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ORIGINAL ARTICLE

# Urine DNA (uDNA) as a non-lethal method for endoparasite biomonitoring: Development and validation

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## Abstract

Changes in environmental conditions alter host–parasite interactions, raising the need for effective epidemiological surveillance. Developing operational, accurate, and cost-effective methods to assess individual infection status and potential for pathogen spread is a prerequisite to anticipate future disease outbreaks in wild populations. For endoparasites, effective detection of infections usually relies on host-lethal approaches, which are barely compatible with wildlife conservation objectives. Here, we used the brown trout (*Salmo trutta*)—*Tetracapsuloides bryosalmonae* host–parasite system to develop a non-lethal method for endoparasite infection detection, hereafter called "uDNA" for urine DNA. The uDNA diagnostic test is based on the amplification of endoparasite DNA from host urine. We sampled wild fish ($N$ = 111) from eight sites, let them excrete in individual buckets filled with mineral water and performed parasite DNA amplification from water filtration. We compared the results of the uDNA diagnostic test for host infection status and parasite load to those from kidney samples (the current standard method). uDNA was sensitive in determining host infection status (even for infected hosts showing no sign of the disease), since up to 90% of fish individuals were correctly assigned to their infection status. The quantity of uDNA detected from the hosts depended on the sampling sites, suggesting a spatial variation in the parasite spread. uDNA was positively, but weakly correlated with parasite load in the kidney. This correlation depended on the severity of macroscopic lesions caused by the disease and was negative in fish with severely damaged kidney, likely due to impaired urine excretion. The uDNA approach provides novel avenues to non-lethally infer infection parameters from wildlife populations at large spatial scales. By targeting parasite transmission stage, uDNA is also valuable to get insights on the parasite fitness and the ecological and evolutionary dynamics of this host–parasite interaction.

**KEYWORDS**
fresh water, non-lethal sampling, parasitology, proliferative kidney disease, salmonids, wildlife

## 1 | INTRODUCTION

Changes in environmental conditions can affect both host and pathogen geographic ranges as well as the outcomes of host–parasite interactions, such as parasite virulence and transmission rate, host susceptibility to infection, and ultimately disease development (Gallana et al., 2013; Lafferty, 2009; Schrag & Wiener, 1995). Investigating the dynamics of parasites in space and time in wild host populations is crucial for management and conservation plans (Smith et al., 2006). However, parasite surveillance in wild populations is notoriously challenging because of sampling difficulties (Ryser-Degiorgis, 2013), especially when it involves endoparasites. For instance, screening for the presence of endoparasites often requires sampling specific organs or tissues, which generally implies killing a substantial number of hosts to reliably monitor key parameters such as parasite prevalence and abundance (e.g., Cilia et al., 2020; McAllister et al., 2016; Sjöberg et al., 2009). These lethal approaches raise ethical issues and often fail to provide sufficient sample sizes in host populations. These approaches can additionally hardly be considered for protected, endangered, or even exploited species for which parasite infection monitoring is particularly important (Breed et al., 2009; Smith et al., 2006). Moreover, lethal sampling impedes repeated individual-based survey, which would be useful to monitor individual infection over time. To overcome this challenge, an increasing number of studies have developed indirect methods to non-lethally detect and monitor endoparasites in wild animal populations. Most of these studies involve parasite morphological identification in feces collection (Kumar et al., 2019; Riepe et al., 2019) and/or the use of environmental DNA (eDNA) to detect the infectious agents and evaluate disease risks (Bass et al., 2015; Honma et al., 2011; Huver et al., 2015; Sengupta et al., 2019). eDNA is defined as free DNA molecules released from living bodies in surrounding environment without having to isolate target organisms, thereby indicating the presence of a species (Bohmann et al., 2014; Pawlowski et al., 2020). eDNA has quickly become a prominent tool due to the increasing availability of powerful genetic devices allowing the detection of species even from slight amounts of DNA (Jerde et al., 2011; Miotke et al., 2014; Shokralla et al., 2012). eDNA can be used to detect parasites from the open environment (i.e., air, water, soil) (Rusch et al., 2018) or from host fluids (i.e., blood, feces, or urine) to measure host individual infection status along the infection course (e.g., Etienne et al., 2012). Using host fluids may be particularly relevant for detecting endoparasites colonizing internal organs, but is still rarely used, especially for aquatic species (but see Berger & Aubin-Horth, 2018; Jousseaume et al., 2021). Moreover, detection of parasite DNA into host fluids mediating parasite transmission could further provide valuable information on the parasite fitness and on its ability to produce infectious stages.

The myxozan endoparasite *Tetracapsuloides bryosalmonae* represents an ideal case study for testing the effectiveness of a non-lethal method to infer infection from its fish host fluids. This parasite has a complex life cycle involving two hosts: a salmonid fish species (intermediate host) and a bryozoan species (primary host). Parasite spores excreted from infected bryozoans enter salmonid fish through gills and skin, circulate through the blood until they reach the kidney and spleen where they settle and develop (Hedrick et al., 1993; Okamura et al., 2011). Parasite multiplication and strong host inflammatory response can lead to renal lesions and impair renal functions, especially blood cell production (Bailey et al., 2020; Hedrick et al., 1993). As a result, fish can suffer from anemia, which is considered to be the most severe health consequence of proliferative kidney disease (PKD), often resulting in losses in aquaculture and declines of several wild salmonid populations (Hedrick et al., 1993; Okamura et al., 2011).

