odour | normal ☐ |
| Oxygen content mg/L | 17,47 | | must ☐ |
| pH-value | 7,63 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 4/2/A-B |

| Notes |
|---|
| |

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Afritschteich | Protocol number | 04 |
| Name | Katharina, Denise | Start | 13:30 |
| Date | 17.6.2017 | End | 13:45 |
| Atmospheric conditions | Cloudy, wind | Air temperature in °C | 20 |
| Atmospheric pressure in hPa | 1019 | Weather previous day | Sun, warm |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Artificial pond, swimming pool, mixed forest, near a settlement zone | | |
| Species | Water frogs, fishes, many watersnails, warty newt | | |
| Size in m² | 30 | Colour | clear ☐ |
| Depth in m | 1-2 | | turbid ☐ |
| Water temperature °C | 25,7 | Odour | normal ☐ |
| Oxygen content mg/L | 8,08 | | must ☐ |
| pH-value | 8,1 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 4/3/A-B |

| Notes |
|---|
| |

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Bombentrichter 1 | Protocol number | 05 |
| Name | Denise | Start | 19:00 |
| Date | 9.5.2017 | End | 19:15 |
| Atmospheric conditions | Rain, cloudy | Air temperature in °C | 9 |
| Atmospheric pressure in hPa | 1015 | Weather previous day | Sun |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Mixed forest arround, natural pond, bomb crater | | |
| Species | Many larvae of fire salamander (Salamandra salamandra) | | |
| Size in m² | 16 | Colour | clear ☐ |
| Depth in m | 0,5 | | turbid ☐ |
| Water temperature °C | 9,6 | Odour | normal ☐ |
| Oxygen content mg/L | 1,06 | | must ☐ |
| pH-value | 7,01 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 5/1/A-C |

| Notes |
|---|
| |

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Bombentrichter 1 | Protocol number | 05 |
| Name | Denise, Nasim | Start | 10:30 |
| Date | 19.5.2017 | End | 10:45 |
| Atmospheric conditions | Sun, wind | Air temperature in °C | 22 |
| Atmospheric pressure in hPa | 1012 | Weather previous day | Sun |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Mixed forest arround, natural pond, bomb crater, water is sinking! | | |
| Species | Many larvae of fire salamander (Salamandra salamandra) | | |
| Size in m² | 16 | Colour | clear ☐ |
| Depth in m | 0,5 | | turbid ☐ |
| Water temperature °C | 16,5 | Odour | normal ☐ |
| Oxygen content mg/L | 2,6 | | must ☐ |
| pH-value | 6,9 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 5/2/A-F |

| Notes |
|---|
| |

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Parapluie Teich | Protocol number | 05 |
| Name | Denise | Start | 18:00 |
| Date | 22.6.2017 | End | 18:20 |
| Atmospheric conditions | Sun, hot | Air temperature in °C | 32 |
| Atmospheric pressure in hPa | 1011,4 | Weather previous day | Sun, hot |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Mixed forest arround, natural pond in a park | | |
| Species | None visible | | |
| Size in m² | 80 | Colour | clear ☐ |
| Depth in m | 2-3 | | turbid ☐ |
| Water temperature °C | 24,7 | Odour | normal ☐ |
| Oxygen content mg/L | 11,76 | | must ☐ |
| pH-value | 8,2 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 5/3/A-D |

Notes: Bombentrichter 1 dried out

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Sieveringer Wehr | Protocol number | 06 |
| Name | Denise | Start | 16:55 |
| Date | 6.5.2017 | End | 17:20 |
| Atmospheric conditions | Sun | Air temperature in °C | 21 |
| Atmospheric pressure in hPa | 1011 | Weather previous day | changeable |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Natural pond near a small dam, less reed, mixed forest | | |
| Species | Fire salamander larvae, water frogs, leap frog | | |
| Size in m² | 30 | Colour | clear ☐ |
| Depth in m | 1,5 | | turbid ☐ |
| Water temperature °C | 17,6 | Odour | normal ☐ |
| Oxygen content mg/L | 7,59 | | must ☐ |
| pH-value | 7,64 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 6/1/A-B |

Notes: beware of sinking in

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Sieveringer Wehr | Protocol number | 06 |
| Name | Denise | Start | 19:45 |
| Date | 20.5.2017 | End | 19:55 |
| Atmospheric conditions | Cloudy, wind | Air temperature in °C | 18 |
| Atmospheric pressure in hPa | 1019 | Weather previous day | Sun, wind |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Natural pond near a small dam, less reed, mixed forest | | |
| Species | Fire salamander larvae, water frogs, common newts | | |
| Size in m² | 30 | Colour | clear ☐ |
| Depth in m | 1,5 | | turbid ☐ |
| Water temperature °C | 14,5 | Odour | normal ☐ |
| Oxygen content mg/L | 8,10 | | must ☐ |
| pH-value | 7,48 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 6/2/A-B |

