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Evidence of *Batrachochytrium dendrobatidis* and other amphibian parasites in the Green toad (*Bufotes viridis*), syntopic amphibians and environment in the Cologne Bay, Germany

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Abstract. Chytridiomycosis, a disease induced by the chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*), has strongly contributed to the ongoing worldwide amphibian conservation crisis. While *Bd* infection has caused amphibian declines for decades on several continents, *Bsal* is a novel threat to Central European salamanders and newts, being responsible for the collapse of Fire Salamander populations in the Netherlands, Belgium, and Germany. However, numerous other parasites causing harm to amphibians exist, yet have received much less attention than the chytrid fungi. The goal of the present study was to contribute to the understanding of declines of the Green Toad, *Bufotes viridis,* at its northwestern distribution border, in the area of Cologne, Germany. We combined the data from four years of *Bd* monitoring with a metabarcoding approach to detect other, mainly unicellular parasites, from amphibian feces and environmental samples, and also report results from *Bsal* testing in 2019. Skin swabs of approximately 280 amphibians were tested for *Bd* and 66 for *Bsal*, and 150 cloacal swabs and environmental samples from five sites were tested for other pathogens and parasites.We found *Bd* in all sampled locations with high prevalences and partly high individual infection loads but without clinical signs of chytridiomycosis. None of the samples tested for *Bsal* was positive for this pathogen. We further detected eight additional potential amphibian pathogens from fecal samples: three metamonads (*Tritrichomonas augusta, Trichomitus batrachorum* and *Hexamita inflata*), three ciliates (*Balantidium duodeni, Nyctotherus cordiformis* and *N. hubeiensis*), one stramenopile (*Blastocystis* sp.) and one metazoan (the nematode *Rhabdias ranae*). In the environmental samples, we detected OTUs of nine organisms potentially harmful for amphibians: *Blastocystis* sp., *Hexamita inflata*, *Tritrichomonas augusta*, *Trichomitus batrachorum*, two oomycetes (*Leptolegnia* sp., *Saprolegnia* sp.), two ichthyosporeans (*Amphibiocystidium ranae*, *Anurofeca* sp.) and the myxozoan *Myxobolus* sp.

Key words. Amphibia, *Bd, Bsal,* chytrid infection, pathogens, North Rhine-Westphalia.

Introduction

Chytridiomycosis has been characterized as the panzootic associated to the greatest recorded loss of biodiversity arising from a disease (Scheele et al. 2019), substantially contributing to ongoing declines of amphibian diversity worldwide. This skin-affecting disease is caused by the chytridiomycete fungi *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*). It affects mainly the epidermis (stratum corneum and stratum granulosum), leading to a strong disturbance of its biological functions (van Rooij et al. 2015). While *Bd* has a broader host-range and can affect representatives of all major amphibian clades, *Bsal* appears to be restricted to urodelan hosts, i.e., salamanders and newts (Stegen et al. 2017), although it recently was shown to likewise be present on anurans, viz. the small-webbed fire-bellied toads (*Bombina microdeladigitora*) (Nguyen et al. 2017). Yet, *Bsal*, in many species seems to be even more aggressively causing mortality of infected animals. According to recent studies (Laking et al. 2017, O'Hanlon et al. 2018) the origin of these pathogens is in Asia. Upon its discovery in the late 1990's *Bd* was mostly studied in the trop-

ics but later also found in temperate zones; in 1999 it was first detected in Germany (Mutschmann et al. 2000), predominantly in captive animals. Since then, several studies have traced its occurrence in the wild in Europe (Garner et al. 2005, Lötters et al. 2018) where most amphibian species appear to be able to coexist with *Bd*, but others are affected by chytridiomycosis (Garner et al. 2005).

Since 2016, *Bd* monitoring has been carried out in the Cologne bay (Western Germany) in the framework of a conservation management project for *Bufotes viridis*. This project, initiated by the NABU (Naturschutzbund Deutschland e.V.), Technische Universität Braunschweig and the Cologne Zoo and subsequently supported also by the Stadtentwässerungsbetriebe Köln (StEB) AöR, targeted the severe population declines of *B. viridis* in this area, including conservation genetics approaches (Vences et al. 2019) and ex-situ rearing (Ziegler et al. 2019). Additionally, in 2018, cloacal swabs of around 150 anuran individuals plus a series of environmental samples were tested for the occurrence of amphibian parasites that may be contributing to the declines of *B. viridis* in Cologne. Intestinal protist parasites or commensals like metamonads or ciliates are quite common in amphibians (Baker 2008) yet their harmfulness is strongly dependent on their grade of infestation and the health state of their host. This especially applies when the animals are burdened with multiple infections. We applied a metabarcoding approach to obtain reliable information on parasites from environmental DNA (Bass et al. 2015, Rytkönen et al. 2018), especially for protists that may be difficult to cultivate in culture media. Since Cologne is close to current *Bsal* outbreaks (Dalbeck et al. 2018, Lötters et al. 2020, Schulz et al. 2020, this issue), we also screened for this second pathogen in 2019.

