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"Detection of the Chytrid-fungus: Biomonitoring using Environmental DNA and its potential planktonic predators in Vienna"

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CONTENTS

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ABSTRACT

Many threats impair the life of amphibians, caused mainly by humans, such as pollution, fragmentation or destruction of their habitats and also the spread of the Chytrid-fungus *Batrachochytrium dendrobatidis* (*Bd).* The occurrence of this pathogen varies depending on environmental influences. Temperature affects reproduction and the growth of the zoospores which infect new hosts, and thus influences infection and mortality rates of amphibians. The seasonal weather in temperate zones has a strong effect on the lifecycle of *Bd*. Under climate change an enhanced prevalence of the pathogen may be expected in certain regions. Coexisting zooplankton such as Cladocera and Rotifera may reduce new infections by consuming free-swimming zoospores.

In this study, two methods were applied to determine zoospores of *Bd*. Firstly by investigation of the environmental DNA (eDNA) and secondly by analysis of skin swabs. Water samples were taken three times at ten sampling sites in Vienna. Two or more filters were used for filtering of 3 liters depending on the clarity of the pond water. Five of the resulting 95 filters indicated *Bd* spores at the quantitative polymerase chain reaction (qPCR). Five skin swabs were taken at each sampling site. Only two of 50 individuals were tested *Bd*-positive. Furthermore, plankton samples were examined for potential predators of zoospores such as Notommatidae or Lecanidae (Rotifera) and Daphniidae (Cladocera). In eight ponds at least, members of one family of planktonic predators were determined.

Key words: Pathogen, eDNA, *Batrachochytrium dendrobatidis*, amphibian, planktonic predator

ZUSAMMENFASSUNG

Das Leben von Amphibien birgt eine Vielzahl an Gefahren, größtenteils durch den Menschen verschuldet, denke man an die Verschmutzung, Fragmentation bzw. Zerstörung ihrer Habitate und die Verbreitung des Chytrid-Pilzes *Batrachochytrium dendrobatidis* (*Bd*). Das Auftreten dieses Pathogens variiert mit den Umwelteinflüssen. Aus Laborversuchen mit dem Pilz geht hervor, dass Temperaturunterschiede die Reproduktion und das Wachstum der Zoosporen stark beeinflusst. Das hat wiederum Einfluss auf Infektions- und Mortalitätsraten der Amphibien. Jahreszeitliche Witterung in den gemäßigten Zonen beeinflusst den Lebenszyklus des Chytrid-Pilzes. Der Klimawandel ist bereits spürbar und kann die Verbreitung von *Bd* beeinflussen und die daraus resultierten Konsequenzen für bestimmte Regionen verändern.

Im selben Habitat koexistiert Zooplankton wie Cladocera und Rotifera die beim Konsum der Pilzsporen beobachtet wurden. Dies führte zur Reduktion der freischwimmenden Zoosporen im Wasser und minimierte somit Neuinfektionen.

In dieser Studie wurden zwei Methoden angewandt, um *Bd*-Sporen zu detektieren. Zum einen wurden Wasserproben über "environmental DNA" (eDNA) und zum anderen wurden Hautabstiche auf *Bd*-Sporen untersucht. Dafür wurden Wasserproben aus zehn Gewässer in Wien, in drei Durchgängen und bei unterschiedlichen Wassertemperaturen entnommen. Dafür wurden jeweils drei Liter Wasser an drei verschiedenen Stellen pro Teich entnommen. Zumindest zwei oder auch mehr Filter, abhängig von der Wasserqualität, wurden pro Filtriervorgang verwendet. Fünf der insgesamt 95 Filter zeigten ein *Bd*-positives Resultat bei der "quantitativen Polymerasen Kettenreaktion" (qPCR). Bei jedem Standort wurden fünf Hautabstriche genommen. Bei nur zwei von insgesamt 50 Individuen wurden Pilzsporen nachgewiesen. Außerdem wurden Planktonproben von jedem Gewässer entnommen, um potenzielle Prädatoren nachzuweisen, wie zum Beispiel aus der Familie Notommatidae oder Lecanidae (Rotifera) und Daphniidae (Cladocera). In acht Teichen wurde zu minderst eine der drei Familien nachgewiesen.

Stichwörter: Pathogen, eDNA, *Batrachochytrium dendrobatidis*, Amphibien, planktonische Prädatoren

I INTRODUCTION

1.1 Amphibian decline

Amphibians are endangered due to different causes such as habitat destruction, pollution, trade for food or as pets, introduction of invasive species, climate disturbance and infections by pathogens (ROOIJ et al., 2015). These aspects contribute to the fact that amphibians are the most threatened vertebrates (LIU et al., 2013). At least 42 % of all amphibian species are threatened according to the IUCN Red List (PETERSEN et al., 2016). According to the "Global Amphibian Assessment" 35 species are considered as "extinct" and more than 130 species as "possibly extinct" (KWET, 2008). About 400 amphibian species have experienced a rapid decline since 1980 to 2004. The extinction of 233 species was attributed to overexploitation or habitat loss (SKERRATT et al., 2007; STUART et al., 2004). About 200 species show an "enigmatic" decline (STUART et al., 2004). The Chytrid-fungus *Batrachochytrium dendrobatidis* (*Bd*) is the major primary cause of extinction and decline of about 200 anurans (SKERATT et al., 2007). It is one of the worst pathogens and can be termed as panzootic (BERGER et al., 1998). The Chytrid fungus is associated with many of the observed catastrophic die-offs and is documented for hundreds of amphibian species worldwide (ROSENBLUM et al., 2008). Already in the year 2004 nine species certainly were considered as extinct by *Bd* and further 113 were possibly extinct since the 1980s. In 2010 already 30 species were lost, including five undescribed species only in Panama (CRAWFORD et al., 2010). Up to now 1.300 amphibian species have been tested for *Bd* – almost 700 of them were *Bd*-positive. Till today Chytridiomycosis was detected in different regions around the world and has reduced the native amphibian populations since the 1970s, in Australia, Central America, South America, the Caribbean islands, the North American Sierra Nevada and the Iberian Peninsula (O´HANLON et al., 2018).

The Chytrid-fungus (*Bd*) was reported in the 1990s as cause for mass extinction of the common midwife toad (*Alytes obstetricans)* in Central Spain (BOSCH et al., 2001). Population collapses were observed in fire salamanders and common toads, too. In a study of GARNER et al. (2005) 1.600 amphibians were tested for *Bd*. The disease-causing agent was detected for 20 species in several European countries (KWET et al., 2008).

The Swiss common midwife toads have lost 64% of their populations since about 1985 and have a high mortality from chytridiomycosis in laboratory experiments (TOBLER et al., 2010). The common midwife toads are threatened also in Germany (PROESS et al., 2015). The Chytrid-fungus was detected in almost all amphibian species in 53 out of 156 populations investigated between 2003 and 2009 in Germany. Except *Rana dalmatina* (small sample size) all autochthonous species were tested *Bd*-positive. In the case of *Pelophylax* sp. annual fluctuations were observed infection rates between 28 % and no infection (OHST, 2011).

The human-mediated transport of infected amphibians is the most likely factor for the global spread of *Bd* (KRIGER et al., 2009). The trade and release of anuras for example *Lithobates catesbeianus* and *Xenopus laevis* was documented. The global trade of *X. laevis* for pregnancy tests in the 1970s, which was considered as carrier of *Bd*, may have contributed to the distribution of the pathogen (MORGAN et al., 2007; RACHOWICZ et al., 2005). A massive import of water frogs for food and pet trade was reported to Western Europe (OHST, 2011). The Chytrid-fungus was widespread among amphibians in terrariums and the most common cause of death (KWET, 2008). Due to the rapid spread, the OIE (World Organization for Animal Health) qualifies Chytridiomycosis as notifiable (SZTATECSNY, 2011).
In Northern Europe a population of common toads was investigated which lived in coexistence with *Bd* (MARTEL et al., 2012; ROOIJ et al., 2015). Also, in Alpine regions the fungus was detected. In Tyrol (Austria) and the Pyrenees (Spain), both localities lie above 1.600 meters altitude, infected amphibians were found, but with different mortality rates. The common midwife toad in the Pyrenees suffered a very high mass mortality whereas the common frogs and water frogs in Tyrol showed less mortality (WALKER et al., 2008, GLASER & SZTATECSNY, 2009). The increasing risk for infection by *Bd* in Alpine regions was correlated with harsh environmental factors (OHST, 2011). The severity of the infection does not only depend on the acclimation status of a species but also on parameters such as temperature and moisture (RAFFEL et al., 2015). One of the most important environmental factors is the temperature, which influences both, the host and the pathogen (BRADLEY et al., 2017). In colder seasons of the year or in high-altitude regions many deaths by *Bd* were observed (PIOTROWSKI et al., 2004). Conversely, better chance to survive of infected amphibians was observed at higher temperatures.

Experiments with newts showed that the Zoospores "GE" (Genome equivalents, Appendix Fig. 17) are twice as high at 15 °C as at a temperature of 25 °C. The amphibians showed a reduced immune defense at lower temperatures under laboratory conditions and at wild populations, too (RAFFEL et al., 2015). The *Bd* outbreaks occurred during cool seasons likely correlating with increased release of zoospores and reduced microbial skin peptide-based immune response of the host (ROHR et al., 2013).

In a laboratory experiment the increased ambient temperature decreased *Bd* infection. Two infected species (12 bullfrogs and 16 northern cricket frogs) were cured by an air temperature of 30 °C for 10 days (CHATFIELD & RICHARDS-ZAWACKI, 2011).
The major causes for Europe's amphibians decline are still habitat fragmentation and destruction (STUART et al., 2004), but the fungus can be an additional problem soon due to climate change. The environmental temperature influenced the physiology of amphibians and pathogen and was jointly responsible for several *Bd* outbreaks and amphibian population declines (ROHR et al., 2013).

1.2 *Batrachochytrium dendrobatidis* (*Bd*)

1.2.1 History of discovery

The fungus was described by BERGER et al. in 1998 and was first detected at infected or dead adult anurans in Australia and Central America. The species name *Batrachochytrium dendrobatidis* (*Bd*) derives from the genus *Dendrobates*, because some captive specimens died due to infection with *Bd* (LONGCORE, 1999). The earliest findings of the pathogen date back to the 1930s. During a survey of preserved specimens *Bd* was detected in clawed frogs, *Xenopus laevis* collected in the year 1938 in Africa. The origin of *Bd* was assumed to be "out of Africa" (WELDON et al., 2004). The evolutionary complexity with a deep phylogenetic diversity was indicated by 49 global isolates of the fungus until 2013 (ROSENBLUM et al., 2013).

Today 234 isolates are known in all continents in which *Bd* has been detected so far. The newly discovered lineage *Bd*ASIA-1 (Korean peninsula) redefined previously accepted lineages *Bd*GPL(global), *Bd*CAPE (Africa), *Bd*CH (Europe), *Bd*BRAZIL (Brazil) and their relationships (O´HANLON et al., 2018). The *Bd*ASIA-1 lineage was also found in Switzerland and a second Asian-associated lineage (*Bd*ASIA-2) was detected in invasive North American bullfrogs in Korea, this lineage is closely related to *Bd*BRAZIL. All isolates were compared pairwise and showed that the average number of segregating sites and the nucleotide diversity were greater for *Bd*ASIA-1 than for any others (O´HANLON et al., 2018). This suggested that East Asia contained the ancestor population of *Bd* (BATAILLE et al., 2013, O´HANLON et al., 2018). The other panzootic lineages have emerged out of them. The Chytrid-fungus was spread globally in the early 20th century and infected nearly 700 amphibian species (O´HANLON et al., 2018).

1.2.2 Biology of the Chytrid-fungus

The Chytrid-fungus *Bd* is one of approximately 1.000 described species of the phylum Chytridiomycota. They all produce motile flagellated zoospores. Chytrid-fungi are found in aquatic or terrestrial habitats and some of them parasitize algae, plankton or vascular plants, others are saprotrophic organisms of refractory substrate including pollen, chitin, keratin and cellulose (JAMES et al., 2006). *Batrachochytrium salamandrivorans* (*Bsal*) and *Bd* are the only two Chytrid-fungi so far known which parasitize vertebrates, especially amphibians. In import of salamanders from Asia, *Bsal* was detected, a pathogen that exclusively infects caudates (ROOIJ et al., 2015). The Chytrid-fungus proliferates within the cells of the stratum corneum and the stratum granulosum (keratinized layers of the epidermis) in amphibians (ROOIJ et al., 2015).

The fungus has two main life stages, a motile free-swimming zoospore with a flagellum and the reproductive thallus in which asexual zoospores are produced (ROOIJ et al., 2015).

The reproductive thallus of the Chytrid-fungus contains mostly a single sporangium. Zoospores develop in a sac-like structure of the sporangium by internal divisions (JAMES et al., 2006). Substrate-independent, free-swimming zoospores show a relatively short activity period in which they are covering a distance of less than 2.0 cm before encysting (PIOTROWSKI et al., 2004).

The migration of zoospores actively towards the mucous layer (part of the upper epidermis) at *X. laevis* was observed. The main component of mucus are mucins or mucin glycoproteins. A spectrum of sugars (derivates of Galactose, Mannose, Fucose etc.), constituting the so-called integumental free sugars, was also found in the epidermis of smooth newt (*Lissotriton vulgaris*) and the edible frog (*Pelophylax esculentus*) (ROOIJ et al., 2015). The waterborne zoospores search for their hosts by chemotaxis (BERGER et al., 2005; ROSENBLUM et al., 2008).

The diameter of a spore is about 3–5 µm and the flagellum has a length of 19–20 µm (LONGCORE et al., 1999). The lifetime is about 24 hours also in distilled water, pond water or 1 % tryptone liquid medium. Already after 18 hours only approximately 50 % of zoospores remained motile and after 24 hours 5 % were still alive (PIOTROWSKI et al, 2004). The fungus endures a range of temperature between 4–25 °C. The pH optimum for *Bd* lies between pH 6 and 7 but the tolerance range is given between pH 5 and pH 8. Although the fungus grows poorly below pH 6 (PIOTROWSKI et al., 2004).

With the encystment in the host skin the substrate-dependent stage of *Bd* starts (ROSENBLUM et al., 2008). After the infection, the flagellum is reduced, and the cell wall is formed. The lifecycle in culture indicated that zoospores take 4 to 5 days at 22 °C to transform to sporangia (BERGER et al., 2005; ROOIJ et al., 2015). The Chytrid-fungus responded susceptibly to warmer temperature despite the optimal growing temperature in culture is about 23 °C. The growth is retarded or discontinued at a temperature above 28 °C or below 10 °C (PIOTROWSKI et al., 2004). The developed germ tube invades the host epidermis and a sporangium is shaped (ROOIJ et al., 2015). The young thalli can develop "Septa" and each compartment grows into a separate sporangium (colonial growth or only one single sporangium) in which flagellated zoospores are developed. The approximately 10 µm long discharge tubes of a sporangium release matured spores in a moist environment. If the environment is too dry, the fungus can retain its zoospores until sufficient moisture is present (BERGER et al., 2005).

1.2.3 Pathogenesis

The global pathogen *Batrachochytrium dendrobatidis* (*Bd*) causes an epidermal disease of amphibians around the world (BERGER et al., 2005). Thus, the Chytrid-fungus *Bd* infects a class with a complex life history, which consists mostly of an aquatic larval stage and subsequently metamorphose to a semiaquatic or terrestrial life stage. The superficial layers of the epidermis are infected by *Bd* which causes hyperkeratosis with cytoplasmic degeneration and vacuolation of the amphibian skin, but there are no lesions visible (BERGER et al., 1998; CAMPBELL et al., 2012). The keratinized structures of the mouthparts of early-stage larvae or the epidermis of adult amphibians are affected (SEARLE et al., 2013). Tadpoles were observed when consuming zoospores. The increased contact rates between spores and mouthparts also increase the infection risk (BUCK et al., 2016).

Depending on the infection in an early or a late stage of development, the progression of the disease can vary. In early stages only the mouthparts are keratinized unlike to late larval stage, where keratin is also found in regions of the skin. After metamorphosis can lead to weight loss, lethargy and death, but some species shed infection. Growth and larval development are often slowed when a pathogen is present, also can influence the intra- and interspecific competitive interactions of the adults (SEARLE et al., 2013).
The mucosome in the amphibian skin can reduce the infection during the first 24 hours (ROOIJ et al., 2015). To infect the remaining skin or new hosts the fungus mostly needs a thin film of water (RAFFEL et al., 2015). The pathogen moves to the Stratum granulosum and matures in the Stratum corneum to a thallus. The mature zoosporangia are transported to the surface of the skin, afterwards they are released by discharge tubes into the water, where the spores survive between 24 and 48 hours before they encyst or die (ROLLINS-SMITH, 2011). The Stratum granulosum is involved in an active electrolyte transport.
Amphibians rehydrate across the ventral skin, especially about the pelvic patch (CAMPBELL et al., 2012). The pathogen *Bd* infects the keratinized cells of the Stratum corneum on the ventral skin and interrupts osmoregulation, electrolyte and fluid homeostasis. The ion transport of the frog skin varies between species and environmental conditions. The different epidermis, typical for several species, could be one factor of the grade of infection (CAMPBELL et al., 2012).

The amount of absorbed water correlates with the rate of Na+ transport which depends upon the activity of the NA+/K+-pump; a key feature of chytridiomycosis is the reduced Na+ transport (LARSEN et al., 2009; VOYLES et al., 2009; CAMPBELL et al., 2012).

The impairment of osmoregulatory mechanisms and the electrolyte imbalance caused by damage on the epidermal skin layers can lead directly to cardiac arrest. Larval and subadult amphibians are also damaged by the fungal metabolites including toxic substances (GERVASI et al., 2013).

Increasing environmental temperature decreases *Bd* infections. In one study, infected frogs were exposed to a temperature of 30 °C for 10 days and nearly all of them were cured (RICHARDS-ZAWACKI, 2009). Also, great barred frogs (*Mixophyes fasciolatus*) were healed to 50 %, if exposed to a water temperature of 27 °C (FORREST & SCHLÄPFER, 2011).

This phenomenon was observed in captive boreal toads (*Anaxyrus boreas*) and also in wild populations of Panamanian golden frogs (*Atelopus zeteki*). The amphibians were all suffering from a "fever" affecting the entire population, so they increased their body temperature by 2,4 °C, the infection rate was reduced. Even minor environmental or behavioral changes could have an influence too (RICHARDS-ZAWACKI, 2009; FORREST & SCHLÄPFER, 2011).