As its development strongly depends upon water temperature and quality, global environmental changes could lead to more recurrent and severe PKD outbreaks (Okamura et al., 2011), emphasizing the need for precise and operational tools for monitoring *T. bryosalmonae* infection in both wild and hatchery-reared salmonid populations. *T. bryosalmonae* spores are excreted from infected fish into the water through urine (Hedrick et al., 2004; Morris & Adams, 2006). Up to date, detection of *T. bryosalmonae* in open waters is based on eDNA approaches targeting free-circulating spores and/or DNA molecules (Fontes et al., 2017; Hutchins et al., 2018). Nonetheless, the amount of *T. bryosalmonae* DNA detected with these approaches does inform neither on its developmental stage (i.e., relative number of spores released by the salmonid fish or by the bryozoan) nor on individual host infection status. The latter information is, however, important, because fish individuals are not equally infected even when exposed to the same concentration of parasite spores due to individual variation in resistance (Debes et al., 2017; Råberg et al., 2007). Current monitoring of fish parasite load and infection status relies on histological observation or *T. bryosalmonae* DNA amplification out of kidney samples (hereafter kDNA for "kidney DNA") (Bruneaux et al., 2017; Hedrick et al., 1993), involving fish euthanasia, and thus relatively small-sample sizes when inferring infection prevalence (Fontes et al., 2017; Okamura et al., 2011). *T. bryosalmonae* DNA detection from brown trout excretion has been explored recently in an experimental setup to approximate the start of spore release by infected fish host (Strepparava et al., 2018), but it has never been used for individual infection status assessment.

Here, we exploited the fact that *T. bryosalmonae* spores are released in fish urine to develop a non-lethal diagnostic test to monitor *T. bryosalmonae* infection and parasite spore release at the individual fish level. This novel approach based on the detection and quantification of *T. bryosalmonae* DNA in the fish urine (hereafter uDNA for "urine DNA"), was developed on wild brown trout (*Salmo trutta*), a species known to be an intermediate host of *T. bryosalmonae* and for releasing infectious spores (only toward bryozoan host) through urine (Okamura et al., 2011). Specifically, we tested (a) whether the probability to detect *T. bryosalmonae* DNA in the excreted urine (uDNA) is positively correlated with the probability of detection directly in the kidney (kDNA) and to a lesser extent with the detection of PKD symptoms, that is, typical gross renal lesions, and (b) whether the quantity of *T. bryosalmonae* uDNA is a good proxy of individual fish parasite load. Infected brown trout should release

*T. bryosalmonae* spores or at least DNA traces in their surrounding water through urine, even though we have no information on the regularity of the release. However, by waiting for several excretion cycles, the uDNA could inform on the infection status (infected or uninfected) and parasite load (relative quantity of parasites in the kidney) of fish individuals and be used to infer parasite prevalence and mean parasite load in fish populations. Moreover, as uDNA measures parasite transmission through fish excretion, it could be used as a proxy for the fitness of the parasite (the higher the quantity of DNA excreted in urine, the higher the fitness of the parasite), which has to our knowledge never been estimated. To assess the reliability of the uDNA method, we compared results of the uDNA test to the detection of *T. bryosalmonae* DNA in fish kidney (kDNA) that we used as a "reference" as it is the classical method currently used to detect fish infection status and parasite load (Bettge et al., 2009; Bruneaux et al., 2017). We expected the infection status inferred directly from kidney (kDNA) to be only partially correlated with the detection of gross PKD lesions, as some individuals can be asymptomatic parasite carriers (Abd Elfattah et al., 2014; Soliman et al., 2018). In addition, previous PKD studies did not find a clear relationship between the level of PKD lesions and the parasite load in the kidney (Bruneaux et al., 2017; Gorgoglione et al., 2013). More importantly, we expected to find a positive correlation between the infection status inferred from DNA detected in the urine and that inferred from DNA detected in the kidney, and that the uDNA diagnostic test would be more efficient than macroscopic examination of gross renal lesions to detect infected fish hosts, as asymptomatic infected fish should also release parasite DNA (Soliman et al., 2018). Moreover, we tested whether the amount of uDNA varied among environmental contexts (sampled sites), as ecological parameters such as water temperature may impact parasite development and disease severity (Okamura et al., 2011), which could influence uDNA detection success through variability in parasite release. We predicted a positive correlation between uDNA concentration and parasite load inferred from kidney, except for fish showing important gross renal lesions and/or fish living in the warmest sites, because of impaired excretion rate due to the disease development.

## 2 | METHODS

### 2.1 | Brown trout sampling

The study area was located in southern France, at the foothills of the Pyrenean mountains and sampling took place in the Bouigane, Lez, Oriège, Arize and Ariège Rivers (Figure 1). PKD was suspected in the area by the Fédération Départementale de Pêche de l'Ariège since late 2000s and confirmed in 2016 after a high mortality of juvenile brown trout was reported through passive surveillance (Garmendia & Lautraite, 2017). Brown trout were sampled at eight sites: six of them were sampled during the first week of September 2018, while the last two sites were sampled during the first week of October 2018 (Table 1, Figure 1). At this time of the year, all the infected fish should be shedding parasite spores (Strepparava et al., 2018). The sampled sites showed contrasted environmental conditions, especially in terms of thermal regime (see Table 1). Fish were sampled through electrofishing by the Fédération Départementale de l'Ariège de Pêche et de Protection des Milieux Aquatiques, in charge of the local angling management and the conservation of aquatic environment. We primarily targeted small juvenile trout (mean = 120 mm, range = 68–169 mm) corresponding mainly to young-of-year (0+) because brown trout are more prone to develop PKD when exposed to infectious spores of *T. bryosalmonae* for the first time (which generally occurs in Spring, some months after trout emergence from the gravel, Okamura et al., 2011). A total of 111 fish were sampled (4 to 16 individuals per site according to local abundances, Table 1).