Thus, we here present *Bd* monitoring data from 2016–2019 and *Bsal* monitoring data from 2019. We also summarize records of amphibian pathogens and parasites detected via metabarcoding in the same populations, and discuss the relevance of these findings for conservation management of amphibians, especially *B. viridis*, in Cologne.

Materials and methods

Sample collection

Skin swabs were collected in 2016–2019 from wild amphibians at nine sampling sites in and around the municipal area of Cologne (Fig. 1, Table 1). Sites were selected depending on amphibian species diversity and the occurrence of our main target, *Bufotes viridis*. A total of 288 samples were taken from five different anuran and urodelan species (*B. viridis*, *Bufo bufo*, *Epidalea calamita*, *Pelophylax* kl. *esculentus* and *Lissotriton vulgaris*). Until further examination, the swabs were kept at -20°C.

Cloacal swabs were taken from anurans from six of the selected sampling sites (not all sites were revisited every year) in 2018. All animals were probed with a sterile small swab (MW113, MWE Medical Wire, Corsham, UK) inserted in the cloaca. The inserted swab was gently rotated around three times, extracted from the cloaca, and immediately transferred to a clean, pre-labeled 1.5 ml microtube (Roth, Karlsruhe, Germany). The tubes were frozen at -20°C until further processing.

Two-liter water samples were taken in 2018 from five sites (where *B. viridis* occured), transferred to the laboratory, pre-filtered with a 100 µm gauze to remove larger debris and, with the help of a hand-pump, up to 600 ml water were pulled over a cellulose nitrate filter with a pore size of 1.2 µm (Sartorius, Göttingen, Germany). Samples were taken in duplicates or triplicates. Filters were preserved individually each in 5 ml DESS (Yoder et al. 2006) at room temperature.

Chytrid detection via quantitative PCR (qPCR)

DNA from skin swabs was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following Bletz et al. (2015). We used the manufacturer's pretreatment protocol for gram negative bacteria, expanding the initial incubation to 1 h and carrying out the second incubation at 70°C (Belden et al. 2015), then followed by the protocol for animal tissues. Samples from 2016–2018 were analyzed for *Bd* with qPCR assays using the *Bd*-specific primers according to Boyle et al. (2004), following a standard mixture using KlearKall Master Mix (LGC genomics, Middlesex, UK) each time investing 5 µl DNA and 20 µl Mix. In 2016 and 2017 we implemented an initial step at 95°C/10 min, followed by 49 cycles of 95°C/15 s and 62°C/1 min on a CFX96 Real-Time System (Bio-Rad Laboratories Inc., Hercules, USA). In 2018 we implemented an initial denaturation step of 95°C for 15 min followed by 43 cycles of 15 s/95°C and 60 s/60°C on a StepOne™ Real-Time System (Applied Biosystems, Foster City, USA). In 2016–2018, we used the *Bd* isolate JEL310 (obtained from the laboratory of D. Woodhams at the University of Massachusetts, originally from that of J. Longcore at the University of Maine) as a standard, in a dilution of 1–10,000 zoospore genome equivalents. For 2016, the threshold was set at 1 zoospore for positive detection. Due to technical difficulties with implementing the standards, no exact loads could be determined for 2017 and 2018. For samples from these two years, we implemented two qualitative approaches considering a sample as a putative positive when the shape of the amplification curve was logarithmic with a certain cycle threshold (Ct-value). Following a very conservative approach according to the Techne (Cole-Palmer, Staffordshire, UK) qPCR test manual for *Bd* detection and our own experience, a sample would then be a putative positive if it had amplified at cycle 28 ± 3. After a more inclusive approach, following a protocol of the Institute for Research in Immunology and Cancer (IRIC) of the University of Montreal, curves that amplify between cycle 15 and 35 would be considered a positive detection for *Bd*.

Since Cologne is spatially close to current *Bsal* outbreaks (Dalbeck et al. 2018), in 2019 we analyzed the samples for *Bd* and *Bsal*. Different than in 2016–2018 we used

Table 1. Sampling sites in the municipal area of Cologne where amphibians were studied for pathogen infections. *Bb* (*Bufo bufo),* *Bv* (*Bufotes viridis*), *Ec* (*Epidalea calamita*) *Lv* (*Lissotriton vulgaris*), *Pe* (*Pelophylax* kl. *esculentus*).