1.3 Detection of *Batrachochytrium dendrobatidis* (*Bd*)

Due to the worldwide declines in amphibian populations caused by *Batrachochytrium dendrobatidis,* it is necessary to implement precautionary investigations to detect the fungus in time. Environmental DNA (eDNA) technique is a method independent on finding and handling individuals to determine the presence of species in lentic and lotic aquatic environments (KAMOROFF & GOLDBERG, 2017; SCHMIDT & URSENBACHER, 2015).

This is a non-invasive technique compared to analyzing skin swabs of amphibians to detect the pathogenic agent *Bd* (KAMOROFF & GOLDBERG, 2017).

1.3.1 Environmental DNA

The DNA of organisms is detected in environmental samples (BOHMANN et al., 2014; SCHMIDT & GRÜNIG, 2017). The DNA can be a free molecule or still be part of the cell. To detain the free DNA-fragments, the filters should be as small-pored as possible. During the filtration of water DNA of all organisms such as bacteria, fungi, insects, algae or fish remain in filters. The species community of the investigated locality DNA is copied by quantitative polymerase chain reaction (qPCR) followed by Next-Generation Sequencing (SCHMIDT & GRÜNIG, 2017). A small volume of about 15 ml could be enough to find a species in a pond (SCHMIDT & URSENBACHER, 2015). It is a dependable method for detecting rare or difficult to sample taxa and to investigate the community diversity in an ecosystem (BOHMANN et al., 2014). Advantages of qPCR are low costs and single or multi species are verifiable. It is a common method to detect pathogens. The investigated sequence should be short to increase the sensitivity of the analysis.

Fragments of mitochondrial DNA normally are investigated for eDNA because each cell contains 1.000 – 5.000 mitochondria and each of these contain a copy of mtDNA (SCHMIDT & URSENBACHER, 2015). The decoded sequences can be compared to a reference gene data bank. The results are influenced by the number of individuals and their distribution in the water, the eDNA-excretion rate of the species and inhibiting substances (SCHMIDT & GRÜNING, 2017). This is a time-saving and economical method to detect species or their pathogens in their environment.

1.3.2 Detection of the fungus in Austria

The fungus was first detected in Tyrol, Austria in the year 2009. GLASER & SZTATECSNY (2009) investigated 11 amphibian species at 24 sampling sites. Skin swabs were taken of 408 individuals and qPCR was applied to determine the Chytrid fungus. The fungus was detected at five sites higher than 500 m sea level in six species: alpine crested newt (*Triturus carnifex*), northern crested newt (*Triturus cristatus*), alpine newt (*Ichthyosaura alpestris*), common frog (*Rana temporaria*), pool frog (*Pelophylax lessonae*) and yellow-bellied toad (*Bombina variegata)*. The affected amphibians showed no signs of disease (GLASER & SZTATECSNY, 2009). For a study were investigated 74 locations in all federal states of Austria.

Approximate 1.500 skin swabs of smooth newt (*Lissotriton vulgaris*), fire and yellow-bellied toad (*Bombina bombina, B. variegata*) alpine newt (*Ichthyosaura alpestris*), crested newts (*Triturus cristatus, T. carnifex, T. dobrogicus*), European tree frog (*Hyla arborea*), moor frog (*Rana arvalis*), common frog (*Rana temporaria*) and water frogs (*Pelophylax lessonae, P. ridibundus, P. esculentus*) were analyzed by qPCR.

Chytridiomycosis was detected at 56 % of the investigated amphibian populations, the highest infection rates were documented in Vienna following by Vorarlberg, Rheintal, eastern and southern Austria. The infection was higher in the Anura, such as fire-bellied toads and green frogs than in Caudata such as great crested newts. There were even documented two dead metamorphosing larvae of *Bombina bombina* and *B. variegata* and also two dead individuals of smooth newts in Austria (SZTATECSNY & HÖDL, 2011).

In all nine federal states of Austria, amphibians were found which were infected by the Chytrid fungus. The pathogen was present in the low elevation flood plains of eastern Austria, in the subalpine valleys of western Austria and with a high prevalence in Vienna (Fig. 1; SZTATECSNY & GLASER, 2011). Almost 80 % of the investigated ponds with commercial exploitation were tested *Bd*-positive. The ponds with infected populations were located near residential areas or streams.

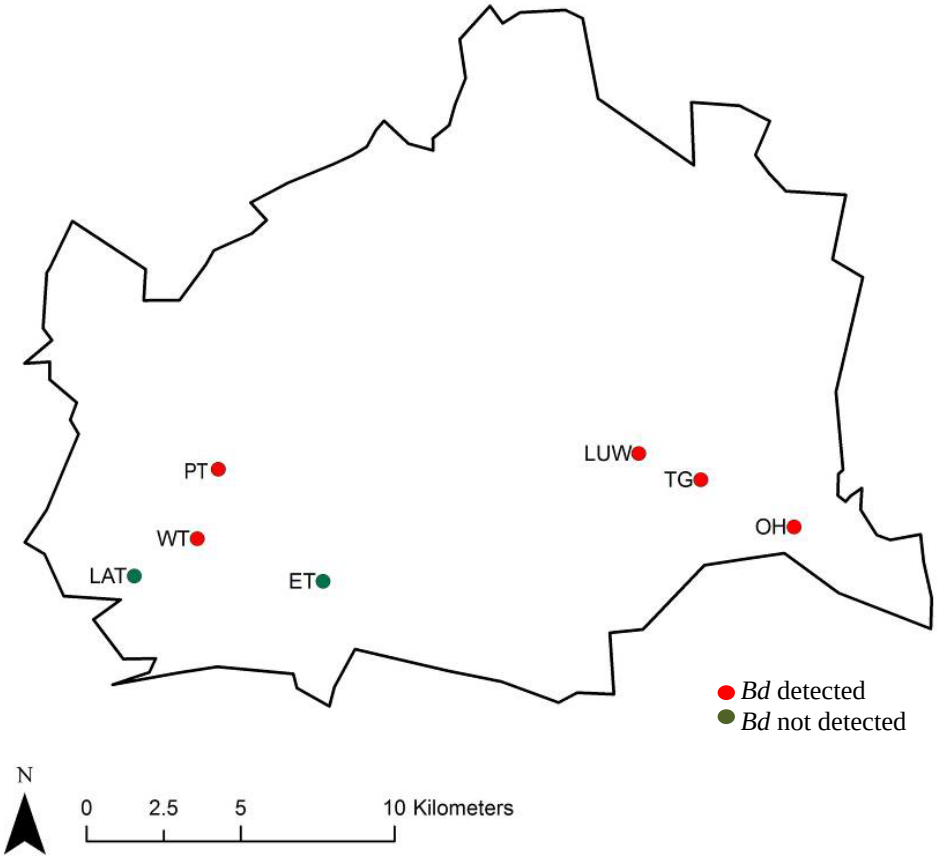

Fig.1: Map of Vienna with red dots (*Bd* could be detected by skin swabs) and green dots (no one of the investigated skin swabs was *Bd* positive tested) where amphibian populations were investigated by SZTATECSNY and HÖDL, 2011; ET private pond in Liesing; LAT Lainzer Tiergarten; LUW Lusthauswassr; OH Ölhafen (Lobau); PT Pappelteich; TG Hüttelteich; WT Afritschteich (SZTATECSNY & HÖDL, 2011).

The pathogen was detected at five of seven investigated ponds in Vienna, all ponds are located along the Danube (Lobau, Danube Island and Prater) and two ponds in western Vienna (Fig.1; "Pappelteich" and "Afritschteich"). Only three of seven investigated species, smooth newt (*Lissotriton vulgaris*), Danube crested newt (*Triturus dobrogicus*) and fire-bellied toad were infected (*Bombina bombina*) (SZTATECSNY & GLASER, 2011). The observation of these amphibian and the investigation of potential breeding sites are therefore of major importance

1.4 Planktonic predators

Some microorganisms consume zoospores and can reduce the infection by *Bd*. In the Pyrenees researchers discovered that in one water sample, only two hours after sampling, significantly less flagellated zoospores were observed if they occurred together with protozoa and microscopic metazoans than without them (SCHMELLER et al., 2014). Fluorescently stained zoospores were reduced by indigenous planktonic organism in different ways such as predation, passive consumption and filtration. Subsequent tests showed that Rotifera of the family Notommatidae caused the greatest reduction of viable zoospores. The abundance of *Bd* also decreased in the presence of Parameciidae (ciliates). In experiments no one tadpole of *Discoglossus scovazzi* were infected by *Bd* in the presence of Notommatida sp. and only a few if ciliates were present. The planktonic communities affect the host/pathogen dynamics. More ecological studies are needed to clarify the correlation of abiotic variables, the planktonic communities and the outcome of the host/pathogen dynamic (SCHMELLER et al., 2014).

Other planktonic organisms such as crustaceans were also observed at consumption of stained free-swimming zoospores. In water storages *Daphnia* are used for water purification of bacteria and algae (up to 99 %). *Daphnia* and some other genus (*Diaphanosoma*, *Chydorus*, *Ceriodaphnia*) consume particles of a diameter between 0,16–4,2 µm or up to 25 µm (GAVIRIA-MELO, 2016). Cladocera feed on zoospores of the Chytrid-fungus thus reduce the threat of *Bd* (BUCK et al., 2011; HAMILTON et al., 2012). The degree of infection depends on several factors. Relevant are the abundance, the food concentration and the grazing period while consuming the zoospores. Some species such as *D. magna* and *D. dentifera* absorbed a similar number of *Bd*-zoospores but amphibians showed an increased reduction of infection in the present of *D. magna* because they digested more of its.

The zoospores can survive the passage through the gut of *Daphnia* sp. better if alternative food (algae) was present in high concentrations (SEARLE et al., 2013). A study showed larval *Xenopus laevis* consumed over 10.000 zoospores in 4,5-hours compared to a single *Daphnia magna* which feed on in the same time almost 200 zoospores (WILSON et al., 2018).

1.5 Targets

This study examined the presence of zoospores of the Chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) using environmental DNA (eDNA). Additionally, amphibians are examined for *Bd* infection by means of skin swabs. The composition of the planktonic communities – potential feeders of the Chytrid-fungus – was investigated. Ten spawning waters were selected in the city of Vienna. The survey aimed to better link abiotic variables to the composition and density of planktonic communities and the intensity of the *Bd*-infection.

Central questions were:

1. Can *Bd* be detected in the study areas?
2. Which are the planktonic predators in the investigated ponds?
3. Is there a connection between environmental parameters (such as temperature, plankton) and the occurrence of *Bd*?

II MATERIAL AND METHODS

For a survey of spawning waters in Vienna in the years 2015 and 2016 approximate 450 localities were documented (GRILLITSCH & SCHWEIGER, 2016). Ten sites from this survey were selected the chytrid fungus. All investigated waters are located in the Eastern part of Vienna.

Field work

The water samples were taken in three steps from April to June 2017. In the same time skin swabs of five amphibian species were taken from each locality. The plankton sampling was conducted until August 2017 (Appendix Tab. 2). The water parameters were noted in a "water sampling protocol" at each investigation (Appendix Fig. 1).

2.1.1 Sampling sites

Some of the investigated ponds are located along the Danube, some in the district Donaustadt and two waters are part of the Donau-Auen-National Park (Fig. 2). Rivers are also a part of the distribution of *Bd*, it was expected that *Bd* is present in spawning waters along the Danube (SZTATECSNY et al., 2011).

"Tritonwasser" (Fig. 2; Appendix Fig. 2) is an about two ha large artificially pond, constructed as wetland in the former flood area on the Danube Island. The pond was designed for ecological compensation. The initial planting such as water lily, yellow flag, reed, sedge, bulrushes, amphibious bistort, purple loosestrife, alisma and buttercup is still present. A big population of *Pelophylax* sp. inhabits this pond. Another pond on the Danube Island is the "Teuflteich", a very small artificially pond in a dog zone (Fig. 2; Appendix Fig. 7). The planting of the about 120 m² pond consists only of reed and willows. A water frog population is present.
Close to the Danube on the mainland is the "Krebsenwasser" (Fig. 2; Appendix Fig. 4). The about 260 m long and 6 m wide pond is an old Danube backwater in the Prater.

The depth is between 1 m and 1,6 m. Reed, yellow flag, hornwort and a deciduous forest in the surrounding are on-site. Numerous water frogs inhabit this pond.
Also, at the west side of the Danube the "Rudolf-Bednar Park" is located in the settlement area of Vienna (Appendix Fig. 15). There are thirteen very low concrete pools with 10 cm high stagnant water bodies and reed. A variety of aquatic Heteroptera are to be found. Green toads (*Bufotes viridis*) visit the ponds in the "Rudolf-Bednar Park" during the mating season.
About four kilometers away from the Danube the central cemetery of Vienna is located. There, a small pond which is totally overgrown by reed was investigated. Water frogs inhabit this pond and common toads are visitors during the mating season.

Two investigated ponds are located in the Donau-Auen-National Park. Only several meters of land separate the natural temporary water from the "Ölhafen Lobau" (Fig. 2; Appendix Fig. 3). In spring the pond is approximately 140 m long, 40 m wide and about 1 m deep during the summer the water dries up. Surrounding the water are deciduous trees and lots of deadwood. On site the reed canary grass is also part of the vegetation. The second investigated pond in the Donau-Auen-National Park is a natural water in the "Untere Lobau" with wide fluctuations in water levels because it is a canal with connection to the Danube. "Zainethau" is near to the border to Lower Austria and amid of a deciduous forest (Appendix Fig. 10). There are also reed belts with breeding areas for birds. The whole Lobau is a fire-bellied toad (*Bombina bombina*) distribution area.

The artificial pond "Badeteich Hirschstetten" in Donaustadt has a length of 540 m, a wide of 280 m, a max. depth of 10 m and a total size of 127.000 m² (Fig. 2; Appendix Fig. 8). In the 70s a gravel pit has been excavated which is filled by ground water. Around the pond there is a meadow with a sparse stand of trees and reed as riverbank vegetation. Next to the Badeteich is located "Blumengärten Hirschstetten" with several small ponds. The vegetation at the "Seerosenteich" in the "Blumengärten Hirschstetten" consists of reed, water lilies, willow and around the pond is a small stripe lawn. The pond is inhabited by fish, water turtles and water frogs (Fig. 2; Appendix Fig. 9). Another pond in Donaustadt is "Himmelteich" in Essling with a volume of 150.000 m³ (Fig. 2; Appendix Fig. 11). In the 30s a gravel pit has been excavated which was filled by household waste and rubble in the 70s. Afterwards the waste has been removed and it has been filled by ground water. The vegetation consists of reed and deciduous wood.

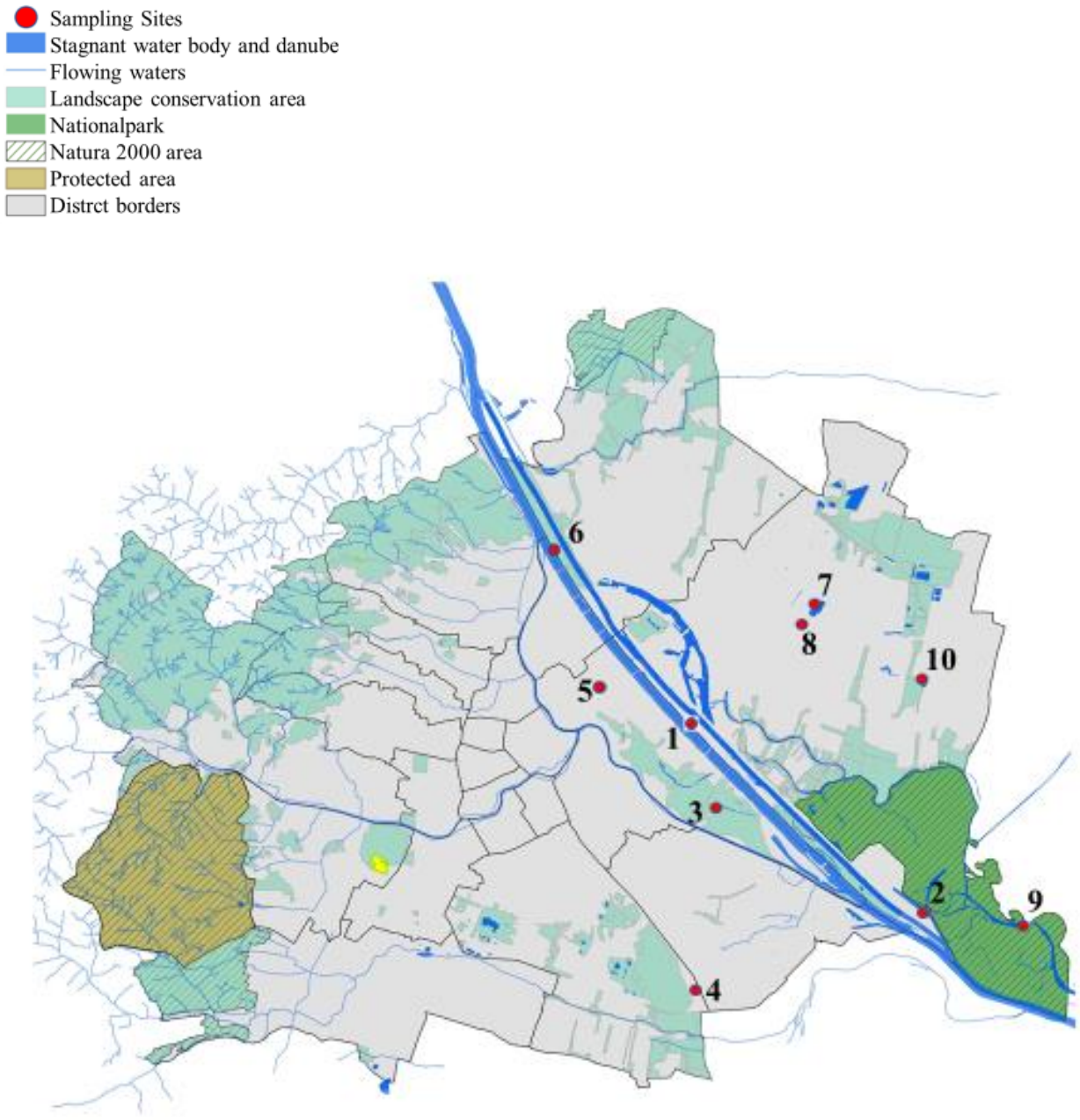

Fig 2: Research Area, City of Vienna: Sampling sites are marked with red dots.1 Tritonwasser (district Leopoldstadt); 2 Ölhafen (Donaustadt); 3 Krebsenwasser (Leopoldstadt); 4 Zentralfriedhof (Simmering); 5 Rudolf-Bednar Park (Leopldstadt); 6 Teuflteich (Brigittenau); 7 Badeteich Hirschstetten (Donaustadt); 8 Blumengärten Hirschstetten (Donaustadt); 9 Zainethau (Donaustadt); 10 Himmelteich (Donaustadt). MAP: viennagis, records: HFDÖ, Natural History Museum Vienna.