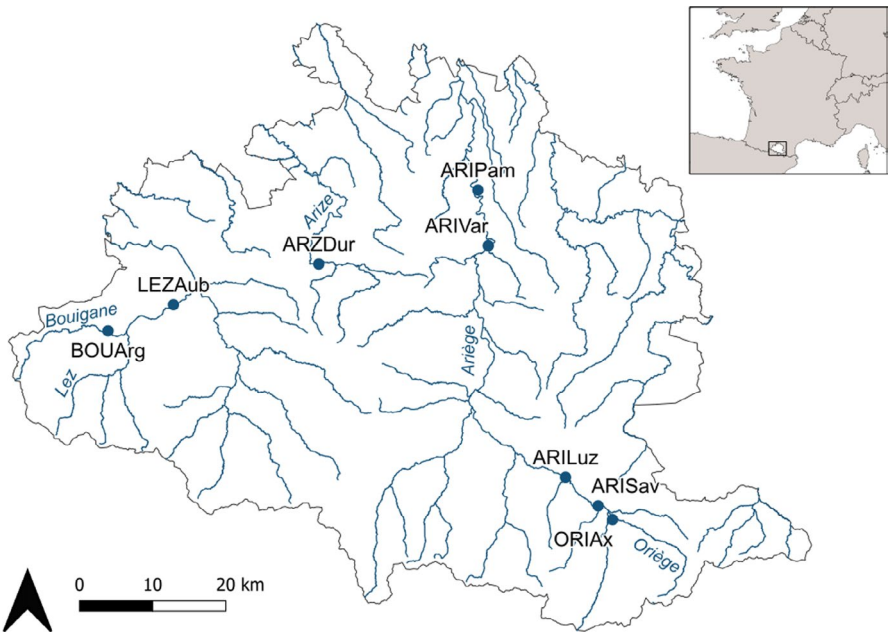


**FIGURE 1** Sampled sites location (blue dots with codes) at the foothills of Pyreneans Mountains, southern France. Main rivers of the sampled area (Ariège department) are also shown

**TABLE 1** Characteristics of sampled sites, date of sampling, and number of brown trout individuals sampled (N). Mean water temperature for summer 2018 was computed with SIEAG (Système d'Information sur l'Eau du Bassin Adour Garonne) and the Fédération de pêche de l'Ariège data when available

| Code | Site | Stream | Altitude (m) | Source distance (km) | Mean summer temperature (°C) | Sampling date | N |
|---|---|---|---|---|---|---|---|
| ARIPam | Pamiers | Ariège | 277 | 102 | – | 9/7/2018 | 16 |
| ARIVar | Varilhes | Ariège | 324 | 87 | 15.6 | 9/7/2018 | 16 |
| ARZDur | Durban | Arize | 351 | 27 | 16 | 9/5/2018 | 16 |
| LEZAub | Aubert | Lez | 418 | 32 | 17.2 | 9/6/2018 | 12 |
| BOUArg | Argein | Bouigane | 531 | 21 | 15.8 | 9/6/2018 | 16 |
| ARILuz | Luzenac | Ariège | 590 | 40 | 15 | 10/10/2018 | 4 |
| ARISav | Savignac | Ariège | 677 | 32 | 14.6 | 10/10/2018 | 15 |
| ORIAx | Ax-les-Thermes | Oriège | 777 | 21 | 12.6 | 9/4/2018 | 16 |

## 2.2 | uDNA collection

After capture, fish recovered for about 30 min in a bucket filled with stream water and aerated by a small air pump. Each individual was "rinsed" with commercial (i.e., uncontaminated) mineral water to avoid false-positive detection of parasite DNA potentially present in the stream water and then placed in a plastic bag filled with 2L of commercial mineral water (as in Raffard et al., 2019; Villéger et al., 2012). To minimize physiological stress, we chose mineral water (Cristaline®) with physicochemical characteristics similar to those encountered in streams and stored at stream temperature. Each bag was maintained in a bucket to allow proper movements of the fish, and buckets were covered and shaded to reduce fish stress. Given that urine excretion occurs by bursts every ~20 min (Curtis & Wood, 1991), fish were kept in bags for at least one hour to ensure they had enough time for at least one excretion cycle. At each site, the experimental design included 1 negative control bucket with mineral water only per 4 sampled fish (i.e., 1–4 controls per site, Table 1).

After excretion, 1L from each bag was filtered onto a 1.2µm cellulose nitrate Sartorius® filter (Ø 50 mm), using a Solinst® peristaltic pump (model 410) and Sartorius® filter holders. Filters were then individually stored in Eppendorf Tubes 5ml in a cooler on the field and then at −80°C until DNA extraction. The material (filter holders, buckets, pipes…) was thoroughly disinfected in a 10% bleach bath overnight and rinsed with clear water after each field session to avoid cross-contamination.

## 2.3 | Gross lesions and kidney parasite prevalence and load (kDNA)

After excretion, fish were euthanized with an overdose of benzocaine, measured to the nearest mm, and weighed to the nearest 0.1g. PKD gross lesions were assessed after dissection by a trained fish veterinarian (AL) through a visual inspection of gills, spleen, and kidney, showing typical PKD gross lesions ranging from 0 (no lesion) to 3 (very high PKD suspicion). A gross lesions score of 3 represents a pale kidney exhibiting severe swelling (due to hyperplasia). Medium kidney samples were collected from each individual, stored in 70% ethanol, and sent to the laboratoire des Pyrénées et des Landes (LPL, Mont-de-Marsan), a certified laboratory for analyses, in order to assess fish kidney parasite load (kDNA) through quantitative polymerase chain reaction (qPCR). 18S *T. bryosalmonae* rDNA was co-amplified together with 12S brown trout mitochondrial DNA to correct for the amount of kidney tissue used for extraction. kDNA is thus expressed as the ratio between the number of 18S *T. bryosalmonae* rDNA copies detected and the number of 12S brown trout mitochondrial DNA detected in the sample. As the LPL is commercially exploiting the kDNA approach described above, they held details about the laboratory protocol confidential. kDNA provides the actual infectious status and parasite load of a fish, that is, the "reference" value (Bruneaux et al., 2017).