Sampling site	Longitude, Latitude	N samples studied for *Bd*	Species studied
Baadenberger Senke (BS)	50.9878, 6.8518	13	*Bb*
Decksteiner Weiher (DW)	50.9070, 6.8992	2	*Bb*
Ginsterpfad (GP)	50.9850, 6.9301	118	*Bb, Bv, Ec, Lv, Pe*
Kiesgrube Esser (KE)	50.8368, 6.9300	6	*Bv*
NBI (NB)	50.8514, 6.9451	37	*Bv*
Porz Wahn (PW)	50.8618, 7.0773	12	*Bv*
R2.12 (R2)	50.8454, 6 9397	13	*Bv, Ec, Lv*
Vogelacker (VA)	50.8449, 6.9535	2	*Bv*
Westhovener Aue (WA)	50.9037, 7.0085	81	*Bb, Bv, Pe*

gBlocks® gene fragments instead of zoospores as standard to account for ITS copy number variations between strains, as described by Rebollar et al. (2017). We implemented a duplex real time PCR, following the protocol of Blooi et al. (2013) to detect *Bd* and *Bsal* simultaneously, again with the use of KlearKall Master Mix (LGC genomics, Middlesex, UK) but with the alteration of 15 min/95°C for the first step and investing 10 µl Mix and 5 µl DNA. Final concentration of the ordered stock solution was 1 ng/µl. We used a logarithmic standard dilution from 10^7 to 10^2 ITS copies/5µl. Given that Rebollar et al. (2017) estimated around 73 copies zsp.$^{-1}$ for strain JEL310, we set the threshold at 100 ITS copies for the 2019 samples (next logarithmic scale to 73) for positive detection.

Metabarcoding of cloacal swab samples

DNA from cloacal swabs was isolated with the Quick-gDNA prep kit (Zymo Research, Irwing, USA) following the swab isolation protocol suggested by the manufacturer. We amplified a fragment of the hypervariable V9 region

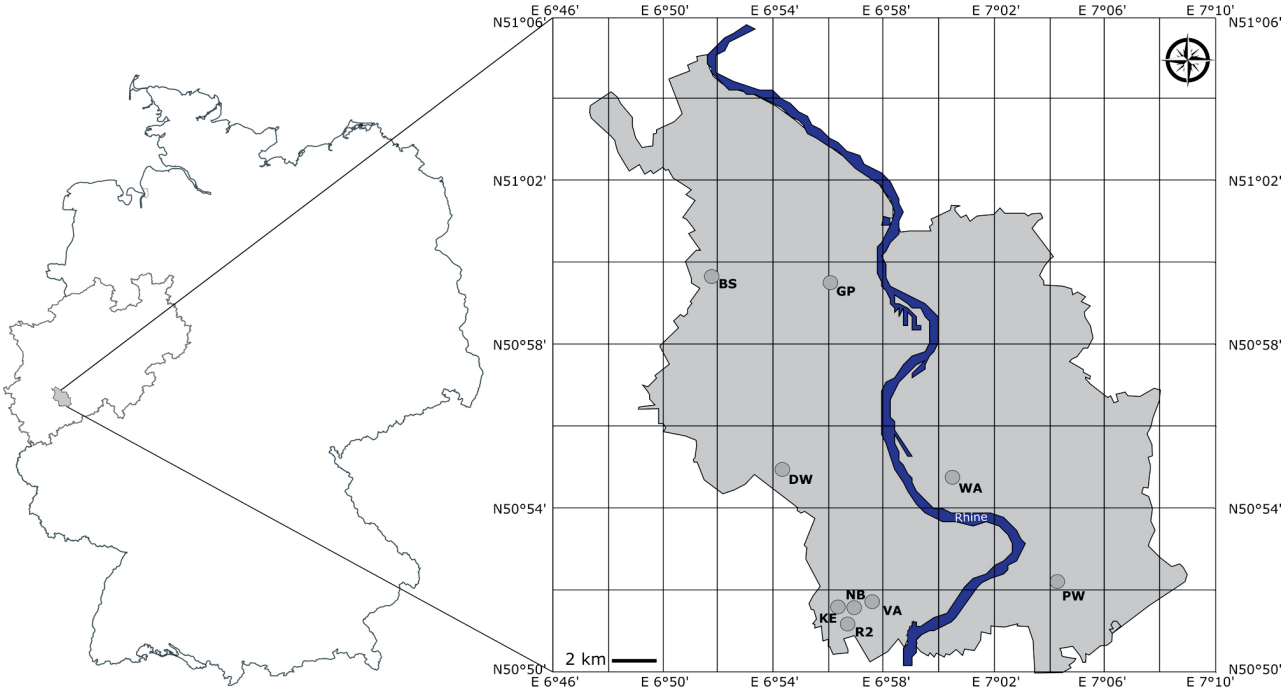

Figure 1. Maps of sampling locations within the area of Cologne. Germany map ("Lage von Köln in Nordrhein-Westfalen, Deutschland") was created by TUBS and is under the Creative Attribution-Share Alike 3.0 Unported license (https://creativecommons.org/licenses/by-sa/3.0/deed.en). It can be found on https://commons.wikimedia.org/wiki/File:Locator_map_K_in_Germany.svg_. The map was re-colored and some elements were removed. BS (Baadenberger Senke), GP (Ginsterpfad), DW (Decksteiner Weiher), KE (Kiesgrube Esser), NB (Neues Biotop Immendorf-NBI), R2 (R2.12), VA (Vogelacker), PW (Porz Wahn), WA (Westhovener Aue).

of the 18S small subunit rDNA (Amaral-Zettler et al. 2009) in duplicate, with uniquely indexed primers that also contained Illumina adapters, as described in Vences et al. (2016). To decrease the amplification of the host species we added a vertebrate blocking primer (Vestheim & Jarman 2008). Samples were pooled in approximately equal concentrations based on band strength on agarose electrophoresis, cleaned up with the PCR clean-up gel extraction kit (Macherey-Nagel, Düren, Germany), quantified using a Qubit fluorometer (Invitrogen, Carlsbad, USA), and sequenced on an Illumina MiSeq instrument (250 bp, paired-end).