Notes: beware of sinking in

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Sieveringer Wehr | Protocol number | 06 |
| Name | Denise | Start | 16:00 |
| Date | 19.6.2017 | End | 16:15 |
| Atmospheric conditions | Sun, hot | Air temperature in °C | 29 |
| Atmospheric pressure in hPa | 1021 | Weather previous day | Wind, warm, cloudy |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Less water than previous times | | |
| Species | Fire salamander larvae, water frogs, common newts | | |
| Size in m² | 30 | Colour | clear ☐ |
| Depth in m | 1,5 | | turbid ☐ |
| Water temperature °C | 22,9 | Odour | normal ☐ |
| Oxygen content mg/L | 3,6 | | must ☐ |
| pH-value | 7,8 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 6/3/A-B |

Notes: beware of sinking in

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Pötzleinsdorfer Schlosspark | Protocol number | 07 |
| Name | Denise, Nasim | Start | 9:45 |
| Date | 7.5.2017 | End | 10:00 |
| Atmospheric conditions | cloudy | Air temperature in °C | 16 |
| Atmospheric pressure in hPa | 1008 | Weather previous day | sun |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Natural pond in a park | | |
| Species | Common newt, tadpoles of common toads | | |
| Size in m² | 200 | Colour | clear ☐ |
| Depth in m | 2 | | turbid ☐ |
| Water temperature °C | 15,5 | Odour | normal ☐ |
| Oxygen content mg/L | 4,95 | | must ☐ |
| pH-value | 7,11 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 7/1/A-B |

Notes:

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Pötzleinsdorfer Schlosspark | Protocol number | 07 |
| Name | Denise, Nasim | Start | 12:40 |
| Date | 19.5.2017 | End | 12:50 |
| Atmospheric conditions | Sun, wind | Air temperature in °C | 25 |
| Atmospheric pressure in hPa | 1011 | Weather previous day | sun |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Natural pond in a park | | |
| Species | Common newt, tadpoles of common toads leeches, ducks | | |
| Size in m² | 200 | Colour | clear ☐ |
| Depth in m | 2 | | turbid ☐ |
| Water temperature °C | 21,7 | Odour | normal ☐ |
| Oxygen content mg/L | 1,53 | | must ☐ |
| pH-value | 7,23 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 7/2/A-B |

Notes:

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Pötzleinsdorfer Schlosspark | Protocol number | 07 |
| Name | Denise | Start | 19:10 |
| Date | 19.6.2017 | End | 19:30 |
| Atmospheric conditions | Sun | Air temperature in °C | 28 |
| Atmospheric pressure in hPa | 1020 | Weather previous day | Cloudy, wind, warm |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Natural pond in a park, juvenile leap frogs, tadpoles, deer | | |
| Species | Common newt, tadpoles of common toads leeches, ducks | | |
| Size in m² | 200 | Colour | clear ☐ |
| Depth in m | 2 | | turbid ☐ |
| Water temperature °C | 24 | Odour | normal ☐ |
| Oxygen content mg/L | 4,45 | | must ☐ |
| pH-value | 8,2 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 7/3/A-C |

Notes:

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Alpenteich | Protocol number | 08 |
| Name | Bettina, Denise, Katharina | Start | 15:30 |
| Date | 3.5.2017 | End | 15:40 |
| Atmospheric conditions | Rain, cloudy | Air temperature in °C | 15 |
| Atmospheric pressure in hPa | 1015 | Weather previous day | Cloudy, wind, warm |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Artificial pond in the zoo of vienna | | |
| Species | fishes | | |
| Size in m² | 15 | Colour | clear ☐ |
| Depth in m | 2-3 | | turbid ☐ |
| Water temperature °C | 14,1 | Odour | normal ☐ |
| Oxygen content mg/L | 8,24 | | must ☐ |
| pH-value | 6,98 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 8/1/A |

Notes:

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Alpenteich | Protocol number | 08 |
| Name | Denise | Start | 12:45 |
| Date | 20.5.2017 | End | 12:55 |
| Atmospheric conditions | Wind, many clouds | Air temperature in °C | 18 |
| Atmospheric pressure in hPa | 1018 | Weather previous day | Sun, warm |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Artificial pond in the zoo of vienna | | |
| Species | Fishes, water frog | | |
| Size in m² | 15 | Colour | clear ☐ |
| Depth in m | 2-3 | | turbid ☐ |
| Water temperature °C | 18,3 | Odour | normal ☐ |
| Oxygen content mg/L | 7,92 | | must ☐ |
| pH-value | 7,21 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 8/2/A-B |

Notes:

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Alpenteich | Protocol number | 08 |
| Name | Denise, Katharina | Start | 16:50 |
| Date | 21.6.2017 | End | 16:57 |
| Atmospheric conditions | sun | Air temperature in °C | 30,1 |
| Atmospheric pressure in hPa | 1013,3 | Weather previous day | Sun |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Artificial pond in the zoo of vienna | | |
| Species | | | |
| Size in m² | 15 | Colour | clear ☐ |
| Depth in m | 2-3 | | turbid ☐ |
| Water temperature °C | 20,9 | Odour | normal ☐ |
| Oxygen content mg/L | 7,02 | | must ☐ |
| pH-value | 8,16 | | different ☐ |

Fotodocumentation (including points of sampling)

## Samples

| Sample identification | |
|---|---|
| | 8/3/A-B |

Notes:

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Nashornteich | Protocol number | 09 |
| Name | Denise, Katharina, Bettina | Start | 15:08 |
| Date | 3.5.2017 | End | 15:20 |
| Atmospheric conditions | Rain, many clouds | Air temperature in °C | 17 |
| Atmospheric pressure in hPa | 1019 | Weather previous day | cloudy |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Artificial pond in the zoo of vienna near the rhino enclosure | | |
| Species | ducks | | |
| Size in m² | 30 | Colour | clear ☐ |
| Depth in m | 1,5-2 | | turbid ☐ |
| Water temperature °C | 12,5 | Odour | normal ☐ |
| Oxygen content mg/L | 11,53 | | must ☐ |
| pH-value | 7,43 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 9/1/A-B |

Notes:

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Nashornteich | Protocol number | 09 |
| Name | Denise | Start | 12:25 |
| Date | 20.5.2017 | End | 12:40 |
| Atmospheric conditions | Wind, cloudy | Air temperature in °C | 18 |
| Atmospheric pressure in hPa | 1018 | Weather previous day | Sun, warm, storm |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Artificial pond in the zoo of vienna near the rhino enclosure, much material in the water | | |
| Species | | | |
| Size in m² | 40 | Colour | clear ☐ |
| Depth in m | 1,5-2 | | turbid ☐ |
| Water temperature °C | 19,1 | Odour | normal ☐ |
| Oxygen content mg/L | 4,43 | | must ☐ |
| pH-value | 7,17 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 9/2/A-C |

Notes:

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Nashornteich | Protocol number | 09 |
| Name | Denise, Katharina | Start | 17:05 |
| Date | 21.6.2017 | End | 17:17 |
| Atmospheric conditions | sun | Air temperature in °C | 30,1 |
| Atmospheric pressure in hPa | 1013,3 | Weather previous day | Sun, warm |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Artificial pond in the zoo of vienna near the rhino enclosure, much material in the water | | |
| Species | rhinos | | |
| Size in m² | 40 | Colour | clear ☐ |
| Depth in m | 1,5-2 | | turbid ☐ |
| Water temperature °C | 20,4 | Odour | normal ☐ |
| Oxygen content mg/L | 4,76 | | must ☐ |
| pH-value | 8 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 9/3/A-B |

Notes:

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Wachsstöckelteich | Protocol number | 10 |
| Name | Denise, Bettina | Start | 11:20 |
| Date | 4.5.2017 | End | 11:43 |
| Atmospheric conditions | cloudy | Air temperature in °C | 15 |
| Atmospheric pressure in hPa | 1016 | Weather previous day | Rain, cloudy |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Artificial pond near the Afritschteich, mixed forest | | |
| Species | Water snails, water-scavenger beetles | | |
| Size in m² | 20 | Colour | clear ☐ |
| Depth in m | 2 | | turbid ☐ |
| Water temperature °C | 13,3 | Odour | normal ☐ |
| Oxygen content mg/L | 4,34 | | must ☐ |
| pH-value | 7,3 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 10/1/A-B |

Notes:

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Wachsstöckelteich | Protocol number | 10 |
| Name | Denise | Start | 15:45 |
| Date | 20.5.2017 | End | 15:55 |
| Atmospheric conditions | Wind, rain, clouds | Air temperature in °C | 21 |
| Atmospheric pressure in hPa | 1019 | Weather previous day | Sun, wind |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Artificial pond near the Afritschteich, mixed forest, many duckweeds | | |
| Species | Water snails | | |
| Size in m² | 20 | Colour | clear ☐ |
| Depth in m | 2 | | turbid ☐ |
| Water temperature °C | 15,4 | Odour | normal ☐ |
| Oxygen content mg/L | 8,99 | | must ☐ |
| pH-value | 7,15 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 10/2/A-B |

Notes:

# Water sampling protocol

| | | | |
|---|---|---|---|
| Identification of sampling point | Wachsstöckelteich | Protocol number | 10 |
| Name | Denise, Katharina | Start | 14:10 |
| Date | 17.6.2017 | End | 14:25 |
| Atmospheric conditions | Cloudy, wind | Air temperature in °C | 20 |
| Atmospheric pressure in hPa | 1019 | Weather previous day | Sun, warm |