Tab. 1: Sampling sites with coordinates in GEOWGS84.

Site No.	Identification of sampling point	pondtype	vegetation	District	WGS84 O	WGS84 N
1	Tritonwasser	artificial,	forest clearing	Leopoldstadt	16,43063	48,21476
		gravel pond	danube island			
			reed			
2	Ölhafen	natural,	forest clearing	Donaustadt	16,51851	48,16668
		flooding area	National park			
			reed			
3	Krebsenwasser	natural	forest clearing	Leopoldstadt	16,44882	48,19341
		pond in Prater-Au	reed			
4	Zentralfriedhof	artificial	meadow	Simmering	16,4328	48,14533
		pond with foil	reed			
			edge of a small forest			
5	Rudolf-Bednar	artificial,	reed	Leopoldstadt	16,39628	48,22664
	Park	concreted tubs	meadow			
6	Teuflteich	artificial	edge of a small forest	Brigittenau	16,37694	48,25778
		pond with foil	danube island			
			reed			
7	Badeteich	artificial	some trees	Donaustadt	16,48118	48,24587
	Hirschstetten	gravel pond	reed			
			meadow			
8	Blumengärten	artificial	reed	Donaustadt	16,47561	48,23868
	Hirschstetten	pond with foil	meadow			
			park			
9	Zainethau	natural,	National park	Donaustadt	16,55933	48,16216
		flooding area	reed			
10	Himmelteich	artificial	edge of a small forest	Donaustadt	16,52037	48,22477
		gravel pond	reed			

2.1.2 Water sampling and filtration

Each pond was sampled three times with intervals of two weeks. Three liters of water were collected with two bottles at different points at each site. The filtration of the water samples was carried out by bottle top vacuum filtration system (Nalgene™ Rapid-Flow™ Sterile Disposable Bottle Top, Thermo Scientific) with membrane filters (Millipore membrane filters, diameter 47 mm, pore size 0,22 µl, CX09.1, Carl Roth) on site (Appendix Fig. 12). The car batterie supplied electricity over the double-pole DC connector (Appendix Fig. 13). This innovative method enabled to filter large volumes in short time in the field (was designed by Dominik RÜNZLER, FH Technikum Wien).

The throughput varied due to turbidity of the water samples. So, it was possible to filter three liters of a clear water sample in only 45 minutes whereas a strong turbidity took several hours. Subsequently the filters were collapsed in a falcon tube and stored in a cooling box on ice. After that, all falcon tubes were transported in a cooling box to the laboratory, where the filters were harbored in a freezer at a temperature of -20 °C. At every pass two or more (up to nine) membrane filters were used; altogether 95 filters were used for further analyzing after the completion of water sampling.

2.1.3 Protocol of parameters

The pH-value, oxygen content and water temperature were documented at each sampling site (Appendix Fig.1). These parameters were measured by a digital portable 2-channel pH-Meter (HQ30D Digital Multimeter-Kit, LDO Sensor, Outdoor). Furthermore, a description of the pond type was noted with a water sampling protocol. During the filtration, the volume of water that was filtered was also recorded (Appendix Fig. 20, 21, 22)

2.1.4 Skin swabs

In April, May and June five amphibians were captured at each site with a landing net and with latex gloves. Samples were taken by sterile cotton swabs (Heinz Herenz, Hamburg- Germany) from the ventral surface, flanks, feet and tail (for newts) in a standardized manner (20 times per individual). All animals were released at the point of capture immediately after sampling. Swabs were replaced in their original containers and stored at room temperature. The collected 50 skin swabs were analyzed by EISENBERG, expert veterinarian for microbiology in a laboratory in Germany (Landesbetrieb Hessisches Landeslabor LHL, Schubertstraße 60, Haus 13, 35392 Gießen). To avoid the transfer of the pathogen all equipment was cleaned by hot water and dried in the sun before reuse. The fire-bellied toad *Bombina bombina* is widespread in the Donau-Auen-National Park. All skin swabs of *B. bombina* derive from "Ölhafen" and "Zainethau", the two ponds, which are located in the National Park (Fig.2).

Almost all skin swabs of the common toad *Bufo bufo* are from individuals of a population in the pond "Zentralfriedhof" in the Viennese central cemetery.
All skin swabs of *B. viridis* were taken from individuals which were searching for partners in the shallow basins of the "Rudolf-Bednar Park" (Fig.2). Most of the skin swabs originated from water frogs (*Pelophylax lessonae, P. ridibundus, P. kl. esculentus*) of the remaining six waters, "Krebsenwasser" in the Prater, "Tritonwasser" and "Teuflteich" on the Danube island, swimming pond and "Blumengärten" in Hirschstetten and "Himmelteich" in the district Essling (Fig. 2). A long aquatic phase such as at water frogs and fire-bellied toads is proportional to the probability of infection by the Chytrid-fungus (SZTATECSNY & GLASER, 2011).

2.1.5 Plankton sampling

As the zoospores were expected near the amphibians the plankton samples were taken standing in the side water close to riverbank vegetation. The collections of zooplankton were realized by a plankton net with a mesh size of 40 µm or water lifter (5.000 ml) in a standardized manner. The net was cast three times per site, once was drawn through the upper water layers transversally, once was pulled vertically (max. deep 2 m if a bridge was present) and once next to water plants or dead wood. The plankton samples were taken from April to September 2017. The sampled 5.000 ml of water by water lifter was filtered by a filter with 20 µm pore size (Appendix Tab. 2). The plankton samples were used for qualitative investigation. Rotifera and Cladocera were determined to species level and Ostracoda and Copepoda to family level with a light microscope (BRESSER LCD-Schülermikroskop 8.9cm (3.5"); objectives: 4x, 10x, 40x (achromatic), enlargement: 50x-500x, 2.000x digital; Art. No.: 5201000 EAN: 4007922151953 and lecture of SANTIAGO GAVIRIA-MELO, MAX VOIGT & WALTER KOSTE.

2.2 Laboratory work

The laboratory work consisted of the following steps:

- DNA Extraction
- DNA Purification
- DNA Quantification using NanoDrop spectrophotometer
- Quantitative PCR
- Gel-electrophoresis I
- Purification (Wizard Kit)
- A-tailing
- Ligation (pGEM)
- Transformation (pGEM Vektor)
- Colony PCR of 10 E. coli colonies
- Gel-electrophoresis II
- DNA purification with Wizard Plus SV Minipreps
- Sequencing
- Blast of the Chytrid DNA with ApE to NCBI
- Determination of Zooplankton

2.2.1 DNA Extraction

For extraction of DNA the Puregene Core Kit A was applied according to KIRSHTEIN et al. (2007) All steps were carried out after the Gentra Puregene Handbook (06/2011). Each membrane filter was cut with a flamed scalpel in a separate tissue culture dish. The shreds were placed into prepared 2ml-tubes with 900 µl Lysis Buffer. A maximum of 24 tubes were placed in a thermomixer at a temperature of 65 °C for 15 minutes. After centrifugation and transfer of the liquids into 1,5 ml tubes 4,5 µl Puregene Proteinase K were added. The tubes were placed again into the thermomixer at 55 °C for one hour. Then 4,5 µl RNase was added and incubated at 37 °C for 1 hour. After a shot cooling on ice, 300 µl Protein Precipitation Solution was added, centrifugated and pipetted to 810 µl Isopropanol in 2 ml tubes (Appendix Tab. 7).

The solution was centrifugated again and the supernatant was discarded carefully, the pellets remained in the tubes which were subsequently washed with ethanol. Every pellet was solved in 50 µl DNA Hydration Solution in the thermomixer at room temperature and 300 rpm overnight. On the next day the tubes were frozen at -20 °C.

2.2.2 DNA Purification

For purification of DNA the QIAquick Gel Extraction Kit was applied. All steps were carried out according to the manual of the kit. At first the sample DNA diluted in 50 µl DNA Hydration Solution was thawed and 150 µl QG-Buffer was added. After 20 minutes in the thermomixer at 50 °C 50 µl of 99 % Isopropanol was added. Subsequently the solutions were transferred to collection tubes. After switching between centrifuge and adding of wash buffer, the purified sample DNA was diluted with 30 µl EB-buffer in the last step of purification (Appendix Tab. 8).

2.2.3 DNA Quantification using NanoDrop spectrophotometer

Before use of the NanoDrop spectrophotometer the settings were defined. The light factor 10 for double strand DNA in ng/µl was selected. Subsequently 4 µl of the DNA solution were pipetted for measurement 3 times for building an average (Appendix Tab. 9).

2.2.4 Quantitative PCR

The quantitative PCR was carried out with Stratagene Mx3005P and MxPro QPCR software. Triplets of purified water samples and control samples (positive, internal positive and negative control) were pipetted in 96-PCR-well-plate (PCR consumables Star Lab). The positive controls (Bd-2/B; Bd-3/B) was extracted from water samples of a 200 L aquarium with *Bd*-infected Lake Patzcuaro salamander *Ambystoma dumerilii* (Vienna Zoo). The internal positive control (IPC; Microsynth, Order No. 570022) was synthesized with following nucleotide sequence:

5'-TTGATATAATACAGTGTGCCATATGTCtacaatagctaagaagcctgaataGCTCAGCTTGTTTcaatttgactttcgccgttctagccgtttatttAACTTTTGACAACGGATCTCTTG-3' (including primer sequences and binding sequence for the probe).

As negative control was used autoclaved Milli-Q water from Millipore Corporation. At each qPCR the purified water samples and control samples were diluted with autoclaved Milli-Q water to 10 µl and a concentration of 12,5 ng/µl. The synthesized IPC had to be diluted much more due to the high degree of purity (dilution 1:100.000.000). Two µl of each sample and 18 µl of the mastermix (Appendix Tab. 10) were pipetted three times in the 96-PCR-well-plate (a total of 20 µl/well). All synthesized reagents were ordered at Mirosynth, the swiss DNA company. For every PCR run the same thermal profile (Appendix Fig. 15) was selected according to SZTATECSNY 2011 and slightly modified by KABRT (instead of 58 °C was set up 60°C). For detection of the genome equivalents (GE) a standard curve was designed (Appendix Fig. 16 & 17). This gives an information about the amount of the zoospores in the water samples, one zoospore contains 169 copies of the sequence which corresponds to 1,0 GE (KIRSHSTEIN et al., 2007). A dilution series with different number of copies was prepared. All the implementation steps followed the same scheme as the evaluation of the samples (Appendix Tab. 10).

2.2.5 Colony PCR

In order to check as early as possible (to avoid wasting resources) whether the qPCR technique delivered correct results, the sample with the only positive result of the first run was further investigated. For the colony PCR and subsequently DNA sequencing the qPCR-product of the sample Bd-2/B was selected. The sequences were separated in a 1,5 % Agarose-gel electrophoresis by length of base pairs (bp) (Appendix Fib.18). A marker of 100 bp (base pairs, N3231S, Thermo scientific) and a marker of 500 bp (base pairs, SM0623, Thermo scientific) were used to identify the correct region of the sample DNA. The region with a length of 120 bp was cut out and cleaned up according to the manuals of the kit Wizard SV Gel and PCR Clean-Up System (Appendix Tab. 12). Membrane binding solution was added to the gel slice and incubated at 55 °C. Like the DNA purification the solution was transferred into the collection

tubes for washing and centrifuge. Subsequently the A-Tailing procedure (Appendix Tab. 13) was carried out on ice after the technical manual of pGEM-T and pGEM-T Easy Vector Systems from Promega. The solution was incubated at 70 °C for 30 minutes into thermomixer. Thereafter as preparation for the transformation, the ligation was done with the pGEM-T Vector of Promega (Appendix Tab. 14). The solution was incubated overnight at 4 °C for increasing the number of transformants to a maximum. For the upcoming transformation LB Agar plates with ampicillin were prepared. Very carefully 2 µl of the ligation reaction was added into 50 µl-tube of NEB 5-alpha Competent *E. coli* cells (High Efficiency C2987H 50 µl-Tubes BioLabs Inc.). After a resting time of 30 minutes on ice the mixture was heat-shocked at 42 °C for 45 seconds into thermomixer.

Together with added 950 µl SOC-Medium (BioLabs Inc.) the mixture was incubated at 37 °C at 300 rpm for 1,5 hours. X-gal and IPTG stock solution were plated on each of two agar plates. Subsequently 100 µl of the transformation culture was distributed on these. The plates were deposited at 37 °C at 150 rpm into incubator overnight (Appendix Tab. 15). After a resting time (overnight) at 4 °C the blue-white-screening became visible thereby picking of the white colonies were easier. The 10 largest colonies were picked and dissolved in 10 tubes with 50 µl of DEPC-water (Diethylpyrocarbonate) and 1,5 µl of the bacterial suspension. All tubes were placed in thermocycler (Eppendorf Master cycler ep Gradient S) (Appendix Tab. 16).
Subsequently a gel electrophoresis (Appendix Fig. 19) was accomplished, and five samples were selected for MiniPrep (Appendix Tab. 18) and sequencing (Appendix Tab. 19). The bacterial colonies (Appendix Tab. 17) of these four samples were placed inside a crystal tube containing around 4 ml of LB Broth with Ampicillin overnight at 37°C, at gently shaking.

All samples were turbid and subjected to Miniprep on the next day according to the instruction for use of Wizard Plus SV Minipreps DNA Purification System (Promega, USA). After centrifugation and washing, the plasmid DNA was eluted with nuclease-free water. The concentrations were measured with a spectrophotometer (NanoDrop, Implen, Typ: Pearl) but only four of the remaining five samples were suitable (concentration above 60 ng/µl) for sequencing performed by Microsynth. The nucleotide sequences were blasted with ApE (A plasmid Editor) to NCBI (National Center for Biotechnology Information) (Appendix Fig. 20).

III RESULTS

Altogether 95 filters were used to take water samples at ten sites in Vienna (Fig. 2). Each site was investigated three times. Due to different water qualities, a different number of filters was used. At the first pass of water sampling 43 filters were used, at the second pass 28 filters and at the third pass 24 filters (Tab. 3). Together with *Bd*-positive water samples (aquatic water of infected *Ambystoma dumerilii*) the samples of the ponds were evaluated in several runs by qPCR. The Chytrid-fungus was detected at three out of ten sampling sites. The ponds "Blumengärten Hirschstetten" (one filter), "Teuflteich" (two filters) and "Krebsenwasser" (two filters) were tested *Bd*-positive. Almost all of the taken 50 skin swabs were *Bd*-negative with the exception of two cases. The Chytrid-fungus was detected at one *Pelophylax* sp. of the pond number three, "Teuflteich" and one infected *Pelophylax* sp. was found at pond number seven, "Badeteich Hirschstetten" (Fig. 2).

3.1 Amplification curves

The results of the qPCR-runs are represented by amplification curves. Positive tested samples indicate three curves (each sample was applied thrice per plate) which exceeding the threshold (green line) up to a maximum Ct-value of 35 cycles. The amplification curves of the samples Bd-2/B and Bd-3/B of the aquarium water with *Bd*-infected amphibians from the Vienna Zoo significantly exceeded the threshold at every qPCR-run with a Ct-value of 26 (Appendix Tab. 18, 19, 20). The figures with amplification curves (Fig. 3, 4, 5) show only a small extract of all runs of qPCR.

Pass 1

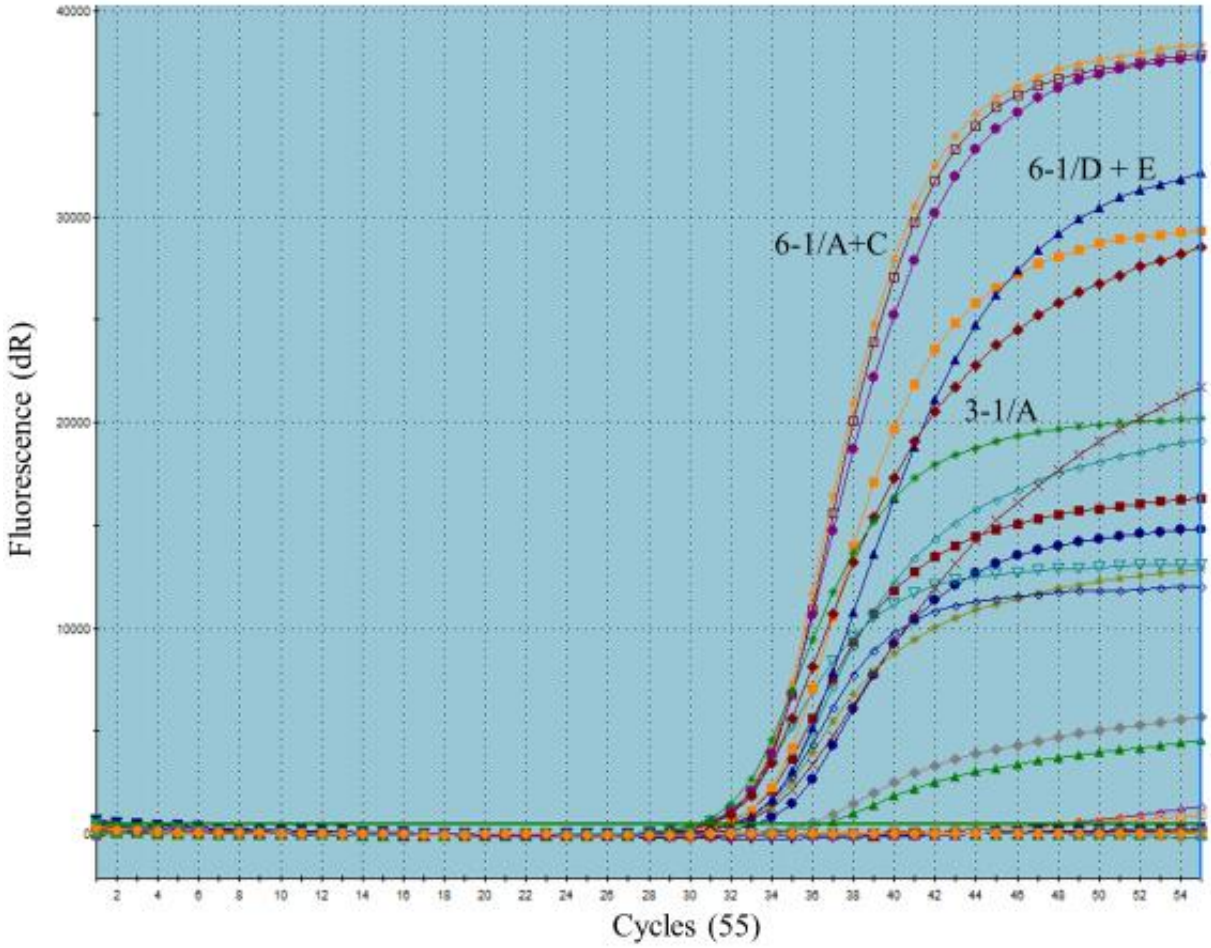

Fig. 3: Amplification curves of water samples: 1-1/A, 1-1/B, 1-1/C, 3-1/A, 5-1/F, 6-1/A+C, 6-1/D+E, 7-1/B, 7-1/C, 8-1/D, 8-1/F, 9-1/AC, 9-1/B, 10-1/B, BD-controls: Bd-1, Neg (Milli-Q-water); green line = threshold; Bd-positive at 3 peaks above the threshold (max. up to Cycle 35); date of qPCR 3.1.2018

The evaluation of the samples collected in April and May indicates that three of 43 filters were clearly tested positive. Each positive detected sample passed the line three times close together and within 35 cycles. "Krebsenwasser" with a sample of filter 3-1/A and "Teuflteich" with samples of filters 6-1/A+C and 6-1/D+E crossed the threshold at about a Ct-value of 30 (6-1/A+C), 31 (6-1/D+E) and 32 (3-1/A) thus located in the *Bd*-positive field (Tab. 2). All remaining samples (1-1/A, 1-1/B, 1-1/C, 5-1/F, 7-1/B, 7-1/C, 8-1/D, 8-1/F, 9-1/AC, 9-1/B, 10-1/B) of "Tritonwasser", "Rudolf-Bednar Park", "Badeteich Hirschstetten", "Blumengärten Hirschstetten, "Zainethau" and "Himmelteich" recorded only single curves (Fig. 3).