Metabarcoding of environmental samples

DNA isolation from water filters took place following a DNAzol (Alfa Aesar, Haverhill, USA) protocol. Filters were rinsed thoroughly with their own supernatants (DESS). The liquids (~5 ml) were transferred to fresh 50 ml tubes (Roth, Karlsruhe, Germany) and centrifuged at 4,000 x g for 20 min at 4°C (Megafuge 2.0R, Heraeus Instruments, Hanau, Germany), followed by the manufacturer's protocol. Pellets were dried in a Speedvac (Concentrator 5301, Eppendorf, Hamburg, Germany) at 45°C and re-suspended with 30 µl elution-buffer from the Quick-gDNA prep kit (Zymo Research, Irving, USA). To remove all remaining salts, all samples were centrifuged at 10,000 x g for 10 min, afterwards discarding the supernatant. The remaining pellet was washed twice with each 1 ml ddH_2O and centrifuged at 10,000 x g for 10 min, each time discarding the supernatant after centrifugation. The remaining pellets were re-suspended with 30 µl elution-buffer. Amplification (in triplicate) of the V9 region of 18S took place with the same primers as used for cloacal swabs, but not containing Illumina adapters. The three replicates of each (filter) replicate were combined and purified with the PCR Purification Kit (Jena Bioscience, Jena, Germany), starting with step 1b for High Yield Sample preparation, and subsequently following the manufacturer's protocol. Samples were quality-checked with Nanodrop (Spectrophotometer ND-1000; Peqlab, Erlangen, Germany) and sent to the sequencing facility of the Cologne Center of Genomics (CCG) for library preparation and a subsequent sequencing on an Illumina HiSeq instrument, sharing a lane with 37 samples in total for a 2 × 150 bp paired-end run.

The raw reads of all metabarcoded samples (environmental and cloacal swabs) were submitted to the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/) and are accessible under BioProject number PRJNA611823.

Statistical data processing

Sequences from cloacal swab metabarcoding were analyzed with MACQIIME version 1.9.1 (Caporaso et al. 2010) using only the forward reads. After demultiplexing, sequences were denoised and quality filtered using Deblur (Amir et al. 2017) with a subsequent clipping to 150 bp. For taxonomic assignment, sequences were aligned to the external SILVA database (Quast et al. 2013, Yilmaz et al. 2014) and clustered into operational taxonomic units (OTUs) at 90% sequence identity. The dataset was rarefied to a minimum read count of 1,000 reads per sample. Because of incompleteness of the SILVA database, around 50% of the OTUs could not be assigned. They were once again blasted to a second database as described for the environmental samples below, which allowed all sequences to be assigned. Sequences identified as OTUs potentially relevant for amphibian health were then once again blasted against the NCBI/GenBank database to verify their identity.

For further statistical analysis we assigned OTUs to categories based on the number of reads per OTU and sample. An OTU with > 500 reads in a sample was considered to be unambiguously detected in this sample, and possibly detected with 1–499 reads. Assuming that technical replicates underlie only small differences due to technical variances associated with Illumina (Marioni et al. 2008), the reads of both (technical) replicates were summed for each OTU and sample. The categories < 500 and > 500 reads per sample and OTU were analyzed with R (Version 3.3.2, R Core Team 2015) regarding their frequency distribution in the different hosts and sampling sites. To test for significant differences between the hosts and sites in terms of frequency distribution of parasite categories we used Exact Fisher tests (all used tests are part of the native R stats package). In cases where the contingency table was too large for the algorithm of the Fisher Test, a Pearson Chi^2 test (also native R stats package) was computed, although it needs to be taken into account that this test is designed for larger sample sizes (and could lead to ambiguous results). In this case, the Exact Fisher test was used as a post hoc test for pairwise analysis between sampling sites and hosts, respectively with a Holm correction as p-value adjustment.

The data received from HiSeq Ilumina sequencing of environmental samples were filtered for high-quality V9 reads and clustered into operational taxonomic units (OTUs). Forward and reverse reads were quality encoded and merged using VSEARCH v2.6.2 (Rognes et al. 2016). Only sequences including the primer sequences were retained, sequences including ambiguous nucleotides (Ns) were discarded. The filtered reads were converted into fasta files, dereplicated with VSEARCH and then clustered with Swarm v2.2.2 (Mahé et al. 2015). Chimeras were identified with VSEARCH and only non-chimeric OTUs were used for further analysis. For taxonomic assignment, OTUs were globally pairwise aligned with VSEARCH using a reference database build from the Tara Ocean V9 database (de Vargas et al. 2015), the Protist Ribosomal Reference database PR^2 (v4.10.0, Guillou et al. 2013), the SILVA All Species Living Tree Project (Quast et al. 2013, Yilmaz et al. 2014), as well as 84 sequences of marine protist strains of the Heterotrophic Flagellate Collection Cologne (HFCC). Amplicons were assigned to their best hit/hits using the Stampa pipeline (Mahé 2016).