## 2.4 | uDNA detection

DNA extraction was performed on excretion filters using the QIAGEN DNeasy PowerWater kit following manufacturer recommendations and under a strict laboratory environment for eDNA extractions. The 518F_Q and 680R_Q primers designed by Fontes et al. (2017) were used to amplify a 182bp fragment of *T. bryosalmonae* 18S SSU rDNA sequence. To accurately measure the quantity of parasite DNA released by fish individuals, DNA amplifications were run through quantitative PCRs (qPCRs). The PCR reactions were performed in a total volume of 20 µl including 10µl SYBR® Green master mix, 2 µl of sample DNA, 1 µl of each primer (10 µM), and 6 µl of DNase/RNase-free water. The PCR program was run with a QuantStudio™ 6 Flex System (Applied Biosystems), under the following thermal conditions: 95°C for 15 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. We performed a qPCR standard curve with one positive sample and a 1:10 serial dilution of this sample ($n$ = 7 concentrations). The linear standard curve revealed an amplification efficiency above 100% suggesting too high concentration of samples. So, we diluted the most concentrated sample in the standard curve and all samples in our study to 1/10$^{th}$. The

7 standards used encompassed the full range of sample tested in this study. The standard curve was applied to all runs to allow comparison of parasite DNA quantification across separately run qPCR plates. Each 96-well qPCR plate included 3 PCR negative controls (water only), 2 field negative controls (buckets with no fish), 2 dilution series, and samples in triplicates. A sample would be considered positive for *T. bryosalmonae* DNA when at least two out of three replicates yielded positive results. Following qPCRs, mean Ct values, representing the number of amplification cycles needed to get to a fluorescence threshold, ranged from 26.8 to 38.8 for positive samples. Ct values exceeding 40 were considered as artifacts and the corresponding assays as negative. After correcting Ct values for among-plates variation in PCR efficiency (see above), the initial relative concentration values (N0) ranged between 0 and 1, 1 being the individual with the highest *T. bryosalmonae* DNA concentration found in urine.

For a comparison purpose, we used a second method of DNA amplification and quantification that is expected to be more sensitive than classical qPCRs and eases comparisons among samples: the droplet digital PCR (ddPCR) (Doi et al., 2015). ddPCR is based on water–oil emulsion droplet technology and transforms the PCR mix into approximately 20000 droplets in which independent PCR reactions occur. If target DNA is present in the droplet, amplification occurs and the droplet fluoresces. This method provides concentration values (hereafter dC for droplet concentration) that are directly interpretable and that corresponds to the ratio of positive fluorescing droplets to total number of droplets, with no need for corrections, nor sample replicates, while limiting trouble with PCR inhibitors (Doi et al., 2015; Wood et al., 2019). ddPCRs were run with a BioRad QX200 Droplet Digital PCR system™ (Bio-Rad, Temse, Belgium), with the following thermal conditions: 95°C for 5 min followed by 40 cycles of 95°C for 30s and 60°C for 1 min; and 4°C for 5 min and 90°C for 5 min. The PCR reactions were performed in a total volume of 22 µl including 11 µl EvaGreen digital PCR Supermix, 2.2 µl of sample DNA, 1.1 µl of primer mix (same primers as for the qPCRs, 2µM), and 7.7 µl of DNase/RNase-free water. Each 96-well run included 4 PCR negative controls (water only), and a total of 18 field negative controls (buckets with no fish) were distributed among the different runs. The baseline threshold for separating positive and negative droplets was manually chosen per run, according to the distribution of the negative droplets from the negative control wells.

Ultimately, because urine excretion depends both on time spent in the plastic bag and on fish body mass (Hunn, 1982), we corrected the resulting uDNA values (N0 and dC for qPCR and ddPCR concentration values, respectively) by the time of excretion (in minutes) and fish body mass (in grams).

## 2.5 | Statistical analyses

Statistical analyses were conducted using the R environment (R 3.6.1, R Core Team, 2019).

## 2.6 | Inferring infection status and parasite prevalence from uDNA

We first used a Cochran's Q test (*RVAideMemoire* R package, Hervé, 2020) to test whether the global prevalence (percentage of infected fish across all populations) varied among the methods (renal lesions examination, kDNA, uDNA amplified using either qPCR or ddPCR). When significant, pairwise Wilcoxon post hoc tests were conducted. Then, we tested whether *T. bryosalmonae* prevalence estimated at the site level using the uDNA diagnostic test correlated (Spearman's rank correlation) with the parasite prevalence estimated using kDNA.

Finally, the sensitivity and specificity of the uDNA test (using either qPCR or ddPCR) were quantified to provide a quantitative reliability of the method. Sensitivity is defined as the proportion of infected trout (i.e., positive with kDNA) that are also detected as infected with the uDNA test, while specificity corresponds to the proportion of non-infected trout (i.e., negative with kDNA) that are also identified as non-infected with the uDNA test (Akobeng, 2007). We thus classified each individual as “true positive” or “true negative” when the status (infected or non-infected) identified through uDNA (using either qPCR or ddPCR) was the same than the status identified through kDNA. On the contrary, we classified each individual as “false positive” or “false negative” when the status identified through uDNA differed from that identified through kDNA. Sensitivity is then calculated as the ratio between the number of true positives and the sum of true positives and false negatives and specificity as the ratio between the number of true negative and the sum of true negatives and false positives.

## 2.7 | Inferring parasite load from uDNA

Our second objective was to test whether the uDNA approach was a reliable method to estimate the parasite load of fish hosts.

We first used a Spearman rank correlation test to assess the relationship between individual *T. bryosalmonae* DNA concentration in urine samples obtained either from qPCR or ddPCR, so as to test whether these two methods yield similar information about *T. bryosalmonae* DNA excretion. A similar approach was used at the site level to assess and test the relationship between *T. bryosalmonae* DNA concentration estimated from kidney and the DNA concentration estimated from urine samples (amplified using either qPCR or ddPCR), in order to provide information about the reliability of uDNA for estimating the mean parasite intensity.