## Water body

| | | | |
|---|---|---|---|
| Description/Vegetation | Artificial pond near the Afritschteich, mixed forest, many duckweeds | | |
| Species | Water snails, ring snake | | |
| Size in m² | 20 | Colour | clear ☐ |
| Depth in m | 2 | | turbid ☐ |
| Water temperature °C | 17,9 | Odour | normal ☐ |
| Oxygen content mg/L | 3,14 | | must ☐ |
| pH-value | 7,88 | | different ☐ |
| Fotodocumentation (including points of sampling) | | | |

## Samples

| Sample identification | |
|---|---|
| | 10/3/A-B |

Notes:

| Sampling site and Date of Filtration | Filternumber | Filtration Quantity (ml) | Concentration (ng/µl) | Date of qPCR | Dilution (µl) | Added Water (µl) | Ct-value average |
|---|---|---|---|---|---|---|---|
| Wienerbergteich | 1/1/A+B | 350+550 | 107 | 09.05.2018 | 1,2 | 8,8 | 39 |
| 02.05.2017 | 1/1/C | 550 | 68 | 11.05.2018 | 1,8 | 8,2 | No Ct |
| | 1/1/D | 400 | 113 | 11.05.2018 | 1,1 | 8,9 | 38 |
| | 1/1/E+F | 500+250 | 174 | 09.05.2018 | 7,2* | 2,8 | 40 |
| | 1/1/G+H | 200+200 | 136 | 09.05.2018 | 9,2* | 0,8 | 39 |
| Silbersee | 2/1/A | 1100 | 255 | 11.05.2018 | 5* | 5 | 37 |
| 03.05.2017 | 2/1/B | 900 | 598 | 09.05.2018 | 2,1* | 7,9 | 39 |
| | 2/1/C | 1000 | 422 | 09.05.2018 | 3* | 7 | 39 |
| Pappelteich | 3/1/A | 800 | 88 | 11.05.2018 | 1,4 | 8,6 | 39 |
| 26.04.2017 | 3/1/B | 700 | 434 | 09.05.2018 | 2,9* | 7,1 | 39 |
| | 3/1/C | 900 | 332 | 09.05.2018 | 3,8* | 6,2 | 42 |
| | 3/1/D | 600 | 374 | 09.05.2018 | 3,4* | 6,6 | 39 |
| Afritschteich | 4/1/A | 600 | 113 | 09.05.2018 | 1,1 | 8,9 | 39 |
| 02.05.2017 | 4/1/B | 550 | 149 | 11.05.2018 | 8,4* | 1,6 | 38 |
| | 4/1/C | 1000 | 73 | 11.05.2018 | 1,7 | 8,3 | 42 |
| | 4/1/D | 850 | 133 | 11.05.2018 | 0,9 | 9,1 | 37 |
| Bombentrichter | 5/1/A | 1500 | 539 | 11.05.2018 | 2,3* | 7,7 | 37 |
| 09.05.2017 | 5/1/B | 1000 | 584 | 11.05.2018 | 2,1* | 7,9 | 37 |
| | 5/1/C | 500 | 452 | 11.05.2018 | 2,8* | 7,2 | 49 |
| Sieveringer Wehr | 6/1/A | 1500 | 251 | 11.05.2018 | 5* | 5 | 39 |
| 06.05.2017 | 6/1/B | 1500 | 388 | 11.05.2018 | 3,2* | 6,8 | No Ct |
| Pötzleinsdorfer Schlosspark | 7/1/A | 1500 | 370 | 11.05.2018 | 3,4* | 6,6 | 39 |
| 07.05.2017 | 7/1/B | 1500 | 582 | 11.05.2018 | 2,1* | 7,9 | 41 |
| Alpenteich 4.5.2017 | 8/1/A | 3000 | 63 | | 1,9 | 8,1 | ? |
| Nashornteich | 9/1/A | 1500 | 326 | 11.05.2018 | 3,8* | 6,2 | 47 |
| 03.05.2017 | 9/1/B | 1500 | 314 | 11.05.2018 | 4* | 6 | No Ct |
| Wachsstöcklteich | 10/1/A | 1500 | 523 | 11.05.2018 | 2,4* | 7,6 | 41 |
| 04.05.2017 | 10/1/B | 1500 | 211 | 11.05.2018 | 5,9* | 4,1 | No Ct |
| positiv control | BD/1/A | 1500 | 43 | 03.01.2018 | 2,9 | 7,1 | 26 |
| 18.05.2017 | BD/1/B | 1500 | 61 | | 2 | 8 | ? |

Figure A1: shows results of the samples of the first sampling round. Samples from the qPCR on 5.9.2017 were diluted to 25 ng/µl instead of 12,5 ng/µl. Samples marked with * had to be pre-diluted.