Other statistical analyses (e.g. ANOVAs) for the years 2016 and 2019 (where exact loads could be determined)

Table 2. Prevalence of *Bd* and *Bsal* in 2016 and 2019 (*Bsal* only tested in 2019) in percent per year, ordered according to year, site, and prevalence. NA = not applicable (not tested).

Year of sampling	Sampling site	N samples	Samples *Bd* positive (%)	Samples *Bsal* positive (%)
2016	Ginsterpfad	41	63	NA
2016	Decksteiner Weiher	2	50	NA
2016	Kiesgrube Esser	6	17	NA
2016	NBI	37	70	NA
2016	Porz Wahn	12	58	NA
2016	R2.12	13	31	NA
2016	Vogelacker	2	100	NA
2016	Westhovener Aue	20	45	NA
2019	Ginsterpfad	22	41	0
2019	Westhovener Aue	44	14	0

were conducted with R (Version 3.3.2) regarding a possible influence of season, species or sampling site on the strength of infection of individuals.

Results

Chytrid infection prevalence and loads

Bd infection was confirmed at all sites sampled, with a persistent presence in the sampled time period between 2016 and 2019 (Fig. 2, Table 2, Supplementary document 1). In 2016 we found almost 60% of the 133 screened animals being positive for *Bd*. In 2016 the three species of toads, *Bufotes viridis* (~57%), *Epidalea calamita* (66%) and *Bufo bufo* (75%, but only 4 specimens tested overall) had the highest prevalence for *Bd* infection (Fig. 3, Table 3). For the years 2017 and 2018 no exact loads could be determined. However, our results indicate that *Bd* was nevertheless very probably present in the system. Using a very conservative approach (only qPCR curves between 28 ± 3 cycles), 5 putative positives were observed in 2017 and 2018 (2 *B. viridis* and 1 *B. bufo* from Ginsterpfad, 2 *B. bufo* from Baadenberger Senke). In a more inclusive approach (qPCR curves between 15 and 35), 18 additional *Bd* positives (Supplementary document 2) were observed (1 *Lissotriton vulgaris* and 9 *B. viridis* from Ginsterpfad, 2 *B. viridis* from Westhovener Aue and 6 *B. bufo* from Baadenberger Senke).

In 2019 the highest frequency of *Bd* positives was found in *B. viridis*, with 22% of 55 specimens being infected. Though the highest *Bd* load in 2016 was detected in May, the season seemed to have no significant influence on the infection load of the individuals ($p > 0.05$).

In 2016 the highest load, with 30,202.46 zoospore equivalents/ swab (zeq/swab) was found on an individual (*B. viridis*) at the sampling site "Ginsterpfad". In this case, species (*B. viridis*) and site were those with overall highest loads in 2016 ($p < 0.05$). Despite methodological differences in the qPCR protocol, similar patterns were observed in 2019. Again, the season seemed to play no statistically significant role in the strength of infection ($p > 0.05$) but the highest load was again detected in May. In 2019 the sampling site with the significantly highest loads was again "Ginsterpfad" ($p < 0.05$), and *B. viridis* was the species with (not significantly) highest loads.

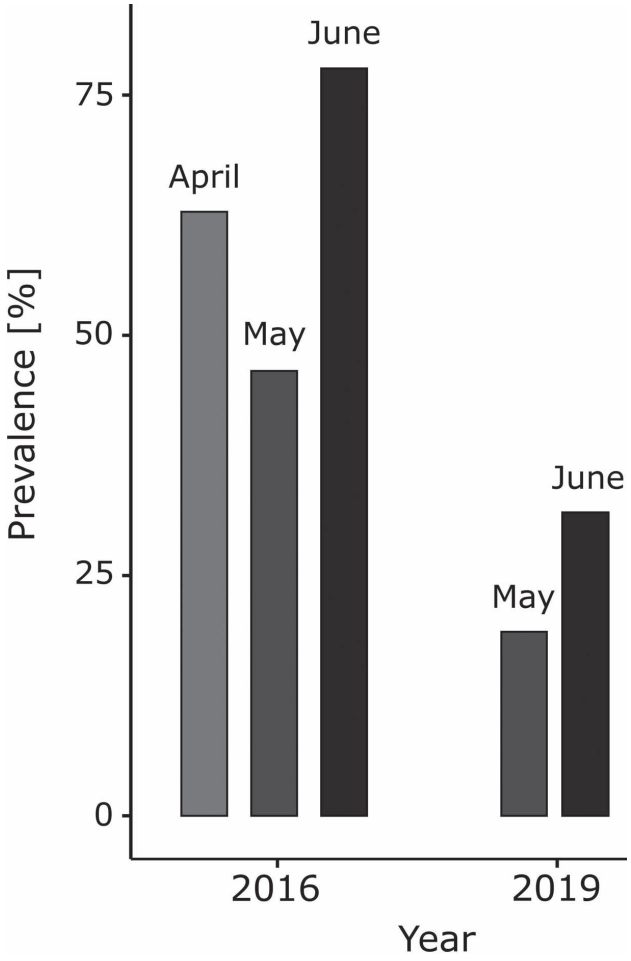

Figure 2. Prevalence [% infected individuals] of *Bd* in Cologne, by month, for the two years 2016 and 2019.