Pass 2

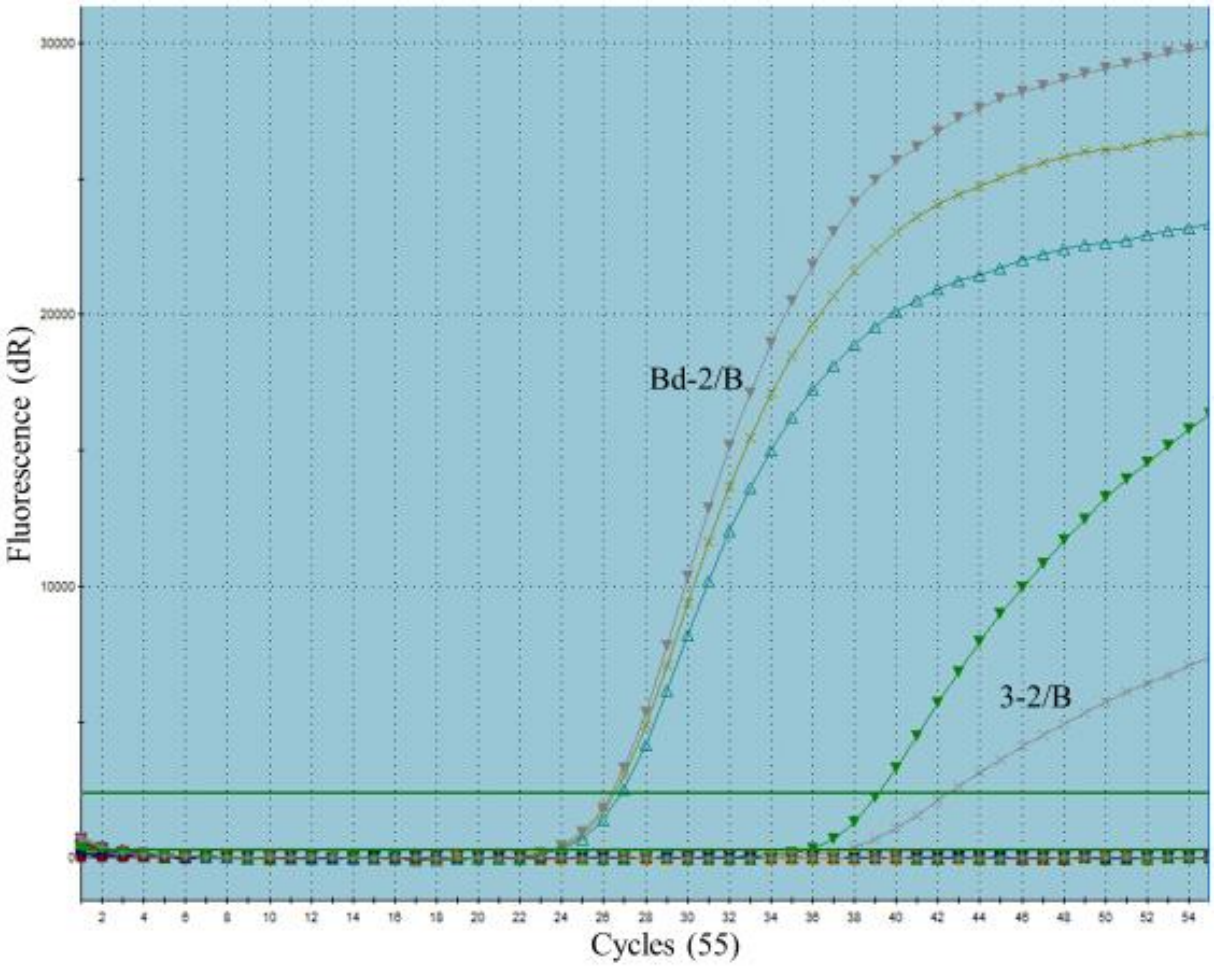

Fig. 4: Amplification curves of water samples: 1-2A, 2-2/A, 2-2/B, 3-2/A, 3-2/B, 4-2/A, 5-2/A, 5-2/B, 5-2/C, 6-2-A, 7-2/A, 8-2/A, 9-2/B, 10-2/A; controls: Bd-2/B, IPC, Neg (Milli-Q-water); green line = threshold; *Bd*-positive at 3 peaks above the threshold (max. up to Cycle 35); date of qPCR 5.9.2017

None of the evaluated samples of the second pass (28 filters) indicated any fluctuations with the exception of sample 3-2/B of "Krebsenwasser", but at only two of possible three deflections are judged as negative result (Fig. 4). A new sample of the same filter extract (3-2/B) was examined a second time and passed the threshold three times (Fig. 5).

Pass 3

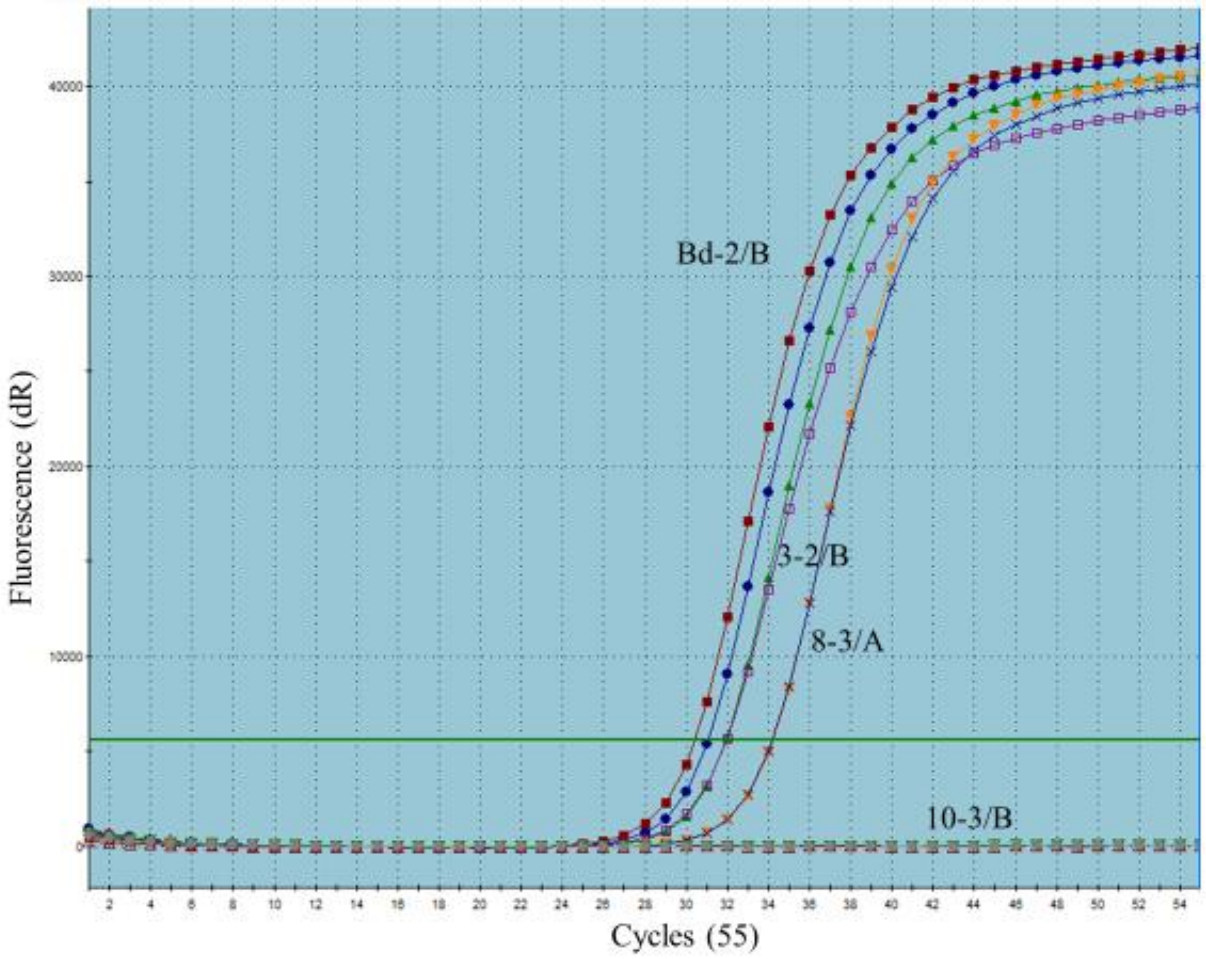

Fig. 5: Amplification curves of water samples: 3-2/B, 6-3/A, 8-3/A, 10-3/B; controls: Bd-2/B, Neg (Milli-Q-water); green line = threshold; Bd-positive at 3 peaks above the threshold (max. up to Cycle 35); date of qPCR 29.12.2017

The evaluated samples of the filters 3-2/B and 8-3/A passed the threshold three times close together and within 35 cycles. The Chytrid-fungus was detected in the water sample 3-2/B of "Krebsenwasser" collected in May and in the water sample 8-3/A of "Blumengärten Hirschstetten" collected in June. The samples of the filters 6-3/A ("Teuflteich") and 10-3/B ("Himmelteich") stayed below the threshold and thus were *Bd*-negative (Fig. 5).

Only for a few samples of the first pass (43 filters) and third pass (24 filters) were detected the Chytrid-fungus. Five (4,75 %) of 95 samples are clearly contaminated by *Bd* (Tab. 2). Using eDNA technique the pathogen was determined at three sampling sites in three different districts of Vienna.

3.2 Filtration and water parameters

Tab. 2: Parameters such as temperature, oxygen content and pH-value at each site (pond with district) and each pass; Date; number and time of filtration; positive results of skin swabs and filters; 1 GE (Genome Equivalents) = 169 copies = 1 zoospore.

Sampling sites	Site No. + sample identification	Water temperatur °C	Oxygen content mg/L	pH-value	Date	Filters	Time of Filtration	positive skin swabs	positive water samples	GE of water samples
Tritonwasser	1-1	17,3	13,36	8,31	05.05.	4	2h			0
Leopoldstadt	1-2	19	4,66	7,5	22.05.	3	1h42min			0
	1-3	27	5,8	7,53	15.06.	2	2h			0
Ölhafen	2-1	16,3	7,44	7,13	05.05.	1	45min			0
Donaustadt	2-2	17,5	7,97	7,59	22.05.	2	1h			0
	2-3	22,5	6,77	8,43	16.06.	1	2h			0
Krebsenwasser	3-1	13,5	9,59	7,55	26.04.	3	1h		positive	7
Leopoldstadt	3-2	18	4,71	7,12	11.05.	3	2h		positive	10
	3-3	24,6	4,6	7,32	15.06.	2	2h30min			0
Zentralfriedhof	4-1	13	11,66	7,57	01.05.	2	30min			0
Simmering	4-2	17,5	10,18	7,25	15.05.	1	1h			0
	4-3	20,6	14,63	8,7	16.06.	2	2h			0
Rudolf-Bednar	5-1	19,8	20,01	8,52	01.05.	8	2h30min			0
Park	5-2	23,1	17,78	8,53	15.05.	3	3h30min			0
Leopoldstadt	5-3	29,2	19,96	9,2	12.06.	4	3h			0
Teuflteich	6-1	12,8	12,28	7,57	29.04.	9	5h		positive (2)	10
Brigittenau	6-2	17,6	14,49	8,01	16.05.	6	3h30min	positive		0
	6-3	24,7	15,16	8,35	12.06.	4	4h30min			0
Badeteich	7-1	15,6	10,67	7,68	30.04.	4	1h	positive		0
Hirschstetten	7-2	19	9,49	8,07	21.05.	2	1h			0
Donaustadt	7-3	26,3	10,15	8,41	13.06.	2	1h			0
Blumengärten	8-1	14,2	11,45	7,68	4.5.	6	2h			0
Hirschstetten	8-2	19,7	10,16	7,86	21.5.	4	2h30min			0
Donaustadt	8-3	25,3	10,3	8,31	13.6.	4	2h40min		positive	7
Zainethau	9-1	15,8	11	7,58	30.4.	2	1h			0
Donaustadt	9-2	26	10,7	7,56	16.5.	2	1h			0
	9-3	26,7	10,11	7,85	15.6.	1	1h30min			0
Himmelteich	10-1	13	11,31	7,28	29.4.	4	1h			0
Donaustadt	10-2	18	9,65	7,48	15.5.	2	2h			0
	10-3	24,2	8,86	7,74	14.6.	2	2h			0

The water temperature at the first sampling pass ranged between 12,8 °C ("Teuflteich") and 17,3 °C ("Tritonwasser"). The oxygen content ranged between 7,44 mg/L ("Ölhafen") and 20,01 mg/L ("Rudolf-Bednar Park"). The pH-value varied between 7,31 and 8,52. Three positive evaluated water samples and two positive tested skin swabs were collected at approximate 13 °C water temperature, 10 mg/L oxygen content and a pH-value of 7,5 (Tab. 3). Two additional *Bd*-positive samples had other parameters at the time of sampling such as higher temperature, lower oxygen content or moderately alkaline pH value.

At the second sampling pass the temperature ranged between 17,5 °C ("Ölhafen") and 26 °C ("Zainethau"). The lowest oxygen content was 4,66 mg/L ("Tritonwasser") and the highest value was 17,78 mg/L ("Rudolf-Bednar Park"). The pH-value varied between 7,12 ("Krebsenwasser") and 8,53 ("Rudolf-Bednar Park"). One of the three filters from "Krebsenwasser" which were emerged on 26th of April (taken at 18 °C, approximate 5 mg/L oxygen content and a pH-value of 7) was Bd-positive tested (Tab. 3).

In the third and final sampling pass temperatures were measured from a minimum of 20,6 °C ("Zentralfriedhof") up to a maximum of 29,2 °C ("Rudolf-Bednar Park"). The oxygen content varied between 4,6 mg/L ("Krebsenwasser") and 19,96 mg/L ("Rudolf-Bednar Park"). The pH-value reached a minimum of 7,32 and a maximum of 9,2 ("Rudolf-Bednar Park"). Only one sample of the final sampling pass was *Bd*-positive (Tab. 3).

The genome equivalents of 7 suggest that the PCR-product of the sample from "Krebsenwasser" filtered on 26th of April contained DNA sequences of seven *Bd*-zoospores. The same applies to the sample from "Blumengärten Hirschstetten" taken at a water temperature of 25 °C, oxygen content of 10 mg/L and a pH value of 8 on the 13th of June. Three PCR-products indicated a value of 10 GE that equates to ten zoospores. Two derived from the nine used filters at "Teuflteich" on the 29th of April. Another Sample of a PCR-product with 10 zoospores was taken at "Krebsenwasser" on 11th of May. The parameters of the two sampling sites indicated slightly differences in temperature and pH value but a significant difference in the oxygen content.

3.3 Colony PCR alignment

For the colony PCR and subsequently DNA sequencing the qPCR-product of the sample Bd-2/B was selected which had the only *Bd*-positive result of the first qPCR-run. This water sample contained zoospores from the aquarium with the *Ambystoma dumerilii* which originally came from Mexico. The follow-up investigation through the colony PCR confirmed the assumption that positive results by qPCR indeed contained DNA sequence of *Batrachochytrium dendrobatidis*.

The result of the sequencing by Microsynth rendered at all evaluated colonies the same DNA sequence which does match to 99% (Appendix Fig. 20) with the isolate UM142 and 5.8S ribosomal RNA sequence of *Batrachochytrium dendrobatidis* in the NCBI database. The isolate UM142 was identical to the *Bd* isolate from a bullfrog collected in Brazil and to the lineage *Bd*ASIA-2 / *Bd*BRAZIL.

3.4 Skin swabs

The independent survey by skin swabs gave additional results. At the ten sampling sites 30 water frogs (*Pelohylax* sp.), nine fire-bellied toads (*Bombina bombina*), six common toads (*Bufo bufo*) and five green toads (*Bufotes viridis*) were caught and after sampling released (Tab. 3). The evaluation of the 50 skin swabs revealed that only two of the individuals were infected. Two adult individuals belonged to the genus *Pelophylax* were tested *Bd*-positive (Tab. 3). All skin swabs were taken between 26th of April and third of July. The two positive tested swabs emanated from "Teuflteich" (Danube Island) and "Badeteich Hirschstetten" (Fig.2). No infected individual showed any skin lesions.