| Sampling site and Date of Filtration | Filternumber | Filtration Quantity (ml) | Concentration (ng/µl) | Date of qPCR | Dilution (µl) | Added Water (µl) | Ct-value average |
|---|---|---|---|---|---|---|---|
| Wienerbergteich | 1/2/A | 950 | 116 | 05.09.2017 | 2,2 | 7,8 | No Ct |
| 16.05.2017 | 1/2/B | 1200 | 24 | 09.05.2018 | 5,2 | 4,8 | No Ct |
| | 1/2/C | 850 | 35 | 09.05.2018 | 3,6 | 6,4 | No Ct |
| Silbersee | 2/2/A | 700 | 383 | 05.09.2017 | 6,4* | 3,6 | No Ct |
| 18.05.2017 | 2/2/B | 700 | 255 | 09.05.2018 | 4,8* | 5,2 | 40 |
| | 2/2/C | 700 | 206 | 09.05.2018 | 6* | 4 | 40 |
| | 2/2/D | 900 | 198 | 09.05.2018 | 6,3* | 3,7 | 39 |
| Pappelteich | 3/2/A | 1000 | 248 | 05.09.2017 | 1 | 9 | No Ct |
| 17.05.2017 | 3/2/B | 800 | 262 | 09.05.2018 | 4,8* | 5,2 | 40 |
| | 3/2/C+D | 600+600 | 207 | 09.05.2018 | 6* | 4 | 39 |
| Afritschteich | 4/2/A | 1500 | 337 | 05.09.2017 | 7,4* | 2,6 | No Ct |
| 17.05.2017 | 4/2/B | 1500 | 235 | 05.09.2017 | 1,1 | 8,9 | 40 |
| Bombentrichter | 5/2/A | 800 | 418 | 05.09.2017 | 6* | 4 | No Ct |
| 19.05.2017 | 5/2/B+C | 600+300 | 659 | 09.05.2018 | 1,9* | 8,1 | 44 |
| | 5/2/D | 600 | 420 | 11.05.2018 | 3* | 7 | 37 |
| | 5/2/E+F | 500+200 | 826 | 11.05.2018 | 1,5* | 8,5 | 39 |
| Sieveringer Wehr | 6/2/A | 1600 | 140 | 05.09.2017 | 1,8 | 8,2 | No Ct |
| 20.05.2017 | 6/2/B | 1400 | 56 | 05.09.2017 | 4,5 | 5,5 | No Ct |
| Pötzleinsdorfer Schlosspark | 7/2/A | 1600 | 132 | 05.09.2017 | 1,9 | 8,1 | No Ct |
| 19.05.2017 | 7/2/B | 1400 | 187 | 05.09.2017 | 1,3 | 8,7 | No Ct |
| Alpenteich | 8/2/A | 1900 | 54 | 05.09.2017 | 0 | 0 | No Ct |
| 20.05.2017 | 8/2/B | 1100 | 22 | 09.05.2018 | 5,8 | 4,2 | 39 |
| Nashornteich | 9/2/A | 1000 | 203 | 05.09.2017 | 1,2 | 8,8 | No Ct |
| 20.05.2017 | 9/2/B | 1000 | 257 | 09.05.2018 | 4,9* | 5,1 | 40 |
| | 9/2/C | 1000 | 334 | 09.05.2018 | 3,7* | 6,3 | 47 |
| Wachsstöcklteich | 10/2/A | 1700 | 325 | 05.09.2017 | 7,6* | 2,4 | No Ct |
| 20.05.2017 | 10/2/B | 1300 | 258 | 09.05.2018 | 4,8* | 5,2 | 54 |
| Positive controle | Bd/2/A | 1500 | 45 | 05.09.2017 | 5,5 | 4,5 | 26-27 |
| 09.06.2017 | Bd/2/B | 1500 | 31 | | 4 | 6 | ? |

Figure A2: shows the results of the samples of the second sampling round. Samples marked with * had to be pre-diluted.