Table 3. Prevalence of *Bd* and *Bsal* in 2016 and 2019 (*Bsal* only for 2019) in percent per year, and sampled species. NA = not applicable (not tested).

Year of sampling	Sampled species	N samples	Samples *Bd* positive (%)	Samples *Bsal* positive (%)
2016	*Bufo bufo* (*Bb*)	4	75.0	NA
2016	*Bufotes viridis* (*Bv*)	91	57.1	NA
2016	*Epidalea calamita* (*Ec*)	24	66.7	NA
2016	*Lissotriton vulgaris* (*Lv*)	9	33.3	NA
2016	*Pelophylax* kl. *esculentus* (*Pe*)	5	40.0	NA
2019	*Bufotes viridis* (*Bv*)	55	21.8	0
2019	*Epidalea calamita* (*Ec*)	6	33.3	0
2019	*Lissotriton vulgaris* (*Lv*)	4	0.0	0
2019	*Pelophylax* kl. *esculentus* (*Pe*)	1	100.0	0

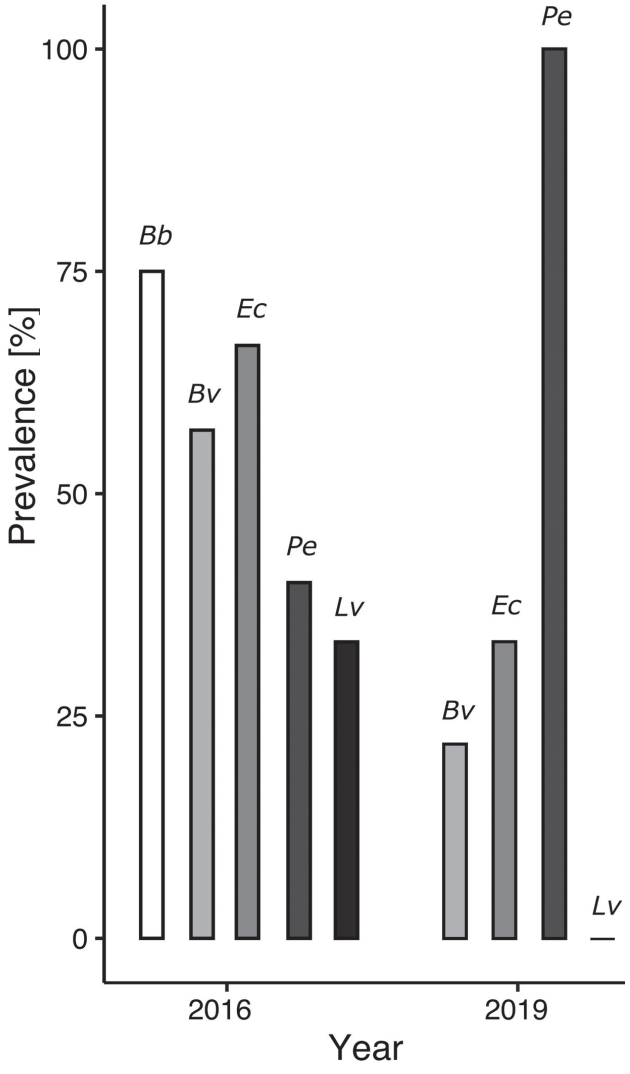

Figure 3. Prevalence [% infected individuals] of *Bd* in Cologne, per species and year, for 2016 and 2019. Abbreviations: *Bb* (*Bufo bufo*), *Bv* (*Bufotes viridis*), *Ec* (*Epidalea calamita*), *Pe* (*Pelophylax kl. esculentus*), *Lv* (*Lissotriton vulgaris*).

Loads ranged from 1.44–30,202.46 zeq/swab in 2016, and from 1,649–2,073,797 ITS copies/swab in 2019. Regardless of the partially high loads of *Bd*, none of the animals showed the typical symptoms of chytridiomycosis (i.e., lethargy, emaciation or skin lesions). From outward appearance, all individuals were found to have lesion-free skin and seemed to be in a healthy, vital condition.

The sample record of 2019 consisted of 66 samples including four *L. vulgaris* and no evidence for *Bsal* was found in these samples.

Pathogen and parasite metabarcoding

Nine different OTUs of potentially harmful organisms were found in the 157 samples originating from cloacal swabs (Table 4, Supplementary document 3): three metamonads (*Tritrichomonas augusta, Trichomitus batrachorum* and *Hexamita inflata*), three ciliates (*Balantidium duodeni, Nyctotherus cordiformis* and *N. hubeiensis*), one stramenopile (*Blastocystis* sp.) and one metazoan (a nematode: *Rhabdias ranae*). Most of these organisms are known as gut commensals with a potential to harm their host, depending on the strength of infestation. *Bd* was only found in one cloacal swab sample with a low read number.