Finally, we tested whether the quantity of *T. bryosalmonae* DNA excretion in fish urine could vary depending on the environmental and epidemiological contexts. We assumed that different sites corresponded to different environmental contexts and that the extent of PKD gross lesions corresponded to different epidemiological contexts, that is, different stages of disease development estimated by a trained veterinarian. We used a linear model with *T. bryosalmonae* DNA concentration estimated from urine at the individual level as the dependent variable, and *T. bryosalmonae* DNA concentration

estimated from kidney as the explicative variable and we included in the full model the site identity, the extent of gross renal lesions, and their two-term interactions as additional fixed effects. Note that three out of the eight sites (LEZAub, ARZDur, and BOUArg) were removed from this analysis because they had too few infected individuals. Based on a full model (all simple terms +the interactions between site identity and kDNA concentration and between renal lesions and kDNA concentration), we used an information-theoretic approach (based on the small-sample size corrected Akaike Information Criterion, AICc) to identify the most likely model(s) (Burnham & Anderson, 2002) using the *MuMIn* package (Barton, 2019).

## 3 | RESULTS

### 3.1 | Inferring infection status and parasite prevalence from uDNA

The overall *T. bryosalmonae* infection prevalence differed significantly among the four methods of detection (Cochran's *Q* test, $Q = 73$, $df = 3$, $p < 0.001$, Figure 2a). We identified more infected individuals with the detection of parasite DNA from kidney (kDNA, 62%) or from urine (uDNA, qPCR = 53%, ddPCR = 56%) than with macroscopic examination of PKD gross lesions (28%, Figure 2a, post hoc tests, $p < 0.001$), meaning that 55% of the infected individuals were asymptomatic. *T. bryosalmonae* prevalence estimated from uDNA was slightly lower than from kDNA (Figure 2a, Figure 3). Nonetheless, this difference was significant when uDNA was amplified using qPCR (post hoc test, $p = 0.02$), but not with ddPCR (post hoc test, $p = 0.08$). Using kDNA individual infection status as reference, uDNA methods provided a correct infection status (true positives + true negatives) for 87% (qPCR) and 90% (ddPCR) of the samples (Figure 3).

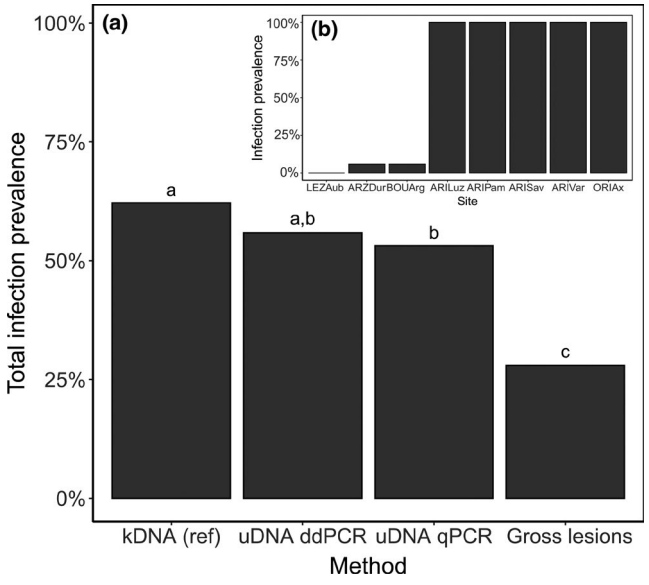


**FIGURE 2** (a) *T. bryosalmonae* infection prevalence recorded among the 111 brown trout sampled according to the detection method. kDNA indicates parasite DNA detected in kidney and serves as a reference. Udna indicates *T. bryosalmonae* DNA detected in urine (either using ddPCRs or qPCRs). Gross lesions indicate suspicion of *T. bryosalmonae* infection based on macroscopic examination of the fish. Different letters above the bars indicate a significant difference after Cochran's Q test and post hoc pairwise Wilcoxon tests ($\alpha = 0.05$). (b) *T. bryosalmonae* kDNA infection prevalence recorded at each site

Infection prevalence (from kDNA) varied markedly among sites with three out of the eight sites (LEZAub, ARZDur, BOUArg) not or barely infected (0 to 6% infection prevalence), whereas five sites (ARILuz, ARIPam, ARISav, ARIVar, ORIAx) had a prevalence of 100% (Figure 2b). In these highly infected sites, PKD gross lesions score varied between 0 and 3, except for ORIAx in which none of the individuals displayed any PKD gross lesions (Figure S1). kDNA and uDNA prevalence measured at the site level were significantly positively correlated (rho = 0.85, $p < 0.01$ for uDNA prevalence estimated from ddPCR; rho = 0.85, $p < 0.01$ for uDNA prevalence estimated from qPCR). However, uDNA underestimated the prevalence for ARIVar and ARISav (Figure 4a).

The specificity of the uDNA diagnostic test was very high (95%) for both amplification methods (qPCR and ddPCR). The sensitivity was slightly lower for uDNA amplified using qPCR than using ddPCR (83% *vs.* 87%) because qPCR yielded more false negatives (11% vs. 8% using ddPCR), even though the difference in distribution of the results categories between both methods was not significant (Stuart-Maxwell marginal homogeneity test, $\chi^2 = 1.8$, $df = 3$, $p = 0.615$) (Figure 3).