| Sampling site and Date of Filtration | Filternumber | Filtration Quantity (ml) | Concentration (ng/µl) | Date of qPCR | Dilution (µl) | Added Water (µl) | Ct-value average |
|---|---|---|---|---|---|---|---|
| Wienerbergteich | 1/3/A | 1200 | 115 | 22.09.2017 | 1,1 | 8,9 | No Ct |
| 18.06.2017 | 1/3/B | 800 | 33 | 22.09.2017 | 3,8 | 6,2 | No Ct |
| | 1/3/C | 1000 | 38 | 09.05.2018 | 3,3 | 6,7 | No Ct |
| Silbersee | 2/3/A | 1600 | 432 | 22.09.2017 | 2,9* | 7,1 | No Ct |
| 22.06.2017 | 2/3/B | 1400 | 391 | 22.09.2017 | 3,2* | 6,8 | No Ct |
| Pappelteich | 3/3/A | 700 | 291 | 22.09.2017 | 4,3* | 5,7 | No Ct |
| 18.06.2017 | 3/3/B+D | 700+400 | 488 | 22.09.2017 | 2,6* | 7,4 | 53 |
| | 3/3/C+E | 600+600 | 316 | 09.05.2018 | 4* | 6 | 39 |
| Afritschteich | 4/3/A | 1500 | 366 | 22.09.2017 | 3,4* | 6,6 | No Ct |
| 17.06.2017 | 4/3/B | 1500 | 393 | 22.09.2017 | 3,2* | 6,8 | 55 |
| Parapluie Teich | 5/3/A+D | 500+500 | 674 | 22.09.2017 | 1,9* | 8,1 | No Ct |
| 22.06.2017 | 5/3/B | 1000 | 545 | 22.09.2017 | 2,3* | 7,7 | No Ct |
| | 5/3/C | 1000 | 594 | 09.05.2018 | 2,1* | 7,9 | 44 |
| Sieveringer Wehr | 6/3/A | 1600 | 263 | 22.09.2017 | 4,8* | 5,2 | No Ct |
| 19.06.2017 | 6/3/B | 1400 | 119 | 09.05.2018 | 1,1 | 8,9 | 39 |
| Pötzleinsdorfer Schlosspark | 7/3/A | 1150 | 308 | 22.09.2017 | 4,1* | 5,9 | No Ct |
| 19.06.2017 | 7/3/B | 1000 | 325 | 09.05.2018 | 3,8* | 6,2 | 38 |
| | 7/3/C | 850 | 272 | 09.05.2018 | 4,6* | 5,4 | 39 |
| Alpenteich | 8/3/A | 1800 | 116 | 22.09.2017 | 1,1 | 8,9 | No Ct |
| 21.06.2017 | 8/3/B | 1200 | 32 | 09.05.2018 | 3,9 | 6,1 | 39 |
| Nashornteich | 9/3/A | 1600 | 254 | 22.09.2017 | 4,9* | 5,1 | No Ct |
| 21.06.2017 | 9/3/B | 1400 | 124 | 09.05.2018 | 1 | 9 | 40 |
| Wachsstöcklteich | 10/3/A | 1900 | 516 | 22.09.2017 | 2,4* | 7,6 | No Ct |
| 17.06.2017 | 10/3/B | 1100 | 393 | 09.05.2018 | 3,2* | 6,8 | 40 |
| Positive control | Bd/3/A | 1500 | 38 | 11.05.2018 | 3,3 | 6,7 | 28 |
| 9.6.207 | Bd/3/B | 1500 | 67 | 22.09.2017 | 1,9 | 8,1 | 22 |

Figure A3: shows the results of the samples of the third sampling round. Samples marked with * had to be pre-diluted.