In the category > 500 reads (considered as unambiguously identified) *E. calamita* showed the highest relative parasite load (Exact Fisher test, $p < 0.01$). In the category < 500 reads per sample and OTU, the Chi² test revealed a significant difference between host species ($\chi^2 = 73.503$, df = 32 and $p = 4.145e^{-5}$), but the post-hoc Exact Fisher test showed no significant differences in pairwise comparisons. For both categories, the Chi² test ($\chi^2 = 102.69$, df = 40, $p < 0.01$, < 500 reads) as well as the Exact Fisher test ($p < 0.01$, > 500 reads) supported significant differences between the sampling sites, with "Ginsterpfad" being the site with the highest frequency of parasitic loads.

In the environmental samples, we detected OTUs of nine organisms potentially harmful for amphibians (Table 5, Supplementary document 4): *T. augusta* and

Table 4. Parasitic organisms from fecal samples of *Bufotes viridis*, *Pelophylax* kl. *esculentus*, *Bufo bufo* and *Epidalea calamita* taken from six sampling locations around Cologne in 2018 and identified using MiSeq amplicon-sequencing of the V9 region of the small subunit rDNA. *Bb* (*B. bufo*), *Bv* (*B. viridis*), *Ec* (*E. calamita*), *Pe* (*P.* kl. *esculentus*); resp. BS (Baadenberger Senke), AV (Am Vogelacker), NB (NBI, Neues Biotop Immendorf), PW (Porz Wahn), GP (Ginsterpfad); OTU (operational taxonomic unit).

OTU	BLAST Identity (%)	OTUs identified (>500 reads)	OTUs (percent)	OTUs included (<500 reads)	OTUs (percent)	Total no reads	Localities	Species
Tritrichomonas augusta	100	43/157	27.30%	109/157	69.40%	60082	BS, AV, NB, PW, GP, WA	*Bb, Ec, Bv, Pe*
Rhabdias ranae	98	9/157	5.70%	74/157	47.13%	10957	BS, AV, NB, PW, GP, WA	*Bb, Ec, Bv, Pe*
Balantidium duodeni	99	2/157	1.30%	102/157	64.96%	6048	BS, AV, NB, PW, GP, WA	*Bb, Ec, Bv, Pe*
Blastocystis sp.	98	5/157	3.20%	20/157	12.74%	5652	BS, AV, NB, PW, GP, WA	*Bb, Ec, Bv, Pe*
Trichomitus batrachorum	94	1/157	0.60%	41/157	26.11%	1795	AV, NB, PW, GP, WA	Bb, Ec, Bv,
Nyctotherus cordiformis	99	0/157	0%	1/157	0.60%	279	NB	*Pe*
Nyctotherus hubeiensis	98	0/157	0%	1/157	0.60%	73	NB	*Pe*
Hexamita inflata	95	0/157	0%	1/157	0.60%	47	AV	*Bb*
Batrachochytrium dendrobatidis	99	1/157	0.60%	1/157	0.60%	2	GP	*Bv*

Table 5. OTUs of parasitic or potentially harmful organisms found in the water samples from 2018 with the help of HiSeq-sequencing of the V9 region of the small subunit rDNA. Occurrence per sampling sites with BLAST identity in percent, total number of reads and localities. Abbreviations stand for: PW (Porz Wahn), GP (Ginsterpfad), NB (NBI), WA (Westhovener Aue), OTU (operational taxonomic unit).

OTU	BLAST Identity (%)	Total no reads	Localities
Trichomitus batrachorum	98	1434	PW, GP
Tritrichomonas augusta	100	397	PW, GP
Leptolegnia caudata	100	292	NB
Anurofeca sp.	98	57	PW
Hexamita inflata	76.9	51	WA
Blastocystis sp.	65.7	18	NB
Saprolegnia sp.	99.2	11	PW, GP, NB, WA
Myxobolus sp.	97	5	PW, GP, NB, WA
Amphibiocystidium ranae	93.9	1	NB

T. batrachorum, the latter one with the highest number of reads, *H. inflata* and *B.* sp. (both with very low BLAST identities), and two oomycetes, *Saprolegnia* sp. and *Leptolegnia* sp. as well as two ichthyosporeans, *Anurofeca* sp. and *Amphibiocystidium ranae*. Furthermore, the myxozoan *Myxobolus* sp. was present in all water samples with a low number of reads (BLAST identity 97%). Overall, the water sample data included only one relevant OTU (*T. batrachorum*) above the chosen threshold of 500 reads, while many OTUs had substantially fewer reads and partially low BLAST identities.