### 3.2 | Inferring parasite load from uDNA

*T. bryosalmonae* DNA concentrations estimated with uDNA from qPCR and ddPCR were strongly and positively correlated (rho = 0.96, $p < 0.001$, Figure S2), indicating that both approaches yielded very similar estimates of DNA parasite concentrations in the urine. Only the results with *T. bryosalmonae* DNA concentrations obtained from ddPCR will be shown hereafter.

kDNA concentration, representing our standard measure of parasite load, ranged from $1.27\ 10^{-6}$ to $9.29\ 10^{-2}$ *T. bryosalmonae* DNA copies per *S. trutta* DNA copy for infected trout. At the site level, mean *T. bryosalmonae* DNA concentrations measured in kidney were positively correlated with mean concentrations measured with uDNA, but this relationship was only marginally significant (rho =0.71, $p = 0.06$). *T. bryosalmonae* kDNA/uDNA concentrations strongly departed from the 1:1 expectation for two sites (ARIPam and ARILuz, Figure 4b) indicating both over- and underestimations of the *T. bryosalmonae* parasite load when measured with uDNA.

The most likely model to explain *T. bryosalmonae* DNA concentration measured in urine at the individual level included the identity of the sampling site, the score of fish PKD gross lesions, *T. bryosalmonae* DNA concentration measured in kidneys, and the two-term interaction involving the two later variables as fixed effects (Table 2, Table S1, AICc = −8.4, W = 0.72). This model revealed

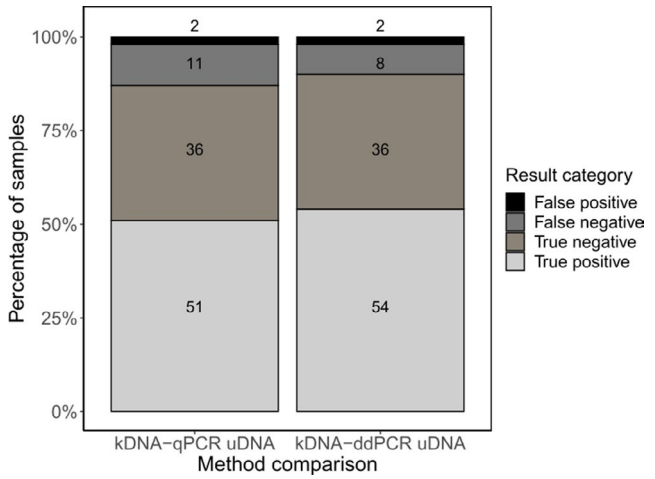


FIGURE 3 Qualitative comparison between *T. bryosalmonae* detection in the kidney (kDNA) and in the urine (uDNA) with qPCR and ddPCR amplifications, respectively. True negative/positive: samples that were found positive and negative both with kDNA and uDNA methods. False negative/positive: samples that were found either positive or negative with uDNA while they were found at the opposite with kDNA (reference method)

TABLE 2 Output of the best linear model explaining the quantity of *T. bryosalmonae* DNA excreted along with infected brown trout (*S. trutta*) urine. kDNA is the quantity of *T. bryosalmonae* DNA detected in fish kidney, Site is the fish site of origin, and gross lesions represents the score of proliferative kidney disease gross lesions estimated through macroscopic examination of the fish. Adjusted $R^2 = 0.38$, $p < 0.001$

| | *df* | *F* value | *p* value |
|---|---|---|---|
| kDNA | 1 | 1.48 | 0.230 |
| Site | 3 | 8.14 | < 0.001 |
| Gross lesions score | 3 | 1.55 | 0.212 |
| kDNA × Gross lesions score | 3 | 5.65 | 0.002 |

that *T. bryosalmonae* DNA concentration measured in urine significantly varied among sites with a highest uDNA quantity excreted by fish from ARIPam (see Figure 4b and Figure 5). More importantly, the relationship between *T. bryosalmonae* DNA concentration measured in urine and *T. bryosalmonae* DNA concentration measured in kidney significantly varied among PKD gross lesions scores (Table 2, Figure 5). The relationship between *T. bryosalmonae* DNA concentration measured in urine and in kidney was positive (as expected) for all gross lesions scores, except for score "3," corresponding to the most affected fish (in which case the relationship was negative, Figure 5). In other words, fish with highly damaged kidneys excreted less *T. bryosalmonae* DNA in their urine than expected despite high concentrations of *T. bryosalmonae* DNA in their kidneys. It is noteworthy that a significant and positive relationship was observed between *T. bryosalmonae* DNA concentration measured in urine and in kidney for the site ORIAx in which no fish exhibited any PKD lesion (see inset in Figure 5).