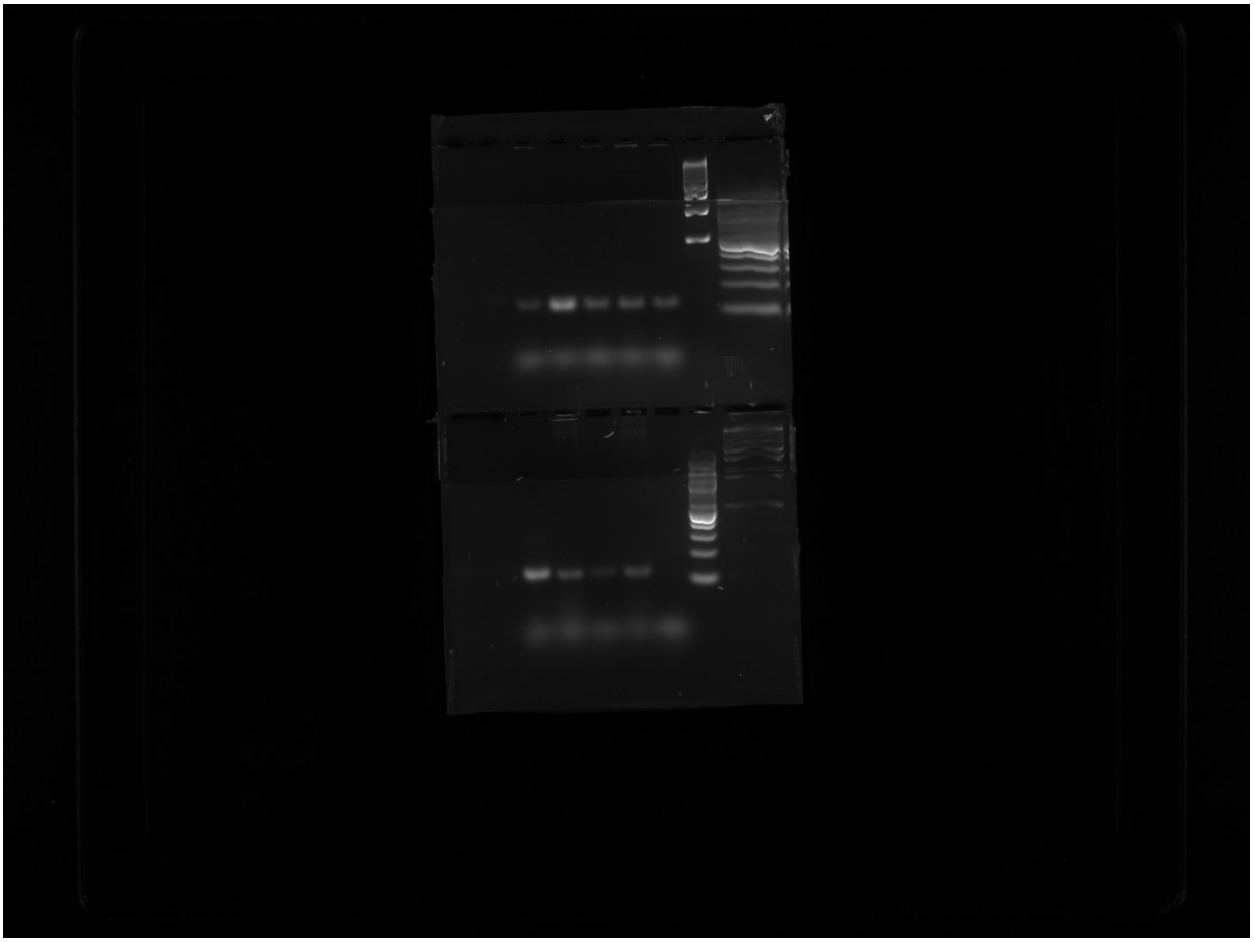

Figure A4: Result of gel-electrophoresis. From right to left: marker 500 bp, marker 100 bp, in the upper part samples 1 to 5, in the lower part samples 6 to 10.

Table A1: skin swab results in detail.

| Sampling site | Date | Swabnumber | Species | Result |
|---|---|---|---|---|
| **Wienerbergteich 01** | 16.05.2017 | 1-1 | *Pelophylax sp.* | negative |
| | 16.05.2017 | 1-4 | *Pelophylax sp.* | negative |
| | 21.05.2017 | 1-3 | *Pelophylax sp.* | negative |
| | 29.05.2017 | 1-2 | *Pelophylax sp.* | negative |
| | 29.05.2017 | 1-5 | *Pelophylax sp.* | negative |
| **Silbersee 02** | 23.06.2017 | 2-3 | *Bufo bufo* | negative |
| | 23.06.2017 | 2-5 | *Bufo bufo* | negative |
| | 29.06.2017 | 2-1 | *Bufo bufo* | negative |
| | 29.06.2017 | 2-4 | *Pelophylax sp.* | negative |
| | 29.06.2017 | 2-2 | *Lissotriton vulgaris* | negative |
| **Pappelteich 03** | 13.04.2017 | 3-1 | *Ichthyosaura alpestris* | negative |
| | 13.04.2017 | 3-2 | *Ichthyosaura alpestris* | negative |
| | 13.04.2017 | 3-3 | *Lissotriton vulgaris* | negative |
| | 17.05.2017 | 3-4 | *Triturus carnifex* | negative |
| | 17.05.2017 | 3-5 | *Ichthyosaura alpestris* | negative |
| **Afritschteich 04** | 17.05.2017 | 4-1 | *Pelophylax sp.* | negative |
| | 08.06.2017 | 4-5 | *Triturus carnifex* | negative |
| | 17.06.2017 | 4-4 | *Triturus carnifex* | negative |
| | 23.06.2017 | 4-3 | *Pelophylax sp.* | negative |
| | 23.06.2017 | 4-2 | *Pelophylax sp.* | negative |
| **Bombentrichter1 05** | 04.06.2017 | 5-1 | *Bufo bufo* | negative |
| | 04.06.2017 | 5-2 | *Bufo bufo* | negative |
| | 04.06.2017 | 5-3 | *Bufo bufo* | negative |
| | 04.06.2017 | 5-4 | *Bufo bufo* | negative |
| | 04.06.2017 | 5-5 | *Rana dalmatina* | negative |
| **Sieveringer Wehr 06** | 15.05.2017 | 6-5 | *Salamandra salamandra salamandra* | negative |
| | 04.06.2017 | 6-3 | *Triturus carnifex* | negative |
| | 19.06.2017 | 6-1 | *Salamandra salamandra salamandra* | negative |
| | 25.06.2017 | 6-2 | *Ichthyosaura alpestris* | negative |
| | 29.06.2017 | 6-4 | *Ichthyosaura alpestris* | negative |
| **Pötzleinsdorfer Schlossp** | 06.06.2017 | 7-1 | *Rana dalmatina* | negative |
| | 06.06.2017 | 7-2 | *Bufo bufo* | negative |
| | 06.06.2017 | 7-3 | *Bufo bufo* | negative |
| | 19.06.2017 | 7-5 | *Rana dalmatina* | negative |
| | 25.06.2017 | 7-4 | *Rana dalmatina* | negative |
| **Alpenteich TGS 08** | 24.05.2017 | 8-1 | *Pelophylax sp.* | negative |
| | 24.05.2017 | 8-2 | *Pelophylax sp.* | negative |
| | 24.05.2017 | 8-3 | *Lissotriton vulgaris* | negative |
| | 24.05.2017 | 8-4 | *Lissotriton vulgaris* | negative |
| | 24.05.2017 | 8-5 | *Lissotriton vulgaris* | negative |
| **Nashornteich TGS 09** | 24.05.2017 | 9-1 | *Pelophylax sp.* | negative |
| | 24.05.2017 | 9-2 | *Pelophylax sp.* | negative |
| | 24.05.2017 | 9-3 | *Bufotes viridis* | negative |
| | 24.05.2017 | 9-4 | *Pelophylax sp.* | negative |
| | 24.05.2017 | 9-5 | *Pelophylax sp.* | negative |
| **Wachsstöcklteich 10** | 23.06.2017 | 10-1 | *Pelophylax sp.* | negative |
| | 23.06.2017 | 10-2 | *Triturus carnifex* | negative |
| | 23.06.2017 | 10-3 | *Pelophylax sp.* | negative |
| | 23.06.2017 | 10-4 | *Pelophylax sp.* | negative |
| | 23.06.2017 | 10-5 | *Triturus carnifex* | negative |