Tab. 3: Results of skin swabs; four tested amphibian species at ten sites; number of positive and negative species; a total of 50 skin swabs

Sampling site	Date	Skin swab No.	Species	Juvenil/ Adult	Skin swabs *Bd* -pos.	Water-samples *Bd* -pos.
Tritonwasser	05.05.	1-1	*Pelophylax* sp.	a	—	—
	22.05.	1-2	*Pelophylax* sp.	a	—	—
	22.05.	1-3	*Pelophylax* sp.	j	—	—
	26.06.	1-4	*Pelophylax* sp.	a	—	—
	26.06.	1-5	*Pelophylax* sp.	j	—	—
Ölhafen	05.05.	2-1	*Bombina bombina*	a	—	—
	05.05.	2-2	*Bombina bombina*	a	—	—
	22.05.	2-3	*Bombina bombina*	j	—	—
	22.05.	2-4	*Bombina bombina*	j	—	—
	22.05.	2-5	*Bombina bombina*	j	—	—
Krebsenwasser	26.04.	3-1	*Pelophylax* sp.	a	—	positive
	11.05.	3-2	*Pelophylax* sp.	a	—	positive
	11.05.	3-3	*Pelophylax* sp.	j	—	—
	15.06.	3-4	*Pelophylax* sp.	a	—	—
	15.06.	3-5	*Pelophylax* sp.	a	—	—
Zentralfriedhof	25.06.	4-1	*Bufo bufo*	j	—	—
	25.06.	4-2	*Bufo bufo*	j	—	—
	25.06.	4-3	*Bufo bufo*	j	—	—
	25.06.	4-4	*Bufo bufo*	j	—	—
	25.06.	4-5	*Bufo bufo*	j	—	—
Rudolf-Bednar Park	04.06.	5-1	*Bufotes viridis*	a	—	—
	12.06.	5-2	*Bufotes viridis*	j	—	—
	12.06.	5-3	*Bufotes viridis*	j	—	—
	16.06.	5-4	*Bufotes viridis*	a	—	—
	16.06.	5-5	*Bufotes viridis*	a	—	—
Teuflteich	29.04.	6-1	*Pelophylax* sp.	a	positive	positive (2)
	16.05.	6-2	*Pelophylax* sp.	a	—	—
	16.05.	6-3	*Pelophylax* sp.	a	—	—
	16.05.	6-4	*Pelophylax* sp.	a	—	—
	16.05.	6-5	*Pelophylax* sp.	a	—	—
Badeteich Hirschstetten	30.04.	7-1	*Pelophylax* sp.	a	positive	—
Hirschstetten	16.05.	7-2	*Pelophylax* sp.	a	—	—
	16.05.	7-3	*Pelophylax* sp.	a	—	—
	16.05.	7-4	*Pelophylax* sp.	a	—	—
	16.05.	7-5	*Pelophylax* sp.	a	—	—
Blumengärten	04.05.	8-1	*Pelophylax* sp.	j	—	—
Hirschstetten	04.05.	8-2	*Pelophylax* sp.	a	—	—
	21.05.	8-3	*Pelophylax* sp.	a	—	—
	29.06.	8-4	*Pelophylax* sp.	a	—	positive
	30.06.	8-5	*Pelophylax* sp.	j	—	—
Zainethau	30.04.	9-1	*Bombina bombina*	a	—	—
	30.04.	9-2	*Bombina bombina*	j	—	—
	30.04.	9-3	*Bombina bombina*	j	—	—
	16.05.	9-4	*Bombina bombina*	j	—	—
	16.05.	9-5	*Pelophylax* sp.	j	—	—
Himmelteich	29.04.	10-1	*Pelophylax* sp.	a	—	—
	03.07.	10-2	*Bufo bufo*	a	—	—
	03.07.	10-3	*Pelophylax* sp.	a	—	—
	03.07.	10-4	*Pelophylax* sp.	a	—	—
	30.06.	10-5	*Pelophylax* sp.	a	—	—

3.5 Identification of Plankton

Rotifera species were found at every sampling site. At eight of ten sampling sites the families Colurellidae and Notommatidae were determined. Synchaetidae was detected at six sites and at five sites the families Brachionidae and Euchlanidae were found. The remaining families such as Asplachnidae, Dicranophoridae, Epiphanidae, Lecanidae, Scaridiidae, Trichocercidae, Collothecidae, Filinidae, Flosculariidae and Hexarthridae occurred much less often. Crustacea such as Cladocera and Copepoda were identified also at few sites. The greatest diversity of Rotifera was detected at "Teuflteich" with 18 species, "Krebsenwasser" with 14 species and "Ölhafen" with ten species. At the remaining sites only seven, four or three species of the phylum Rotifera were determined. Crustaceans were identified most often at "Zainethau" (Appendix Tab. 4).

Tab. 4: Number of species of rotifers families and of crustacea families per sampling site from April until September 2017.

Sampling site: / Zooplankton:	Tritonwasser	Ölhafen	Krebsenwasser	Zentralfriedhof	Rudolf-Bednar Park	Teuflteich	Badeteich Hirschstetten	Blumengärten Hirschstetten	Zainethau	Himmelteich
Rotifera:										
Asplachnidae		1	1							
Brachionidae	1	1	3			4		1		
Colurellidae	1	1	1	1	1	1			2	
Dicranophoridae			1							
Epiphanidae			1			1				
Euchlanidae	1	1	1				1	2		
Lecanidae		2	1							1
Notommatidae	1	2	1			2	1	2	2	1
Scaridiidae			1							
Synchaetidae		1	2	1		2	1	1		
Trichocercidae			1		1	4				
Collothecidae						2				
Filinidae						1		1		
Flosculariidae		1				1				
Hexarthridae						1				
Crustacea										
Cladocera:										
Bosminidae									1	
Chydoridae		1							2	1
Daphniidae									1	1
Copepoda:										
Polyphemidae				1					1	
Diaptomidae									1	
Cyclopidae		1								
Ostracoda:										
Podocopida						1	1			

3.6 Occurrence of *Bd*-positive tested sites and potential planktonic predator

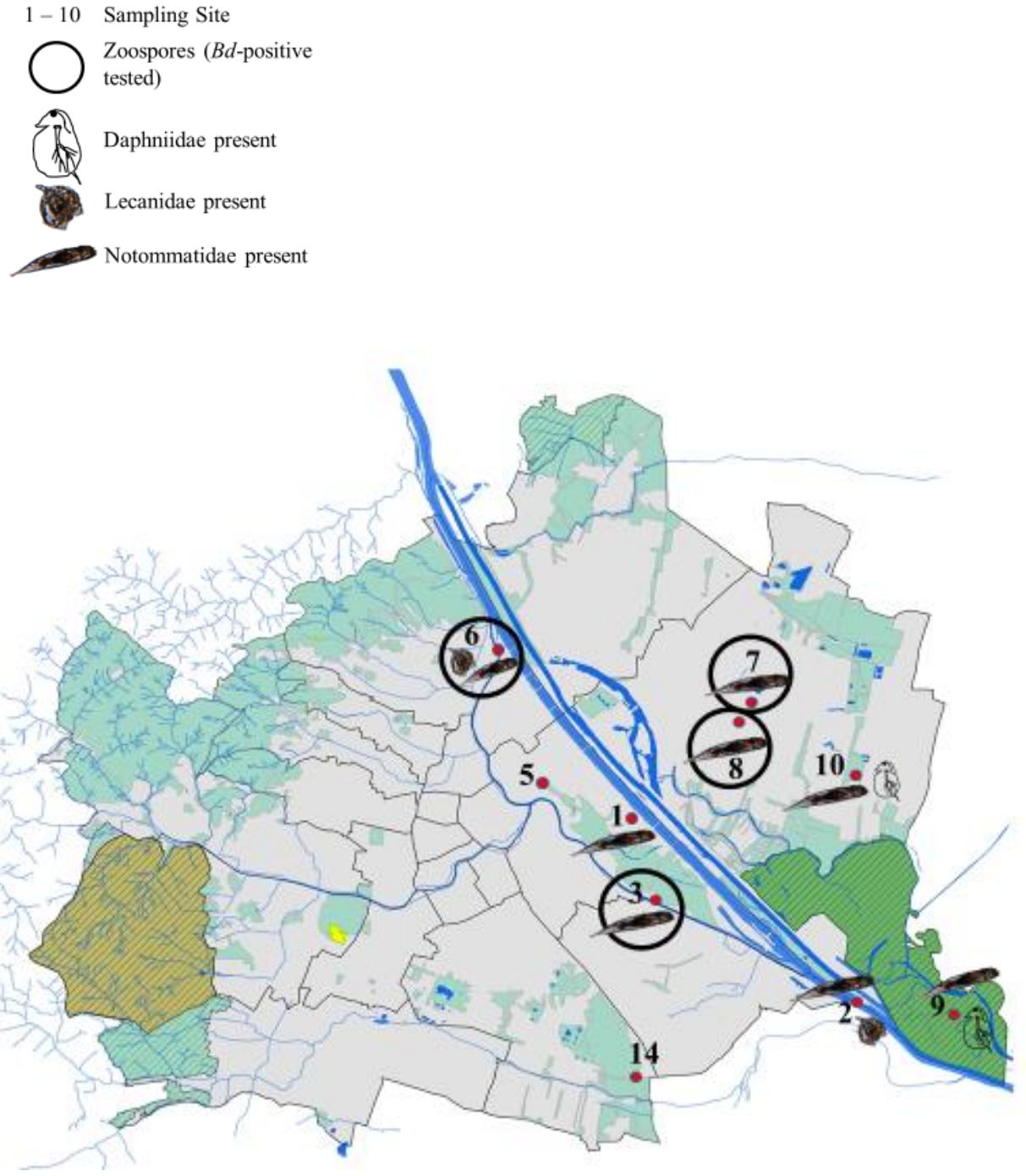

Fig. 7: Map of Vienna with sampling sites from pond 1 to pond 10; potential planktonic predator like rotifers detected at all encircled numbers; *Daphnia* present at site 9 and 10; zoospore within the circle identified *Bd*-positive sites. MAP: viennagis, records: HFDÖ, Natural History Museum Vienna.

In the water samples of all ten ponds rotifers and in almost all crustaceans were found (Tab. 4; Fig. 7). Potential planktonic predators such as Notommatidae and Lecanidae were detected for eight locations and four (50 %) of these were *Bd*-positive. Daphniidae was determined in only two ponds.

IV DISCUSSION

The occurrence of the pathogen *Bd*, was only confirmed at four of ten sites (five positive filters and two positive skin swabs). The remaining 90 filters and 48 skin swabs were tested *Bd* negative (Tab. 2). Multiple sampling of the ponds was important to detect *Bd* by water filtration (HYMAN & COLLINS, 2012) and to mitigate biases (MOSHER et al., 2017). The positive results in the eDNA technique were more significant under laboratory conditions. The water samples (Bd-2/3 B) of the aquarium containing *Bd*-positive amphibians indicated constant results (26 Ct) compared to the samples from the nature. The *Bd*-positive samples taken from the field contained only a few zoospores (about 10) whereas the samples of the aquarium with a volume of about 150 liters contained approximately 100 spores (100 GE; Appendix Fig. 20, 21 & 22). Biases in *Bd* quantification can be persistent (MOSHER et al., 2017). Inhibitors in the qPCR reaction can reduce affinity between probe and binding site, changes in structural conformation or mutations can affect probe/primer binding sites or the copy number/genome size can vary (LONGO et al., 2013). The efficiency (102 %) of the standard curve and the constant results of the internal positive control (aquarium water samples) in each qPCR can eliminate the possible fault causes.

The water volume of the different ponds was larger, the oxygen content, pH-value and the temperature varied depending on locality and size (Tab. 2). The standardized quantity of each water sample was 1,5 l per filter (in laboratory), but in nature these were difficult to carry out due to the turbidity of the water. The most filters were used for "Teuflteich" und "Rudolf-Bednar Park" with a minimum flow of 200 ml up to a maximum of 3 l per filter (Tab. 2; Appendix Tab. 20, 21, 22). A larger volume of water (200 ml to 3 l, mean 1.140 ml) was filtered by the vacuum pump than with the method through a syringe (range: between 20 ml and 2.4 l, mean 350 ml) (CHESTNUT et al., 2014). The fact that only 4 % of the skin swabs indicated *Bd*-positive results matches the small amount of detections by eDNA. Only two of the 30 investigated water frogs were infected but no skin lesion such as loosing of sloughed skin (VOYLES et al., 2009) or abnormal posture of legs (ROOIJ et al., 2015) were visible. Even the two infected frogs (Tab. 3) seemed to be healthy. Apparently, the susceptibility to get infected by *Bd* depends on other factors such as seasonal differences including environmental variables, host immunological, behavioral and ecological characteristics, virulence of different *Bd* strains or a combination of all (CAMPELL, 2012).

The host responses can be defined as susceptible, tolerant or resistant depending on the species. Some infected amphibians die, other recover and develop pathogen-inhibiting factors (ROOIJ et al., 2015). The amphibian skin mucus disposes defense factors like antimicrobial peptides (AMPs), lysozymes and mucosal antibodies and symbiotic skin bacteria producing antifungal metabolites against colonization by pathogens (ROLLINGS-SMITH, 2009). Many species of the families Bombinatoridae and Ranidae synthesize and release cationic alpha-helical antimicrobial peptides, which are able to disturb biological membranes (ROLLINGS-SMITH, 2009).

The mucous glands of the edible frog (*Pelophylax esculentus*) produce mucins including polysaccharides such as N-acetylglucosamine, which is a natural substrate of lysozyme with potential fungicidal potential. The dermal granular glands produce Antimicrobial peptides (AMPs), as first line defense against *Bd*, reducing the infection load on amphibian skin to tolerable levels or even clearing them from infection. The efficacy of skin peptide defenses may vary at species and population (WOODHAMS et al., 2007; ROOIJ et al., 2015). Most of the investigated individuals belonged to the *Pelophylax* group, which usually develops this type of defense.

Another factor may, which influence the outbreak of infections is the glucorticoid stress hormone corticosterone. Several studies have shown that an increasing stress level influences the outbreak of infections (ROOIJ et al., 2015).
In this study, direct human contact was noted at four sampling sites, but whether the stress level of the animals was increased, need further investigation. "Badeteich Hirschstetten" (Donaustadt; Tab. 1; Fig. 2) was visited by numerous bathers and many dogs. The "Blumengärten Hirschstetten, " is a highly frequented recreation space of the city of Vienna and accordingly there are many visitors. "Teuflteich" (Brigittenau; Tab. 1, Fig. 2) on the Danube Island is located in a dog area where during this investigation several dogs were taking a bath. Only at the sampling site "Krebsenwasser" (Leopoldstadt; Tab. 1; Fig. 2) disturbances by humans were hardly noticeable unlike the other three positive ponds. At the sites "Rudolf-Bednar Park" (Leopoldstadt) and "Himmelteich" (Donaustadt) high fluctuation and road traffic probably affect amphibian behavior patterns, but the fungus was not present there. All other sampling sites are less visited by humans and there are more hiding spots for amphibians such as abundant reed and other vegetation.

In general, *Bd* outbreaks occur in colder seasons, a weakened amphibian immune defense and an increased zoospores release was observed. Drops in temperature correlate more to amphibian declines than increases in temperature (RAFFEL et al., 2015).
The Chytrid-fungus exhibited temporal and spatial heterogeneity in detection and density in amphibian habitats. In temperate areas, there is *Bd* seasonal variation in infection prevalence. In a study in North America was detected *Bd* in all months surveyed, but not in all water samples and with a clear signal of increased *Bd* density in the spring and in the summer month August. (CHESTNUT et al., 2014)
In this study, the water temperatures of the ponds increased continuously between April and June. Almost all positive tested samples were taken at a water temperature between 12,8 °C and 18 °C, and contrary to prediction one sample was tested positive taken at a temperature of 25,3 °C. Temperatures ranged between 20,6 °C and 29,2 °C (at eight ponds over 24 degree) at the last pass of this investigation (Tab. 2). Therefore, during the hot summer months, the fungus has no optimal environmental conditions in the investigated ponds. It is not surprising that there was almost no *Bd* evidence, because average temperature prevailed of 25 °C in the ponds at the last survey (RAFFEL et al., 2015; WOODHAMS et al., 2008; Tab. 2).

The climatic variability and temperature shift have an influence on the hosts and their pathogens. According the climate variability hypothesis the pathogens may have an advantage in adaptation to temperature change due to their smaller size and faster metabolisms (RAFFEL et al., 2015). An increased occurrence of *Bd*-related disease was related to rising temperatures in moderately severe winters in the northern temperate zones (BOSCH et al., 2007; XIA et al., 2014). Between years 1948 and 2010, the strongest rates of spring and winter warming have been observed to occur more frequently in the Northern Hemisphere (XIA et al., 2014).
Before 2017, winters in Austria were less harsh. In January and February 2017, just before the survey, the sampling sites were covered by thick ice layers for several weeks. The samples, collected in spring 2017 contained only some *Bd* zoospores, this could be due to preceding unusual cold periods. The occurrence of *Bd* does not only depend on the temperature but also on the amount of precipitation (XIE et al.; 2016). In future, because of climate change, the distribution of *Bd* will shift into higher latitudes and altitudes (XIE et al.; 2016). The model by XIE et al. (2016) showed a broad expansion of areas which are particularly suitable for the fungus in the temperate zones, but it does not consider the effects of coevolution or local adaptations.

The planktonic communities in intact ecosystems may have a positive influence on the prevention of chytridiomycosis. Some planktonic species of Rotifera (Notommatidae and Lecanidae) are effective consumers of *Bd* zoospores. An experiment with the tadpoles of the Moroccan painted frog (*Discoglossus scovazzi*) by SCHMELLER et al. (2014) showed that none of the individuals developed infections by *Bd* if they occur together with species of the family Notommatidae. Grazing crustaceans such as Daphnia sp. consume also the zoospores and so reduce the infection rate of amphibians (HAMILTON et al., 2012).

Despite the occurrence of Rotifera at all sampling sites, *Bd*-zoospores were detected at four of the ten investigated ponds (Tab. 2; Fig. 7). "Teuflteich" is the smallest pond with the highest number of species of Rotifera (19) and a triple detection of the Chytrid-fungus. All observed frogs at this pond appeared to be healthy. It is possible that the existing zooplankton community reduced the number of free-swimming spores in the water, providing an explanation for the low number of detected zoospores. That applies to the other three *Bd*- positive tested ponds ("Krebsenwasser", "Badeteich Hirschstetten" and "Blumengärten Hirschstetten"; Tab. 1; Fig. 2). Plankton sampling took place at different times because the equipment needed to be completely cleaned and dried before reuse to avoid transmission of *Bd*. All data are collected from April to September (temperature range between 12,8 °C and 29,2 °C; Tab. 2). All ponds show differences in relation to size, depth, temperature, oxygen content, pH-value and solar radiation (Tab. 2). Also, ultraviolet radiation could reduce the zoospores but also the density of plankton (HITE et al., 2016). In the eutrophic pond "Teuflteich" the visible depth was under a meter caused by high density of unicellular algae (Fig. 2; Tab. 1). This high concentration may have diminished the disease risk for amphibians, because the chemical substances that *Ankistrodesmus falcatus* (green algae) gives off destroy the zoospores. An additional positive effect at high density of algae is the increased digestion rate of *Daphnia* (SEARLE et al., 2013).