Discussion

Our data show that *Bd* is common in amphibian populations in the Cologne area, and was detected across the whole sampling period from March to June (at least in 2016 and 2019), without clear seasonal trends. Seasonal fluctuations of the occurrence of *Bd* can be explained based on its temperature optimum between 17–25°C (van Rooij et al. 2015) which supports its prevalence being higher in spring. Individual *Bd* loads in our study are difficult to compare across sampling years due to methodological differences, and technical qPCR problems in 2017 and 2018. Reliable data are available for 2016 and 2019 but were obtained with different qPCR standards. Applying the estimate of 73 ITS copies/zoospore for strain JEL310 (Rebollar et al. 2017), the highest loads for 2019 (2,073,797 ITS copies/73 ~28,408 zeq/swab) are comparable to those of 2016 (~30,202 zeq/swab). These values are similar to those detected in other amphibians (e.g., Briggs et al. 2010), but their interpretation is limited by our lack of knowledge on the *Bd* strain involved. This also impedes precise comparison of loads which can be over- or underestimated without strain-specific custom standards for qPCR. The use of gBlocks® gene fragments can be seen as a methological improvement as it allows a correlation between ITS copies and zoospores. Vredenburg et al. (2009) set the threshold for a population collapse at an average ~10^4–10^5 zoospores; in 2016 we measured an average *Bd* load of 579.44 (± 3,311.64) zeq/swab, a value that is by far lower than the threshold.

The city of Cologne is geographically situated in between the two documented *Bsal* hotspots in Germany, i.e., the Eifel Mountains and the Ruhr district (Spitzen-van der Sluijs et al. 2016, Dalbeck et al. 2018), both at a dis-

tance of 60–70 km from Cologne. Since *Bsal* is apparently spreading in western Germany (Dalbeck et al. 2018), screening for this pathogen in Cologne is highly relevant. In the limited number of samples studied in 2019 we could not detect this pathogen, but it can be expected to reach the area in the next years, for example via the many hikers that frequently visit the Eifel Mountains from Cologne.

In addition to *Bd*, we found amphibian populations in Cologne to be regularly infected with a variety of mostly unicellular organisms that may be harmful to their hosts. Our data suggest that protists such as *Tritrichomonas* very frequently inhabit the intestines of amphibians in the study area, and it is known that these, if present in high densities and depending on the physical condition of the host, can be harmful for amphibians (Baker 2008). Despite the high prevalence of *Tritrichomonas*, we observed no signs of illness or malnutrition in the infected amphibians. The same applies for the infection with the nematode *Rhabdias* that is known to be quite common in the lungs of anurans and can cause pneumonitis as well as tumors when its larvae migrate and form cysts (Baker 2008). Still, many Green toads, as in our case, stay asymptomatic and pathogenic changes only occur when the individual is heavily burdened. In the water samples, other organisms were present that could have an effect on amphibian health. The so called 'watermolds', like *Saprolegnia* or *Leptolegnia*, can have strong impact on amphibian eggs, causing significant egg mortality (Blaustein et al. 1994) and increasing mortality of larvae (Romansic et al. 2009); but again, we have no evidence for negative effects on amphibians in Cologne. In case of the environmental samples, one must also take in account that due to the methodical choices (pre-filtering), there is a possibility that some organisms of interest may have been removed prior to analysis.

Unfortunately, we carried out the parasite metabarcoding on samples collected in 2018, one of the years in which our *Bd* screening was handicapped by technical difficulties; a correlative analysis of parasite infection and *Bd* infection loads was therefore not possible.

So far, no disease-driven mass mortality in amphibians has been observed in our study area. Yet, populations of *B. viridis* have dwindled and disappeared at various sites in Cologne. This decline is especially well documented for the population "Ginsterpfad", which currently is one of the northernmost populations of the species in western Germany. Despite intensive habitat management, only few individuals currently occur at this site, while a large population was present some decades ago. Our data suggest that, in this population, prevalence of *Bd* and of additional potential amphibian parasites is particularly high. Furthermore, we found that the highest loads in both 2016 and 2019 indeed corresponded to *B. viridis*. This agrees with a recent study from Sweden, where *E. calamita* and *B. viridis* (formerly considered as *B. variabilis*; see Dufresnes et al. 2019) had substantially higher *Bd* prevalences than other tested anurans (Kärvemo et al. 2018), possibly related to their highly keratinized skin. While during our surveys, all sampled adult toads appeared to be in good health, future attention should be directed to juveniles, in particular directly after metamorphosis. Studies on North American toads suggest that these early terrestrial stages may be particularly vulnerable to chytridiomycosis (Kueneman et al. 2015), possibly due to a major restructuring of the immune system and skin during metamorphosis (Rollins-Smith et al. 2011). While the high prevalence of *Bd* and other pathogens in the "Ginsterpfad" and other Cologne populations probably has little effect on adult toads, we speculate that it may drive "silent" declines (Pasmans et al. 2018) via increased juvenile mortality, highlighting the need to test this hypothesis in future studies.

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Supplementary data

The following data are available online:

Supplementary document 1. Overview of *Bd* sampling in 2016 and 2019.

Supplementary document 2. Overview of *Bd* sampling in 2017 and 2018.

Supplementary document 3. Overview of amphibians sampled for the gut parasite metabarcoding study in 2018.

Supplementary document 4. Sampling sites of environmental samples in 2018.