# Appendix B

## 1. DNA extraction

1. Add 900 µl lysis buffer into the prepared and labelled 2 ml tubes and 810 µl Isopropanol (99 %) in other 2 ml tubes.
2. Cut the filters on a cooled metal plate.
3. Add the filter pieces into the tubes with the lysis buffer and incubate for 15 minutes at 65 °C, at thermomixer, then centrifuge them 5 minutes at 10.000 x g.
4. Transfer the supernatant into prepared and labelled 1,5 ml tubes.
5. Add 4,5 µl Proteinase K solution into the tubes and invert 25 times, then incubate for 60 minutes at 55 °C.
6. Add 4,5 µl RNase A solution into the tubes and invert 25 times, then incubate for 60 minutes at 37 °C and place the tubes immediately on ice for 1 minute.
7. Add 300 µl protein precipitation solution into the tubes and vortex 20 seconds, then centrifuge for 5 minutes at 16.000 x g.
8. Fill the supernatant into the 2 ml tubes with isopropanol and invert 50 times. Discard the 1,5 ml tubes with the protein pellet.
9. Centrifuge for 5 minutes at 16.000 x g and discard the supernatant.
10. Wash the DNA pellet with 300 µl ethanol (70 %) and invert, then centrifuge for 5 minutes at 16.000 x g and discard the supernatant.
11. Air dry the opened tubes for 5-10 minutes on a paper towel then add 50 µl hydration solution and incubate for 60 minutes at 65 °C. Carefully snip the tubes after 30 minutes.
12. Incubate at 20 °C overnight and gently shaking at 300 rpm. Next day spin the tubes and store them at -22 °C or start the purification.

## 2. DNA purification

1. Add 150 µl QG-buffer to each 2 ml tube, containing the unfreezed 50 µl extracted DNA.
2. Incubate at 50 °C for 10 minutes.
3. Add 50 µl Isopropanol (99 %) to each sample, invert several times, spin, and transfer the liquid into prepared and labelled 2 ml collection tubes.
4. Centrifuge at 18.000 x g for 1 minute and deflate the discharge.
5. Add 500 µl QG-buffer to each tube, centrifuge at 18.000 x g for 1 minute and deflate the discharge.
6. Add 750 µl PE-buffer to each tube, wait for 5 minutes, then again centrifuge at 18.000 x g for 1 minute and discard the discharge.
7. Repeat centrifugation, afterwards discard the lower parts of the collection tubes and put the upper part into prepared and labelled 1,5 ml Eppendorf tubes.
8. Add 30 µl EB-buffer to each tube, wait for 4 minutes, centrifuge at 18.000 x g for 1 minute, discard the collection tubes and store the 1,5 ml tubes containing 30 µl purified DNA at -20 °C.

## 3. Quantitative PCR

1. Dilute the samples with Milli-Q water to a concentration of 12,5 ng/µl.
2. Prepare the mastermix with the following components:

    - Primer fwd.: 6 µl
    - Primer rev.:6 µl
    - Probe: 5 µl
    - Fast mix: 1 ml
    - Milli-Q-water: 783 µl

3. Add 2 µl of a diluted sample in each of three wells.
4. Add 2 µl respectively of the positive control and the negative control in separate three wells.
5. Add 18 µl of mastermix in each well.
6. Cover the 96 well plate with a plastic foil.
7. Centrifugate the well plate for a few seconds at 1000 rpm.
8. Enter the following thermalprofile to the thermocycler and start the program:

    - 5 minutes at 95 °C (segment 1, 1 cycle)
    - 15seconds at 95 °C (segment 2, 55 cycles)
    - 30 seconds at 56 °C (segment 2, 55 cycles)
    - 30 seconds at 60 °C (segment 2, 55 cycles)