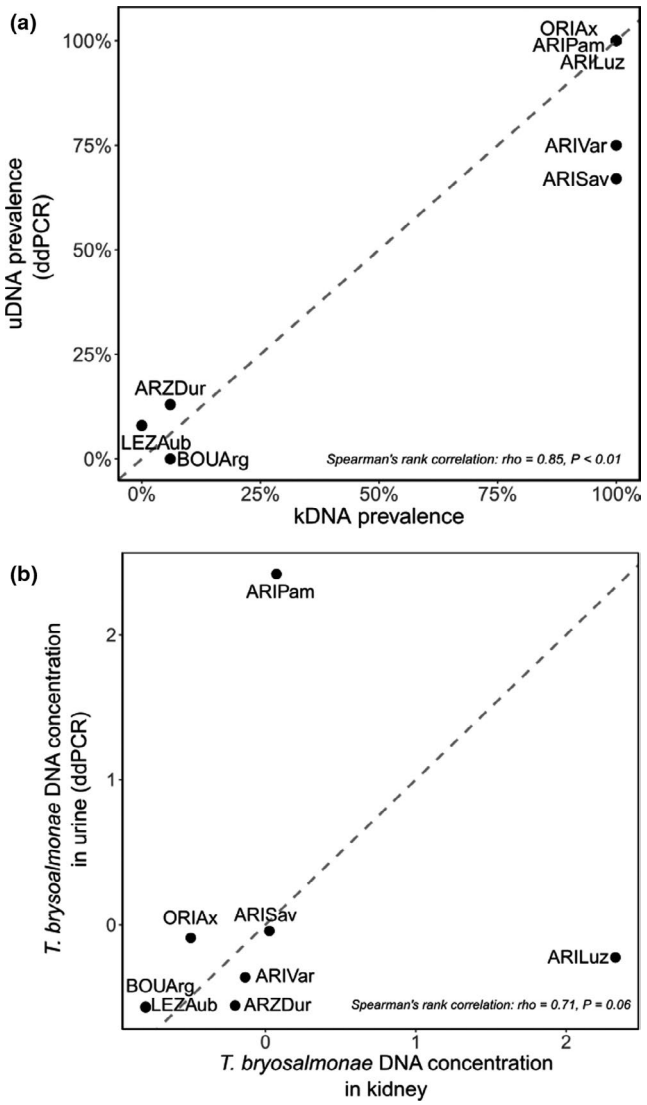


FIGURE 4 (a) Relationship between *T. bryosalmonae* prevalence per site inferred from the parasite DNA contained in the urine (uDNA diagnostic test) and prevalence inferred from the parasite DNA contained in the kidney (kDNA, reference method). (b) Relationship between the mean *T. bryosalmonae* DNA concentration detected in urine (uDNA diagnostic test) per site and the mean parasite DNA concentration detected in kidney (kDNA). Values are scaled to the mean. Each dot represents the values for a single site, and letters above dots are the code of each site (see Figure 1 for site location)

## 4 | DISCUSSION

The aim of the present study was to develop a non-lethal method for monitoring endoparasite infection in wild host populations. Detection of endoparasite infection is usually performed through tissue sampling, which is often lethal for the host (e.g., Cilia et al., 2020; Waldner et al., 2020). However, the recent development of molecular techniques now permits to detect endoparasites DNA in the environment, feces, or other host fluids (Bohmann et al., 2014). Recently, Berger and Aubin-Horth (2018) developed an

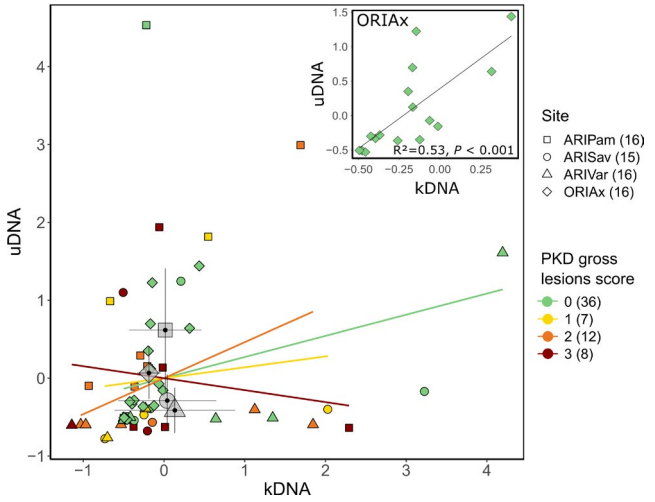


**FIGURE 5** Relationship between the quantity of *T. bryosalmonae* DNA (parasite load) detected in urine (uDNA) and that detected in kidney (kDNA) for 63 infected individuals. Values are scaled to the mean for the different intensities of the proliferative kidney disease gross lesions scores. The different symbols represent different sites, and the different colors are for different gross lesions scores. Mean values for each site are shown, along with their 95% confidence intervals (error bars). The inset shows the linear regression between uDNA and kDNA parasite load for the site ORIAx at the individual level

elegant non-lethal method to detect a large endoparasite using DNA shed by parasites via swabbing the host abdominal cavity and Jousseaume et al. (2021) used DNA from fecal samples to detect endoparasite infections in eels. Here, we followed this logic by hypothesizing that the brown trout microparasite *T. bryosalmonae*, which proliferates in fish kidney, could be non-lethally detected from fish urine excretion (Strepparava et al., 2018), therefore enlightening individual host infection parameters. In line with our initial hypothesis, we successfully inferred parasite prevalence and occurrence from urine DNA ("uDNA") and we were able to determine individual fish parasite load, although this latter inference was strongly context dependent.

Based on individual infection status measured with a conventional (but lethal) approach as a reference (see Bruneaux et al., 2017; Hedrick et al., 1993), our uDNA diagnostic test correctly identified host infection status for 87% and 90% individuals with qPCR and ddPCR amplifications, respectively, proving its efficiency for detecting *T. bryosalmonae* infection. Accordingly, test specificity was high and test sensitivity was slightly higher for ddPCR amplifications. Specifically, when an individual was not infected, the uDNA test was negative in 95% of the cases (specificity), and when an individual was infected, the uDNA test was positive in 83% and 87% cases (sensitivity) considering qPCR and ddPCR amplifications, respectively. One limitation in the validation of the uDNA method is that our sampled populations showed extreme infection prevalence (either 0–5 or 100% of infected individuals), so that we could not validate the method for intermediate values of prevalence.