The eDNA technique was successful under constant laboratory conditions and in small volumes but in nature only a few traces of *Bd* were detected. This can be caused by the variety in environmental conditions or the different volumes of the ponds or just by a lower occurrence of the pathogen at the sampling sites outside the laboratory. A climate change is to notice in Austria due to the generally increase in temperature with heat waves in summer and severely diminished precipitation with drought especially in eastern Austria including Vienna. Due to the amphibian decline and the threat of *Bd*, it will be required to continue observations.

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CONTENTS (APPENDIX)

IV. LABORATORY VALUES

I. FIELD WORK

1.1 Water sampling Protocol

At each sampling site three liters of water were taken and a "water sampling protocol" was filled out. One of them is attached below as example (Tab. 2).

Water sampling protocol

Identification of sampling point	Krebsenwasser	Protocol number	13
Name	Vesely, Disacke, Nickzad	Start	12:10
Date	26.4.2017	End	13:30
Atmospheric conditions	cloudy	Air temperature in °C	16
Atmospheric pressure in hPa	1006	Weather previous day	Sun, warm

Water body

Description/Vegetation	natural pond, reed, wood		
Species	*Pelophylax ridibundus*		
Size in m²		Colour	clear ■
Depth in m			turbid □
Water temperature °C	13,5	Odour	normal ■
Oxygen content mg/L	9.59		must □
pH-value	7.55		different □

Photo documentation: (including points of sampling)

1. Filter: 1000 ml; 13-1/A
2. Filter: 1100 ml; 13-1/B
3. Filter: 900 ml; 13-1/C

1400 ml for investigation of Plankton-community

Samples

Sample identification	Notes
13-1	Skin swab of one *Pelophylax ridibundus* (adult) Plankton sampling by self-made water lifter

Fig. 1: Water sampling protocol of Krebsenwasser in the Praterau

1.2 Photos of sampling sites

Fig. 3: Ölhafen; temporary water in the National Park Donau-Auen; Tab. 1.

Fig. 4: Krebsenwasser; in the Prater Au; Tab. 1.

Fig. 5: Zentralfriedhof; a small artificial pond in the Vienna central cemetery; Tab. 1.

Fig. 6: Bednar Park; eight basins of concrete; see tab.1

Fig. 7: Teuflteich; on the Danube Island; artificial pond; Tab. 1.

Fig. 8: Badeteich Hirschstetten; big swimming pond; a gravel pit filled by ground water; Tab. 1.

Fig. 9: Blumengärten Hirschstetten; pond in a park; Tab. 1.

Fig. 10: Zainethau; a channel with connection to the Danube; Tab. 1.

Fig. 11: Himmelteich; a gravel pit filled by ground water; Tab. 1.

1.3 Water sampling & filtration

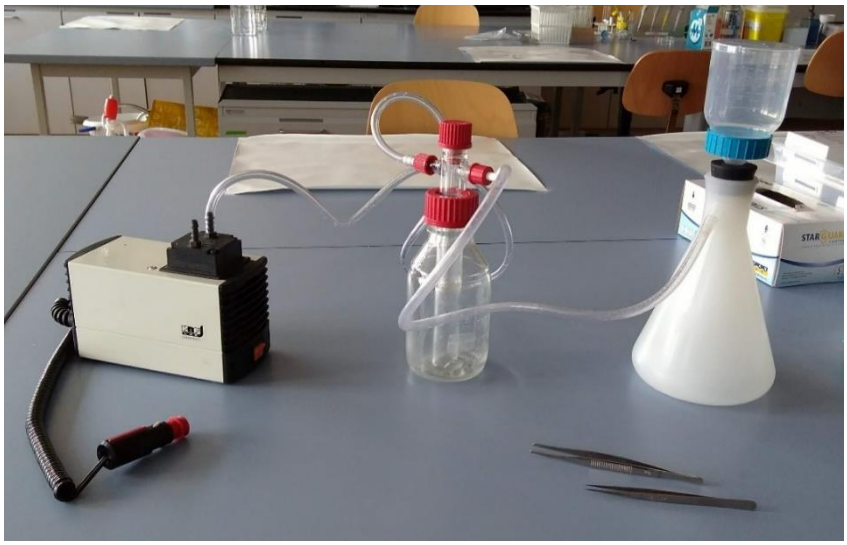


Fig. 12: Vacuum-pump with 12-volt DC adapter plugs into car outlets, Glass bottle as overflow protection and a Sterile Disposable Bottle Top

Fig. 13: Vacuum-pump runs on the car battery

Fig. 14: Vacuum-pump runs by transformer on socket

1.4 Filtration method

Tab. 1: Materials, reagents and equipment of water sampling and filtration.

Materials:	Styrofoam Icebox little, for cooling tubes forceps 1,5 ml tubes (and marker) Plastic bottles Millipore membrane filters, diameter 47 mm, pore size 0,22 µl, CX09.1 (Carl Roth) Nalgene™ Rapid-Flow™ Sterile Disposable Bottle Top (Thermo Scientific)
Reagents:	70% Ethanol
Equipment:	Vacuum-pump (LABOPORT® 12V Field Filtration Pump) with 12-volt DC adapter plugs into car outlets, car or a transformer with electricity connection; Glass bottle as overflow protection

Procedure:

1. Millipore Membrane filter position in Bottle-Top and fill in 50 ml of water with the plastic bottles (one bottle per pond and day to avoid contamination)
2. Filter max. 1500 ml of the pond water through each membrane filter (min. two filters per pond and day)
3. Water sampling at 10 ponds three times
4. Altogether: 95 filters of 10 sites of three times of periods

II. PLANKTON

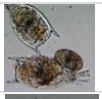
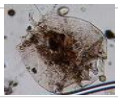
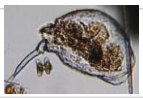
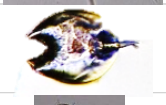
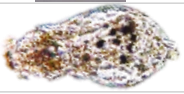
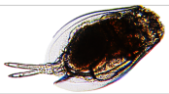
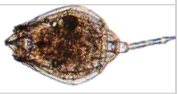
2.1 Sampling

Tab. 2: Plankton sampling

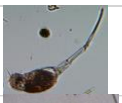
Identification of sampling point	Date	Volume of filtered water [ml]	water lifter	Plankton net	mesh wide
Tritonwasser	09.06.			x	40 µm
	15.06.	3000 ml	x		20 µm
	27.07.	5000 ml	x		20 µm
Ölhafen	09.06.			x	40 µm
	16.06.	3000 ml	x		20 µm
	11.07.	50 ml	x		20 µm
Krebsenwasser	26.04.			x	40 µm
	15.06.	3000 ml	x		20 µm
	22.07.	10 000 ml	x		20 µm
Zentralfriedhof	15.05.			x	40 µm
	28.08.	5000 ml	x		20 µm
Rudolf-Bedner-Park	01.05.			x	40 µm
	22.07.	5000 ml	x		20 µm
Teuflteich	29.04.	2500 ml	x		40 µm
	12.06.			x	40 µm
	27.07.	2500 ml	x		20 µm
Badeteich	21.05.			x	40 µm
Hirschstetten	21.07.	5000 ml	x		20 µm
Blumengärten	4.05.			x	40 µm
Hirschstetten	21.07.	10 000 ml	x		20 µm
Zainethau	30.04.	1500 ml	x		20 µm
	15.06.			x	40 µm
	11.09.	5000 ml	x		20 µm
Himmelteich	29.04.			x	40 µm
	07.06.			x	40 µm
	09.08.	6000 ml	x		20 µm

2.2 Plankton determination

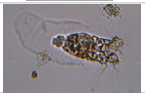
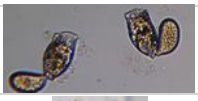
Tab. 3: Detected Zooplankton; Rotifera; sampling sites.

Phylum or Subphylum; Order or Family Suborder or Subclass	Sampling site: Zooplankton:	Tritonwasser	Ölhafen	Krebsenwasser	Zentralfriedhof	Rudolf-Bednar Park	Teufelteich	Badeteich Hirschstetten	Blumengärten Hirschstetten	Zainethau	Himmelteich	
Rotifera: Ploima:												
Asplachnidae:	*Asplachna priodonta*		x	x								
Brachionidae:	*Brachionus caudatur*						x					
	Keratella cochlearis var. irreg.		x									
	Anureaopsis fissa			x			x		x			
	Platyias quadricornis	x					x					
	Brachionus patulus			x			x					
	Branchionus sp.			x								
Colurellidae:	*Colurella logima*	x		x						x	x	
	Squatinella mutica f. tridentata		x									
	Lepadella ovalis				x					x		
	Colurella uncinata					x	x					
Dicranophoridae:	*Itura aurita*			x								
Epiphanidae:	*Epiphanes senta*			x								
	Epiphanes macrourus						x					
Euchlanidae:	*Euchlanis incisa*	x	x						x			
	Euchlanis dilatata			x				x	x			

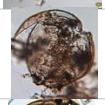
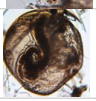
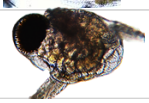
Tab. 4: Detected Zooplankton: Rotifera; sampling sites.

Phylum or Subphylum; Order or Family Suborder or Subclass	Sampling site: Zooplankton:	Tritonwasser	Ölhafen	Krebsenwasser	Zentralfriedhof	Rudolf-Bednar Park	Teufelteich	Badeteich Hirschstetten	Blumengärten Hirschstetten	Zainethau	Himmelteich
Lecanidae:	*Lecane* sp.		x								
	Lecane cornuta cornuta		x								
	Lecane submagna			x							
Notommatidae	*Notommata copeus*								x		
	Notommata tuba						x				
	Cephalodella sp.	x	x	x			x	x		x	x 
	Cephalodella auriculata		x								
	Monommata arndti								x		
	Cephalodella ventripes								x	x	
Rotifera: Ploima:											
Scaridiidae	*Scaridium longicaudum*			x							
Synchaetidae	*Polyarthra dolichoptera*		x	x	x		x	x	x		
	Ploeosoma sp.						x				
	Synchaeta litoralis			x							

Tab. 5: Detected Zooplankton: Rotifera; sampling sites.

Phylum or Subphylum; Order or Family Suborder or Subclass	Sampling site: Zooplankton:	Tritonwasser	Ölhafen	Krebsenwasser	Zentralfriedhof	Rudolf-Bednar Park	Teufelteich	Badeteich Hirschstetten	Blumengärten Hirschstetten	Zainethau	Himmelteich
Trichocercidae	*Trichocerca inermis*					x					
	Trichocerca cylindrica						x				
	Trichocerca fussilo						x				
	Trichocerca capucina			x							
	Trichocerca weberi						x				
	Trichocerca longiseta						x				
Collothecacea:											
Collothecidae	*Collotheca undulata*						x				
	Collotheca mutabilis						x				
Floscullariacea:											
Filinidae	*Filinia aseta*						x		x		
Floscullariidae	*Ptygura furcillatus*		x								
	Limnias ceratophylli						x				
Hexarthridae	*Hexarthra* sp.						x				

Tab. 6: Detected Zooplankton: Crustacea; sampling sites.

Phylum or Subphylum; Order or Family Suborder or Subclass	Sampling site: Zooplankton:	Tritonwasser	Ölhafen	Krebsenwasser	Zentralfriedhof	Rudolf-Bednar Park	Teufelteich	Badeteich Hirschstetten	Blumengärten Hirschstetten	Zainethau	Himmelteich	
Crustacea: Cladocera:												
Bosminidae:	*Bosmina longirostris*								x			
Chydoridae:	*Pleuroxus* sp.		x						x			
	Chydorus ovalis								x			
	Alona elegans									x		
Daphniidae:	*Ceriodaphnia pulchella*									x		
	Simocephalus vetulus								x			
Polyphemidae:	*Polyphemus pediculus*				x				x			
Copepoda:												
Calanoida	*Calanoida*								x			
Cyclopidae	*Macro-cyclops* sp.		x									
Ostracoda:												
Podocopida						x	x					

III. LABORATORY WORK

3.1 DNA Extraction using the Gentra Puregene Tissue Kit

Tab. 7: Materials, reagents, equipment and procedure of extraction.

Materials:	Styrofoam Icebox big for cooling metal plate (Mat.No: 1042601) Styrofoam Icebox little for cooling tubes Scalpel & forceps Tissue culture dishes 2 x 2 ml tubes (Eppendorf) 1,5 ml tube (Eppendorf) 200 µl, 1000, 10 od. 20er µl Micropipettes and tips
Reagents:	Lysis Puffer, RNase, Proteinase K, Protein Precipitation Solution, DNA Hydration Solution (Gentra Puregene Tissue Kit), Isopropanol, 70% ethanol,
Equipment:	Vortexer, Eppendorf Thermomixer, Thermo Scientific Heraeur Centrifuge (16.000 g), freezer

Procedure:

1. Label 24 x 2 ml tubes and fill in 900 µm Lysis Buffer. Pre-program the thermomixer to 65 °C.
2. With flamed Scalpel and forceps cut the filters, following give shreds into the prepared Lysis Buffer. Work quickly and keep tissue on ice all the time.
3. All tubes in thermomixer at 65 °C for 15 minutes. (after that pre-program thermomixer to 55 °C)
4. Centrifuge for 5 minutes at 10.000 x g
5. Transfer liquid in 1,5-ml tube,
6. Add 4,5 µl Puregene Proteinase K, mix by inverting 25 times, and incubate at 55 °C for one hour. Invert tube in 10 miutes intervals during the incubation. (after that pre-program thermomixer to 37 °C)
7. Inverting 25 times
8. Add 4,5 µl RNase A Solution and inverting 25 times. Incubate at 37 °C for 1 hour.
9. On ice to quickly cool the samples for 5 minutes.

10. Add 300 µl Protein Precipitation Solution and vortex vigorously for 10 seconds.
11. Centrifuge for 5 minutes at 16.000 x g. The precipitated proteins should form a tight pellet. If the protein pellet is not tight, incubate on ice for 5 minutes and repeat the centrifugation.
12. Pipet 810 µl Isopropanol in 2-ml tube (3-fold amount minus 10 %)
13. Add the liquid of the samples and mix by inverting gently 50 times.
14. Centrifuge for 5 minutes at 16.000 x g.
15. Carefully discard the supernatant and drain the tube by inverting on a clean piece of absorbent paper, the pellet remains in the tube.
16. Add 900 µl 70 % ethanol and invert several times to wash the DNA pellet.
17. Centrifuge for 5 minutes at 16.000 x g.
18. Carefully discard the supernatant and allow to air dry.
19. Add 50 µl DNA Hydration Solution and flick.
20. Heat in thermomixer at 65 °C for 1 hour, flick after a half hour.
21. Incubate at room temperature (20 °C) overnight with gentle shaking at 300 rpm
22. Centrifuge briefly and store in the freezer at – 20 °C.

3.2 DNA Purification using QIAquick Gel Extraction Kit

Tab. 8: Materials and equipment of purification.

Materials:	Kit with collection tubes (GTIN 04053228005810, REF28706) QG-Buffer PE-Buffer EB-Buffer
Equipment:	Eppendorf Thermomixer, Thermo Scientific Heraeur Centrifuge (16.000 g), freezer

Procedure:

All steps are according to the instruction of the factory manuals (QIAquick)

1. Dilute PE-Buffer (wash buffer) with 40 ml 100 % ethanol.
2. Label 24 x QI Aquick spin column tubes and 24 x 1,5 ml tubes.
3. Pre-program the thermomixer to 50 °C. Add 3 volumes of GelBind to DNA solution. That is 150 µl QG-Buffer (50 µl DNA solution x 3 volumes). Flick them.
4. Incubate at 50 °C for 10 minutes. The color should be yellow. All our solutions did not become discolored.
5. Add 1 volume of 100 % Isopropanol of the DNA solution. Add 50 µl and flick it.
6. Spin and transfer into a spin column tube.
7. Centrifuge at 17.900 x g for 1 minute.
8. Discard the supernatant.
9. Add 500 µl of QG-Buffer and centrifuge at 17.900 x g for 1 minute.
10. Add 750 µl of PE-Buffer and wait for 2 – 5 minutes. Then centrifuge at 17.900 x g for 1 minute
11. Discard the supernatant and centrifuge again for 1 minute.
12. Transfer the spin column into new 1,5 ml tube.
13. Pipet 30 µl EB-Buffer onto the center of spin column membrane. Let it absorb for 4 minutes.
14. Centrifuge at 17.900 x g for 1 minute.
15. Discard the spin column

3.3 DNA Quantification using Nanodrop Spectrophotometer

Tab. 9: Materials of quantification.

Materials:	DNA solution, DNA Hydration Solution, DNA Nanodrop spectrophotometer Implen Typ Pearl, Micropipettes and tips,

Procedure:

1. Before use, choose the light factor 10 and the presets double-strand DNA and ng/µl as unit.
2. Pipet 4 µl of the DNA solution into Nanodrop for measurement.
3. Repeat it twice. Calculate the average concentration of the three results.
4. Between the measurements, clean the lens of the Nanodrop and adjust to blank.

3.4 Quantitative PCR

Tab. 10: Materials and Preparation of qPCR.

Materials:	Autoclaved Milli-Q water from Millipore Corporation Primer, Probe and Positive Control of Chytrid fungus: Primer fwd – Microsynth: TTGATATAATACAGTGTGCCATATGTC Primer n-r – Microsynth: CAAGAGATCCGTTGTCAAAAGTT Taqman Probe: VIC-CGAGTCGAACAAA-MGB-NFQ Synthesized internal positive control (IPC; Microsynth, Order No. 570022): 5'-TTGATATAATACAGTGTGCCATATGTC tacaatagctaagaagcctgaataGCTCAGCTTGTTT caatttgactttcgccgttctagccgtttattt

	AACTTTTGACAACGGATCTCTTG-3' (including primer sequences and binding sequence for the probe). Discovery comfort Micropipettes Multistep pipette Eppendorf Greiner bio-one filter tips (100 µl, 200 µl, 20 µl, 10 µl) Culture plate 96 well StarLab (whit) Centrifuge Thermo Scientific Heraeus Internal positive control (purified filters of aquarium water of Bd-positive tested Lake Patzcuaro salamander *Ambystoma dumerilii*)
Preparation:	Dilution samples and positive control to an amount of 10 µl with a concentration of 25 ng/µl Add water (autoclaved) to primers: Primer fwd.: 100 µM in 382,3 µl water Primer n-r: 100 µM in 378,1 µl water <u>Mastermix:</u> Prepare 10 µM stock solution of primers, probe (Taqman), Milli-Q water (autoclaved) and Fastmix: 1000 µl Fastmix (Kapa Probe Fast Universal) + 6 µl Primer fwd. + 6 µl Primer n-r. + 5 µl Probe (Taqman) + 783 µl Milli-Q water (autoclaved) 1.800 µl for 100 wells, but only 96 wells are used

Procedure:

1. Label 32 x 1,5 ml tubes + 1 x 2ml tube for the Mastermix.
2. Get out the DNA samples, primers, probe (protect for light) and Fastmix of the freezer. Start the lamp for the qPCR. Label the samples at the qPCR-program and choose filter HEX.