The overall patterns of individual infection and population prevalence were very similar when using the qPCR and ddPCR approaches. Moreover, parasite DNA concentrations found in urine with both amplification methods were strongly correlated (as in Koepfli et al., 2016; Mulero et al., 2020), suggesting that both qPCR and ddPCR can be reliably used to infer key epidemiological parameters from hosts' urine. Nevertheless, in agreement with recent studies (Koepfli et al., 2016; Wood et al., 2019), we found a higher sensitivity for ddPCR due to a lower percentage of false negative (i.e., fish found uninfected using uDNA while actually infected), but the difference between both methods was not significant (8% *vs*. 11% for qPCR). It is noteworthy that false negatives found with ddPCR were individuals with relatively low parasite load in their kidney (ranging between 1.27 $10^{-6}$ and 1.45 $10^{-3}$ copies of *T. bryosalmonae* 18S rDNA/one copy of *S. trutta* 12S rDNA), so that some of these false negatives could be attributed to a limit of detection, or to false positives of qPCR detection in the kidney. However, the uDNA diagnostic test was efficient even for very low parasite load in kidney (as low as 3.65 $10^{-6}$ *T. bryosalmonae* DNA copy/*S. trutta* DNA copy), showing its high sensitivity, and when infected hosts showed no sign of PKD (i.e., asymptomatic infected fish). Identifying these asymptomatic infected individuals is particularly relevant as they can act as an invisible reservoir for potential future disease outbreaks. This is for instance the case when asymptomatic infected individuals move (or are moved by humans) into new locations with susceptible bryozoans. Besides, as the disease development depends—among other environmental factors—on water temperature, the presence of asymptomatic infected individuals can point out populations that have to be closely monitored if environmental conditions are changing as they would be in the frontline for developing PKD outbreaks (Okamura & Feist, 2011).

Overall, we found a positive relationship between the quantity of parasite DNA detected in urine and that detected in kidney, but this relationship was weak and strongly dependent upon fish gross PKD lesions score. The relationship between uDNA and kDNA was positive for fish showing none or moderate gross lesions and particularly strong in ORIAx, a site in which all fish were infected but not diseased (no PKD gross lesion detected). One potential explanation could be that parasite development and/or fish hosts immune responses (responsible for kidney hyperplasia in diseased fish) were likely less triggered in ORIAx compared to the other sites due to a lower water temperature or other local environmental factors (Okamura et al., 2011). By contrast, uDNA and kDNA were poorly correlated for fish with severe gross lesions, suggesting that high level of kidney damage may impair urine excretion and hence the quantification of parasite load in this fluid. Furthermore, we found that uDNA quantity varied among sampled sites. Specifically, mean uDNA quantity was significantly higher in fish from ARIPam compared to all other sites, while parasite load inferred from kDNA was similar among all sites. This illustrates that among-site variation in parasite load inferred from uDNA is yet complex to explain and must be carefully interpreted. Furthermore, another potential bias in our uDNA quantification approach is that we do not know the actual quantity of urine excreted by fish in the bags. Indeed, salmonid fish

urine flow rate can be influenced by factors such as nutrition, health status, water quality and temperature, handling, and hypoxic stress (Hunn, 1982). In the future, we propose using an "excretion control" such as urea concentration in the bag, to correct for the quantity of urine excreted. Future studies examining urine excretion may be necessary to refine this promising uDNA method for quantifying parasite load.

Finally, our results show that highly infected individuals do not release spores proportionally to their parasite load in the kidney, which suggests that kDNA may not necessarily be a good proxy for the infectious potential of fish. In other words, our results strongly suggest that fish with high concentration of *T. bryosalmonae* in the kidney do not substantially contribute to the excretion of spores in the open water, likely because of impaired kidney function and decreased excretion. These highly infected fish may therefore not be advantageous for the parasite life cycle and fitness. Our uDNA method actually provides the possibility to quantify what is excreted by fish in the open water, which can be used as a proxy of the number of released parasite spores. These two measures are thus complementary: kDNA informs on host–parasite load, and uDNA could be used as a proxy of fish infectious potential, informing on the parasitic cycle dynamics. For example, in this study, the high uDNA excretion in fish from ARIPam (compared to other populations) seems to indicate a higher fitness of the parasite in this site. It could be explained by favorable environmental conditions for the parasite development, including the fish host's characteristics. The heterogeneity of uDNA release and thus of parasite spore shedding in this site could indeed highlight the presence of super-spreaders, that is, infected fish hosts that highly contribute to the parasite cycle (Stein, 2011; Stephenson et al., 2017). This is particularly relevant as the current detection of *T. bryosalmonae* in streams through eDNA cannot discriminate between parasite DNA stemming from fish spores and that from spores excreted from the primary host (bryozoans) (Fontes et al., 2017), so that uDNA gives access to new information on this infectious disease dynamics.

To conclude, we demonstrated the efficiency of a non-lethal method for endoparasite detection in the context of an emerging infectious disease in salmonid fish. The uDNA diagnostic test proved to be efficient in determining individual infection status and prevalence at population levels and provided encouraging results to estimate individual and population parasite loads. The ease and fast implementation of this new method opens fascinating perspectives for dissecting the eco-evolutionary dynamics of this host–parasite interaction considering large spatial and temporal scales. For instance, individual surveys along the infection course can be performed in the wild through capture–recapture to determine the ecological and evolutionary impacts of this emerging parasite on host populations. We thus encourage the future development of such non-invasive approaches based on host fluids for endoparasite detection to provide a better knowledge about wild animal population infection status and infectious potential, improve the surveillance of wildlife emerging diseases, and take appropriate decisions for management actions.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

SB, GL, and LJ designed the method and coordinated the study. LG and AY conducted fish sampling. ED, SB, GL, LJ, EQ, CV, and JC handled the fish and the experiment. AL performed fish dissections and lesions diagnostic of proliferative kidney disease. GL, CV, NP, and ED performed laboratory work at EDB. ED ran the statistical analyses. ED, SB, EQ, and GL interpreted the data. ED, SB, EQ, GL, and LJ wrote the first draft of the manuscript. CV, NP, AL, LG, AY, and JC read, commented, and corrected the initial draft, and all authors gave final approval for publication.

## ETHICS APPROVAL

Authorization to collect brown trout were provided to the Fédération de l'Ariège de pêche et de protection du milieu aquatique by the « Arrêté préfectoral 2018–7».

## DATA AVAIALABILITY STATEMENT

Raw data from the main analyses are available on Figshare at the following link: https://figshare.com/articles/dataset/raw_data_uDNA_paper_txt/14605770.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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