3. Pre-program the thermal profile of qPCR according to Sztatecsny.

   for 55 cycles:

   15 min. at 95°C

   15 sec. at 95°C

   30 sec. at 56°C

   30 sec. at 60°C

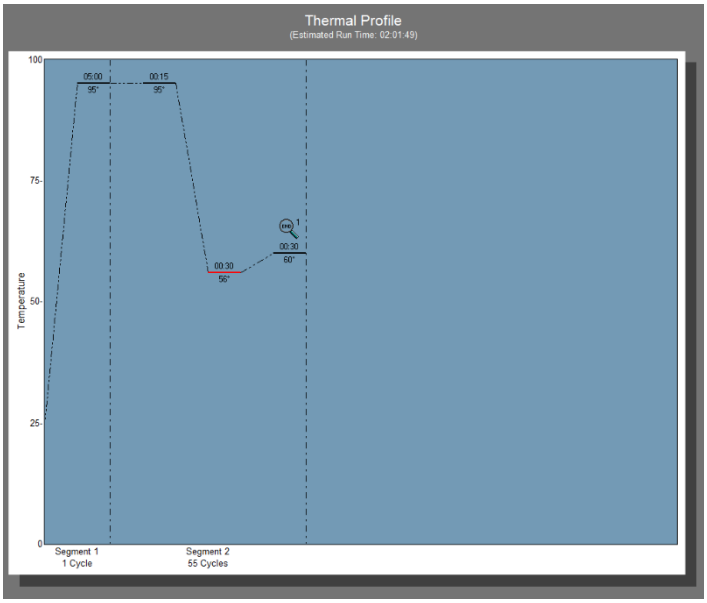


Fig. 15: Thermal Profile at qPCR

4. Prepare dilution of all samples and controls. For example: filter 12-1/B has a nanodrop concentration of 150 ng/µl. (25ng/µl/150 ng/µl) x 10 = 1,7 µl; Dilution: 1,7 µl of the sample DNA or positive control and the remaining amount to 10 µl is filled with water (autoclaved).
5. Flick and spin all tubes.
6. Pipet triplets. Take up 10 µl with the Multistep pipette and drop in each well 2 µl of the sample DNA, discard the supernatant (4 µl). The last nine wells are for the controls. Three times drop the internal positive control, three times drop the positive control (diluted *Bd*-DNA) and three times drop the negative control (autoclaved water).
7. Pipet into each well 18 µl of the mastermix with a Multistep pipette to the DNA samples and controls.
8. Flick the 96-well plate carefully and seal up with foil.
9. Centrifuge at 10.000 rpm for several seconds (code 2014 at the centrifuge).
10. Start qPCR ran with the thermal profile. (Stratagene's Mx Real-Time PCR)

3.5 Standard Curve

The procedure is the same as qPCR, but it was used a dilution series containing 16.000, 1.600, 160, 16 and 1,6 copies.

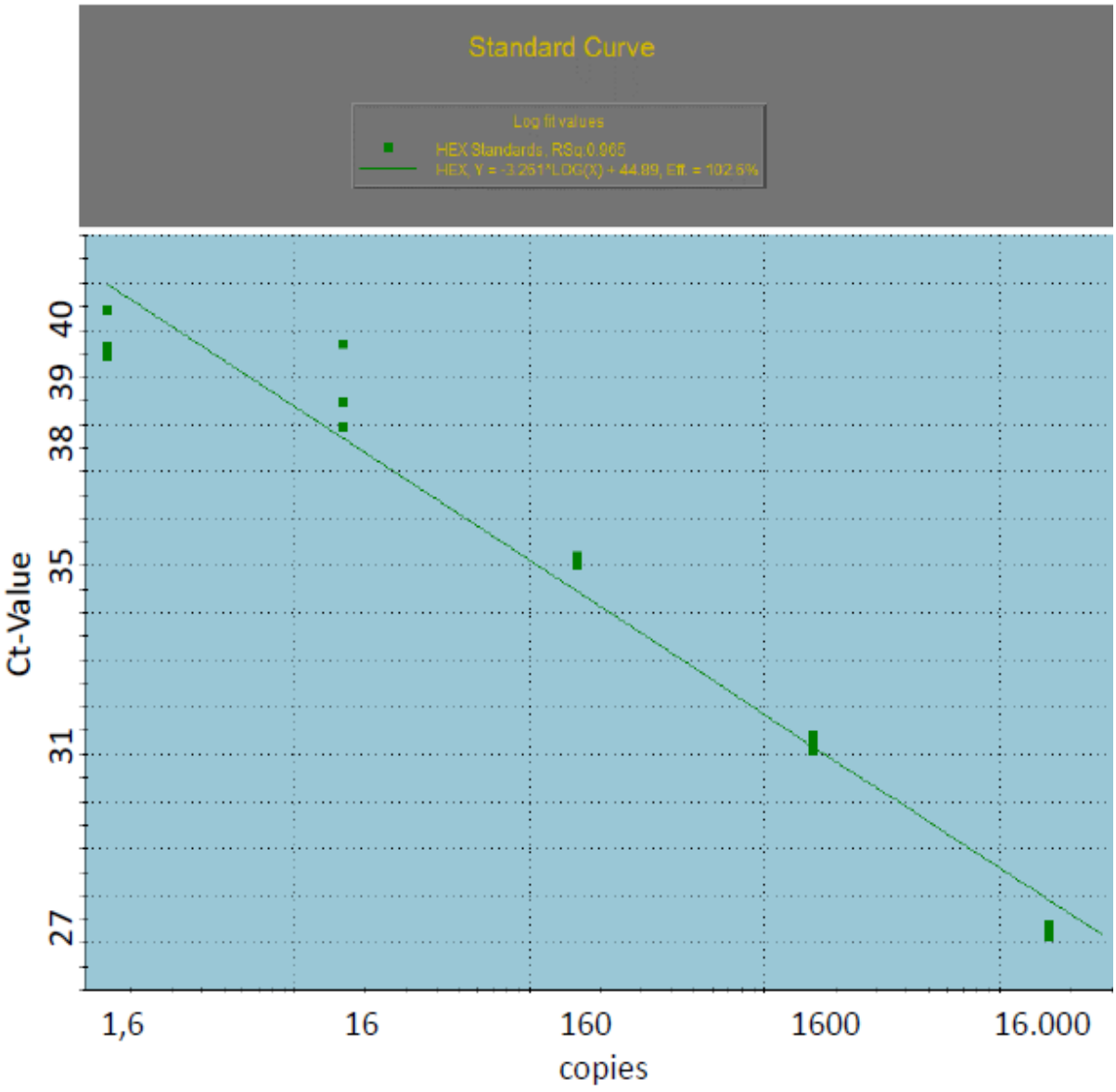


Fig. 16: Standard curve; Ct-value at about 35 (160 copies) => no *Bd;* 169 copies is one Genome Equivalents (GE) = 1 zoospore of *Bd;* samples with a Ct-value of 27 contain 16.000 copies which correspond about to 100 GE; 1600 copies are about 10 GE; 160 copies are just below 1 GE, 16 and 1,6 copies are both below 1 GE

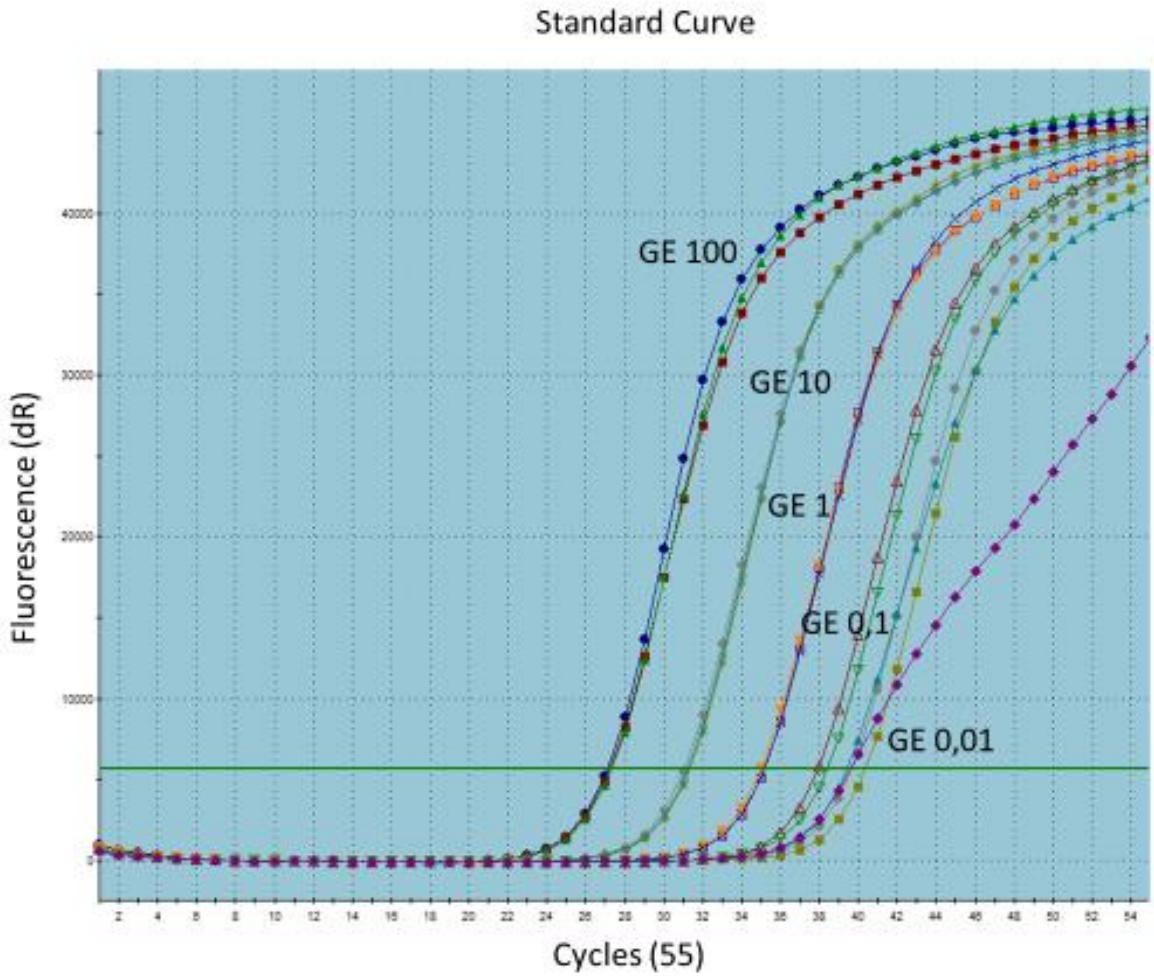


Fig. 17: Amplification plots of the standard curve; five dilutions contain 16.000, 1.600, 160, 16 and 1,6 copies of synthetic *Bd* sequences; there are peaks at a Ct-value of 27 (100 GE), at 31 (10 GE), at 35 (1 GE), at 38 (0,1 GE) and at 40 (0,01 GE); green line is the threshold;

The standard curve provides insight into the Genome equivalents (GE). One GE of *Bd* contains 169 copies of the DNA sequence. Five dilutions with different numbers of copies of the synthetic *Bd* sequence have been applied to receive similar values to the amplification plots of the water samples. The point of intersection between the threshold and the amplification curve is the Cycle threshold (Ct). If the Ct value lies below 35 than *Bd* zoospores are detected (see appendix fig. 16). The Genome Equivalents (GE) could be proven at all water samples by means of a standard curve. Ninety filters indicated no Ct value that means all of them were Bd negative (see appendix tab. 20, 21, 22). Only five of the 95 filters achieve a value of 7 or 10 GE would equal about 1690 copies of the Bd-sequence (KIRSHTEIN ET AL., 2007). Based on that 169 GE is the copy number of one

zoospore would correspond 1690 copies are approximate ten zoospores which have been detected in water samples of “Krebsenwasser”, “Teuflteich” and “Blumengärten Hirschstetten”.

3.6 Gel-electrophoresis

Tab. 11: Materials of electrophoresis.

Materials:	Universal-Agarose; PegGOLD; 1,5 % 50 ml glass bottles Microwave oven 1xSB-Buffer; 50ml + Precision scale GelGreen Nucleic Acid Stain, 10,000X in DMSO: (0,5 mL), Item No. 41004; 5 µl DNA Gel Loading Dye (6X), FERMENTAS, Thermo Scientific, Item No. R0611 O’RangeRuler 100 bp DNA Ladder, Catalog number: SM0623, Thermo Fisher Scientific O’RangeRuler 500 bp DNA Ladder, Catalog number: SM0643, Thermo Sc. Gel electrophoresis apparatus Power Pack P25, Biometra (031744) Scalpel

Procedure:

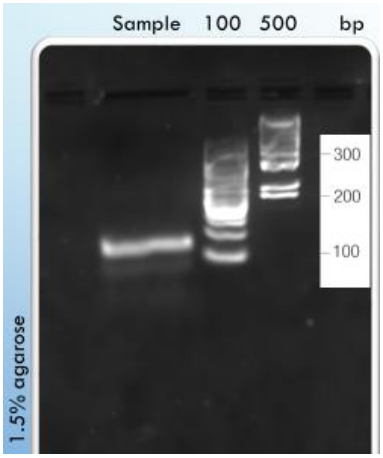


Fig. 18: separated bands by gel electrophoresis of Bd-2/B

1. Preparation of the 1,2 % Agarose – gel:
   0,72g of Universal Agarose, PegGOLD
   + 50 ml 1x SB-Buffer
   The mixture should be heated in microwave oven until the powder is complete dissolved to a clear solution. Then allow to cool.
2. Preparation of the Gel electrophoresis apparatus: Select a comb with 3 teeth (for Bd-2/B, 100 bp & 500 bp LADDER)
3. Fill in the cooled Agarose into the inner tub and wait for become firm
4. Open the ends of the inner tub and fill in 1xSB-Buffer until just below the edge
5. Prepare 1,5 ml tube with 20 µl of the qPCR Product Bd-2/B and add 4 µl of DNA Gel Loading Dye (6X),
6. Fill in the first chamber the mixture of Bd-2/B and loading dye, into the other two chambers the 100 bp and 500 bp markers
7. Connect with black (negative) and red (positive) wires to the power pack at the right terminals
8. Start the current flow at 0.80 Volt for about 40 minutes
9. After the run, the gel block can be investigated in C600 azure biosystems, the separated band is made visible by them,
10. Subsequently over UV-light the DNA-band is cut out by scalpel and transferred into a 1,5 ml tube

3.7 Purification with Wizard SV Gel Clean-Up System

Tab. 12: Materials of purification at colony PCR.

Materials:	Wizard SV Gel and PCR Clean-Up System kit (Promega, USA, Catalog No. A9281) Centrifuge (Thermo Fisher Scientific) Pipettes Precision scale Thermomixer (Eppendorf)

Procedure:

All steps are according to the Instructions of factory manuals (Promega, USA)

1. Add an equal volume of Membrane Binding Solution to the gel slice, therefor the exact weight has been determined; Add 10 µl to 10 mg of gel slice and incubate at 55°C for 10 minutes
2. Transfer the dissolved gel mixture to the Minicolumn and wait for 1 min.
3. Centrifuge at 16.000 x g for 1 min. Discard flowthrough
4. Add 700 µl Membrane Wash Solution and centrifuge at 16.000 x g for 1 min. Discard flowthrough
5. Add 500 µl Membrane Wash Solution and centrifuge at 16.000 x g for 5 min.
6. Discard flowthrough and centrifuge again for 1 min with lid open
7. Transfer minicolumn to a 1,5 ml tube and add 50 µl of Nuclease-Free Water. Centrifuge again at 16.000 x g for 1 min.
8. Discard Minicolumn and store DNA at -20°C

3.8 A-Tailling using pGEM-T Vector Systems of Promega

Tab. 13: Materials of A-Tailling for colony PCR.

Materials:	PCR-product (Bd-3/B) Taq DNA Polymerase 10x Reaction Buffer $MgCl_2$ dATP Taq DNA Polymerase Enhancer 5x Crashed Ice Discovery comfort Micropipettes

Procedure:

All steps are carried out on ice according to the technical manual of pGEM-T and pGEM-T Easy Vector Systems from Promega.

1. Fill in a 1,5 ml tube on ice, following reagents (also on ice):

PCR-product (Bd-3/B)	5,4 µl
Taq DNA Polymerase 10x Reaction Buffer	1 µl
$MgCl_2$	0,6 µl
dATP	1 µl
Taq DNA Polymerase	1 µl
Enhancer 5x	1 µl
Total of:	10 µl

2. Incubate the mixture at 70 °C for 30 minutes into thermomixer.

3.9 Ligation using the pGEM-T Vector Systems of Promega

Tab. 14: Materials of ligation for colony PCR.

Materials:	2xRapid Ligation Buffer pGEM-T Vector (50 ng) PCR product (Bd-3/B) T4 DNA Ligase

Procedure:

All steps are carried out according to the technical manual of pGEM-T and pGEM-T Easy Vector Systems from Promega.

1. Briefly centrifuge the pGEM-T Vector. Pipette following ligation reagents:

Reaction Component for a Standard reaction:	
2xRapid Ligation Buffer	5 µl
pGEM-T Vector (50 ng)	1 µl
PCR product (Bd-3/B)	3 µl
T4 DNA Ligase	1 µl
Total of	10 µl

2. Incubate the reaction overnight at 4°C (max. number of transformants is expected)

3.10 Transformation using the pGEM-T Vector Ligation Reaction

Tab. 15: Materials of transformation for colony PCR.

Materials:	LB Broth + Agar (lennox, SIGMA, catalog No. L2897-250G) Ampicillin 250 ml glass bottles Autoclave NEB 5-alpha comp. E. coli (High efficiency in 50 µl tubes, Biolabs Inc., England, catalog No.: C2987H) SOC-Medium (Biolabs Inc.) 100 mm non-treated petri dishes

	Ethanol Glass rod Gas cartridge Pipettes X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-glactoside) IPTG stock solution (0,1 M) Distilled water Magnetic stirrer

Procedure:

All steps of the Transformation were carried out according to the technical manual of pGEM-T and pGEM-T Easy Vector Systems of Promega, USA

1. Prepare 10 LB-Broth + Agar plates: 200 ml distilled water
   + 6,28 g LB Broth + Agar powder
   Dissolve the powder in water and mix them by magnetic stirrer. Autoclave the bottle with the mixture.
2. After a cooling time, add 400 µl ampicillin and stir again
3. Pour 10 plates, once the agar plates are solidified, store 8 plates closed by cling film at 4°C, two plates are plated by 80 µl x-gal and 40 µl IPTG-stock solution. Incubate at 37°C
4. Add very careful 2 µl of ligation reaction to the 50 µl tube of the High Efficiency Competent Cells.
5. Gently flick the tube to mix and place them on ice for 30 minutes.
6. Heat-shock them at 42°C for 45 seconds in thermomixer
7. Return the tube on ice for 5 minutes
8. Add 950 µl SOC medium
9. Incubate at 37°C at 150 rpm for 1,5 hours in incubator
10. Plate 100 µl of the transformation culture onto a LB-plate and plate 100 µl of a mixture of 50 µl culture and 50 µl SOC medium onto a LB-plate
11. Incubate overnight at 37°C
12. If the colonies have grown, store the plates overnight at 4°C for good results of a blue-white screening

3.11 Colony PCR

Tab. 16: Materials of colony PCR.

Materials:	Thermocycler (Eppendorf Mastercycler ep Gradient S) Pipettes PCR-tubes and 1,5 ml tubes Distilled water Yield buffer $MgCl_2$ Enhancer P dNTP's (10 mM) Primer-n-fwd (10 mM) Primer-n-r (10 mM) Taq Polymerase (50/µl)

Procedure:

1. Cut out 10 white colonies by sterile tipps and dissolve each colony in a tube with 50 µl distilled water
2. Prepare Hot Taq for the PCR:

Colony PCR components for 10 bacterial suspensions:	
Yield buffer	25 µl
$MgCl_2$	15 µl
Enhancer P	50 µl
dNTP's (10 mM)	5 µl
Primer-n-fwd (10 mM)	2,5 µl
Primer-n-r (10 mM)	2,5 µl
Taq Polymerase (50/µl)	1,25 µl
Distilled H_2O	133,75 µl
Volume	235 µl

3. In each of the 10 PCR-tubes is filled in 8,5 µl of the solution and 1,5 µl of the bacterial suspension. All tubes are placed in thermocycler (Eppendorf Mastercycler ep Gradient S) at the following thermal profile:

98°C for 5 min
95°C for 45 s
56°C for 45 s
72°C for 1 min
72°C for 10 min
4°C hold

x28 cycles

3.12 Gel-electrophoresis II

Materials and procedure are according to step 2.5 of this protocol except:

- For 10 samples are needed 14 chambers in the Gel block
- Select 2 combs with à 7 teeth (together 14 chambers) and put them into the tub at a wide distance from each other
- Five samples were selected for purification by MiniPrep and sequencing. No. 3, 4, 7, 9 & 10

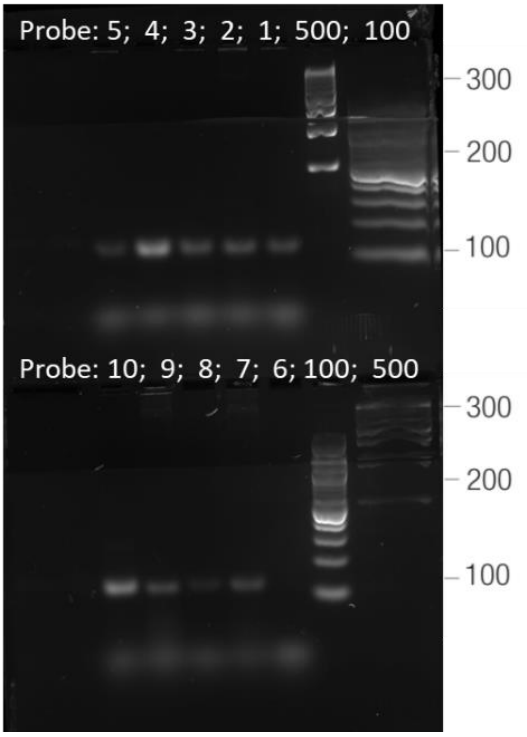


Fig. 19: Gel- electrophoresis of 10 colonies of Bd-2/B (water sample of Bd-pos. water); 500 bp (base pairs, SM0623, Thermo scientific); 100 bp (N3231S, Thermo scientific)

3.13 Bacteria growing in LB-Broth

Tab. 17: Materials of bacteria growing for colony PCR.

Materials:	LB Broth Powder (lennox, SIGMA, catalog No. L3022250G) Ampicillin 5 crystal tubes Incubator Centrifuge

Procedure:

1. Prepare the LB-Broth solution of:

   200 ml dH_2O

   + 4g LB-Broth Powder

2. Dissolve the Powder in dH_2O in a 250 ml bottle and into autoclave
3. After a cooling time add the Ampicillin

4. Pipette 4 ml in each of the five crystal tubes
5. Add 20 µl of dissolved colonies; No.: 3, 4, 7, 9, 10
6. Incubate at 37°C at 300 rpm overnight in incubator
7. Centrifuge in five labeled 2 ml tubes in two steps the 4 ml of each bacterial suspension
8. Discard the fluid and store the pellets at -20°C or wash them by Miniprep

3.14 Miniprep by Wizard plus SV DNA Purification System (Promega)

Tab. 18: Materials of Miniprep for colony PCR.

Materials:	Wizard Plus SV Minipreps DNA Purification System from Promega, USA, catalog No. A1330 Wash Solution, Collection tubes with Spin Column, Nuclease-Free Water Lysis Solution, Alkaline Protease Solution, Cell Resuspension Solution Centrifuge

Procedure:

1. Add 250 µl Cell Resuspension Solution, invert
2. Add 250 µl Cell Lysis Solution, invert
3. Add 10 µl of Alkaline Protease Solution, invert
4. Add 350 µl Neutralization Solution, invert
5. Centrifuge at top speed for 10 minutes
6. Transfer the mixture into Spin Column
7. Centrifuge at top speed for 1 min, discard the flow through
8. Add 700 µl Wash solution and centrifuge at top speed for 1 min
9. Discard flow through
10. Add 200 µl Wash solution centrifuge at top speed for 2 min
11. Transfer Spin Column to a sterile 1,5 ml tube
12. Add 100 µl Nuclease-Free Water and centrifuge at top speed for 1 min
13. Discard column and store DNA at – 20°C or send for sequencing

3.15 Sequencing by Microsynth

Tab. 19: Materials of sequencing

Materials:	1,5 ml tubes Spectrophotometer (NanoDrop, Implen, Typ: Pearl) Nuclease-Free water Pipettes Microsynth Barcodes

Procedure:

1. Measure the DNA concentration of the selected samples
2. Compare the conc. with the company requirements (Microsynth) for sequencing
3. Sample Amounts Per Sequencing Reaction & Concentrations for Plasmid is a concentration between 60-100 ng/µl² (the concentration of No. 9 was to low)
4. Dilute all samples that they meet the specification
5. Fill out an order form on Homepage of Microsynth
6. Stick a Microsynth Barcode on each tube and throw them into Microsynth postbox

3.16 Nucleotide BLAST

The nucleoid sequence can be modified and blasted directly at the GenBank of NCBI with a software named AbE. The following sequence was found by this means:

Batrachochytrium dendrobatidis isolate L820609 internal transcribed spacer 1 and 5.8S ribosomal RNA gene, partial sequence

Ttagttgttggggtggatgggagttttattgatgtgtaaatgttgatggaatgacccattgtttttttcaaaaaacacccttgatataatac
agtgtgccatatgtcacgagtcgaacaaaatttatttatttttttcgacaaattaattggaaattgaatgattttaatttaattgaaaaaaatt
gaaaataaatattaaaaacaactttgacaacggatctcttggctctcgcaacgatgaagaacgcagcgaaatgcgatacgtaatgt
gaattgcaaacctttgtgaatcattaaatctttgaacgcacattgcactcgtaaaagagtatacatgtttgagaattataaaaatacattg
tccgaattgactggacagatatgaaccatgtcaaaattgtttgacaatttgacaggttttaaaagtagtagtaaatgagtgatacaaaa
agtagtgggtctaaacaaccccgtccatcacaccataca

Fig. 20: primer: green labeled sequences (2x23 bases) + filling sequence: grey labeled (27 bases) = Oligonucleotide.

IV. LABORATORY VALUES

Tab. 20: Filtration pass 1 (26.4.- 5.5.2017); Amount of filtered water, DNA concentration after purification; Dilution for a lower conc.; dilution information; date of passed qPCR; results of them; Ct-values of them

Sample No.	Filter Volume [ml/filter]	Pre-Dilution (at conc. >125 ng) (1 µl sample + 9 µl H²O)	Average con. [ng/µl] after pre-dilution	Dilution for qPCR (12,5ng/µl)* 10µl)	Adding Water (RNA-free, autoclaved)	Bd-tested (Date)	Bd-positiv yes/no	Ct-Value	GE (169 copies = 1 GE)
Bd-1/B	1.500		14	undiluted		14.9.	yes	26, 26, 26	100
1-1/A	600	pre-dil.	19	6,5	3,5	03.1.	no	>35	0,1
1-1/B	900		21	6,1	3,9	03.1.	no	>35	0,1
1-1/C	700	pre-dil.	19	6,6	3,4	03.1.	no	>35	0,1
1-1/D	800	pre-dil.	23	5,4	4,6	14.9.	no	>35	0,1
2-1/A	3.000	pre-dil.	108	1,2	8,8	14.9.	no	>35	0,1
3-1/A	1.000	pre-dil.	12	undiluted		03.1.	yes	31,32,31	7
3-1/B	1.100		89	1,4	8,6	9.5.	no	>35	0,1
3-1/C	900		16	7,9	2,1	14.9.	no	>35	0,1
4-1/A	1.500		5	undiluted		9.5.	no	>35	0,1
4-1/B	1.500		86	1,5	8,5	14.9.	no	>35	0,1
5-1/A	300		26	4,8	5,2	9.5.	no	>35	0,1
5-1/B+C	600		67	1,9	8,1	9.5.	no	>35	0,1
5-1/D	300		98	1,3	8,7	14.9.	no	>35	0,1
5-1/E	600		26	4,9	5,1	9.5.	no	>35	0,1
5-1/F	600		76	1,6	8,4	03.1.	no	>35	0,1
5-1/G+H	600		9	undiluted		9.5.	no	>35	0,1
6-1/A+C	400	pre-dil.	31	4,0	6,0	03.1.	yes	30,30,31	10
6-1/B	200		70	1,8	8,2	9.5.	no	>35	0,1
6-1/D+E	900	pre-dil.	31	4,0	6,0	03.1.	yes	32,31,30	10
6-1/F+G	900		28	4,5	5,5	14.9.	no	>35	0,1
6-1/H+I	600	pre-dil.	33	3,8	6,2	9.5.	no	>35	0,1
7-1/A	800	pre-dil.	13	undiluted		9.5.	no	>35	0,1
7-1/B	700	pre-dil.	13	undiluted		03.1.	no	>35	0,1
7-1/C	900	pre-dil.	13	undiluted		14.9.; 03.1.	no	>35	0,1
7-1/D	600		25	5,0	5,0	14.9.	no	>35	0,1
8-1/A	600		3	undiluted		9.5.	no	>35	0,1
8-1/B	650		86	1,5	8,5	9.5.	no	>35	0,1
8-1/C	470	pre-dil.	19	6,4	3,6	14.9.	no	0,13;0	0
8-1/D	430		108	1,2	8,8	03.1.	no	>35	0,1
8-1/E	430		100	1,3	8,8	9.5.	no	>35	0,1
8-1/F	450		108	1,2	8,8	03.1.	no	>35	0,1
9-1/A	1.500		81	1,5	8,5	14.9.; 03.1.	no	>35	0,1
9-1/B	1.500		60	2,1	7,9	03.1.	no	>35	0,1
10-1/A	700	pre-dil.	20	6,3	3,7	14.9.	no	0;0;11	0
10-1/B	900	pre-dil.	36	3,4	6,6	03.1.	no	>35	0,1
10-1/C	900	pre-dil.	31	4,1	5,9	9.5.	no	>35	0,1
10-1/D	500		51	2,5	7,5	14.9.	no	0,0,20	0
mean	830								

Tab. 21: Filtration pass 2 (11.5. – 22.5.2017); Amount of filtered water, DNA concentration after purification; Dilution for a lower conc.; dilution information; date of passed qPCR; results of them; Ct-values of them

Sample No.	Filter Volume [ml/filter]	Pre-Dilution (at conc. >125 ng) (1 µl sample + 9 µl H^2O)	Average con. [ng/µl] after pre-dilution	Dilution for qPCR (12,5ng/conc.ng)*10 µl	Adding Water (RNA-free, autoclaved)	Bd-tested (Date)	Bd-postitiv yes/no	Ct-Value	GE (169 copies = 1 GE)
Bd-2/B	1.500		31	4,0	6,0	5.9.	yes	26, 26, 26,	100,0
1-2/A	900	pre-dil.	17	7,4	2,6	5.9.	no	>35	0,1
1-2/B+C	2.100		109	1,1	8,9	11.5.	no	>35	0,1
2-2/A	1.500	pre-dil.	19	6,6	3,4	5.9.	no	>35	0,1
2-2/B	1.500		82	1,5	8,5	5.9.	no	>35	0,1
3-2/A	1.300	pre-dil.	37	3,4	6,6	5.9.	no	>35	0,1
3-2/B	1.100	pre-dil.	44	2,8	7,2	5.9. 29.12.	no yes	39;42;0 30;30;31	0,1 10
3-2/C	600	pre-dil.	24	5,2	4,8	11.5.	no	>35	0,1
4-2/A	3.000		113	1,1	8,9	5.9.	no	>35	0,1
5-2/A	1.300		50	2,5	7,5	5.9.	no	>35	0,1
5-2/B	700		68	1,8	8,2	5.9.	no	>35	0,1
5-2/C+D	1.000		90	1,4	8,6	5.9.	no	>35	0,1
6-2/A	250	pre-dil.	17	7,4	2,6	5.9.	no	>35	0,1
6-2/B	250	pre-dil.	14	8,9	1,1	11.5.	no	>35	0,1
6-2/C	300		20	6,3	3,8	11.5.	no	>35	0,1
6-2/D+E	1.700	pre-dil.	51	2,5	7,5	11.5.	no	>35	0,1
6-2/F	500	pre-dil.	33	3,8	6,2	11.5.	no	>35	0,1
7-2/A	1.500	pre-dil.	17	7,4	2,6	5.9.	no	>35	0,1
7-2/B	1.500		6	undiluted		11.5.	no	>35	0,1
8-2/A	400		113	1,1	8,9	5.9.	no	>35	0,1
8-2/B	1.100		70	1,8	8,2	11.5.	no	>35	0,1
8-2/C	800	pre-dil.	14	8,9	1,1	11.5.	no	>35	0,1
8-2/D	700	pre-dil.	14	8,9	1,1	11.5.	no	>35	0,1
9-2/A	1.500	pre-dil.	19	6,6	3,4	5.9.	no	>35	0,1
9-2/B	1.500	pre-dil.	14	8,9	1,1	5.9.	no	>35	0,1
10-2/A	2.400	pre-dil.	22	5,7	4,3	5.9.	no	>35	0,1
10-2/B	600	pre-dil.	14	8,9	1,1	11.5.	no	>35	0,1
mean	1.167								

Tab. 22: Filtration pass 3 (12.6 - 16.6.2017); Amount of filtered water, DNA concentration after purification; Dilution for a lower conc.; dilution information; date of passed qPCR; results of them; Ct-values of them and the

Sample No.	Filter Volume [ml/filter]	Pre- Dilution (at conc. >125 ng)	Average con. [ng/µl] after pre-dilution	Dilution for qPCR (12,5 ng/ng)*10µl	Adding Water (RNA-free, autoclaved)	Bd-tested (Date)	Bd-postitiv yes/no	Ct-value	GE (169 copies = 1 GE)
Bd-3/B	1.500		67	1,9	8,1	3.1./ 9.5.	yes	26, 26, 26	100
1-3/A	1.500		32	3,9	6,1	22.9.	no	>35	0,1
1-3/B	1.500	pre-dil.	21	6,0	4,0	11.5.	no	>35	0,1
2-3/A	1.500	pre-dil.	27	4,6	5,4	22.9./11.5.	no/no	>35	0,1
2-3/B	1.500	pre-dil.	18	6,9	3,1	11.5.	no	>35	0,1
3-3/A	1.500	pre-dil.	53	2,4	7,6	11.5.	no	>35	0,1
3-3/B	1.500	pre-dil.	22	5,7	4,3	22.9./11.5.	no/ no	>35	0,1
4-3/A	3.000		70	1,8	8,2	22.9.	no	>35	0,1
5-3/A	550	pre-dil.	15	8,3	1,7	11.5.	no	>35	0,1
5-3/B	850	pre-dil.	17	7,4	2,6	22.9.	no	>35	0,1
5-3/C+D	1.600	pre-dil.	13	9,6	0,4	11.5.	no	>35	0,1
6-3/A	700	pre-dil.	92	1,4	8,6	22.9. / 29.12./11.5.	no/no	(0;37;0); (0;0;0); (38,0,0)	0
6-3/B+C	1.500	pre-dil.	16	7,8	2,2	22.9./11.5.	no/no	>35	0,1
6-3/D	600	pre-dil.	18	6,9	3,1	22.9./11.5.	no/no	>35	0,1
7-3/A	1.500		113	1,1	8,9	22.9.	no	>35	0,1
7-3/B	1.500		86	1,5	8,5	22.9.	no	>35	0,1
8-3/A	500	pre-dil.	16	7,8	2,2	22.9. 29.12. 11.5.	no yes no	40;0;0 31;33;33 46,36,37	0 7 0
8-3/B+C	800	pre-dil.	15	8,3	1,7	11.5.	no	>35	0,1
8-3/D	1.700	pre-dil.	22	5,7	4,3	22.9./11.5.	no/no	>35	0,1
9-3/A	3.000	pre-dil.	19	6,6	3,4	11.5./11.5.	no/no	>35	0,1
10-3/A	1.500	pre-dil.	14	8,9	1,1	22.9./11.5.	no/no	>35	0,1
10-3/B	1.500	pre-dil.	17	7,4	2,6	22.9. /29.12./11.5.	no/no/no	(0,49;0); (0;0;0); (0,0,0)	0
mean	1.423								