Relationship between the prevalence of *Dibothriocephalus latus* (Cestoda: Diphyllobothriidea) and the load of *Escherichia coli*: New findings in a neglected fish-borne parasitic zoonosis

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Abstract

The sub-Alpine lakes of Switzerland, Italy and France have long been reported as an endemic area of diphyllobothriosis, a parasitic zoonosis caused by *Dibothriocephalus latus*. With this study, we explored the hypothesis for a relationship between the prevalence of *D. latus* in *Perca fluviatilis* and the *Escherichia coli* load in lake water. To do this, we identified eleven sampling sites in three areas (north, centre and south) of Lake Iseo (north Italy) to determine *E. coli* load and the prevalence of *D. latus* in *P. fluviatilis*. Prevalence and 95% confidence interval (CI) of *D. latus* infestation ranged from 0% (95% CI: 0.71–0.0) in Sarnico (southern area) to 20% (95% CI: 33.0–11.2) in Pisogne (northern area). There were significant differences in prevalence between the sites ($\chi^2 = 31.12$; *p*-value = .0006) and in *E. coli* load (Kruskal–Wallis test; *p*-value = .0005). There was decreasing gradient of *E. coli* load and prevalence of *D. latus* infestation from north to south. A significant positive correlation ($r = .881$; *p*-value = .003) was found between *E. coli* load and prevalence of *D. latus*. Also, linear regression showed a significant relationship between *E. coli* load and prevalence of infestation ($R^2 = .775$). Our findings offer an explanation for the link between *E. coli* load in water and *D. latus* prevalence. The potential factors in this link are the efficiency of the local wastewater treatment plant, the bathymetric profile of the lake and the life cycle of *D. latus*, which is mainly affected by light and water temperature.

KEYWORDS

Italy, Lake Iseo, *Perca fluviatilis*, prevalence of infestation, wastewater treatment plant

1 | INTRODUCTION

Humans contract a variety of parasitic fish-borne zoonoses, many of which are caused by helminths such as cestodes, trematodes and nematodes (Chai et al., 2005). Infections occur through the consumption of raw, undercooked or improperly processed fish products (salted or marinated fish fillets) harbouring viable larval stages of zoonotic parasites (EFSA, 2010). The type and the number of parasites in fish products depend on the environment where the fish was farmed or fished (Iwamoto et al., 2010). The large sub-Alpine lakes of Switzerland, Italy and France have long been reported an endemic area of diphyllobothriosis, a parasitic zoonosis caused by *Dibothriocephalus latus* (previously named *Diphyllobothrium latum*). According to recently published data, an estimated 20 million people are infected worldwide; however, the global prevalence of *D. latus* is unknown (Scholz et al., 2009). A recent survey funded by the Italian

Ministry of Health (RF-2010–2311360) about human diphyllobothriosis in Italy reported that nine faecal samples of patients from the endemic area tested positive for the presence of eggs and/or proglottids of *Diphyllobothrium* spp. (Prearo et al., 2016). Data on human diphyllobothriosis are scant, human cases are underestimated, and hospital reports are not generally published. In the endemic area around the Italian lakes, *carpaccio* is a popular specialty prepared with raw or undercooked European perch (*Perca fluviatilis*) (Scholz et al., 2009). So culinary tradition may play a key role in exposure to the parasite (Macpherson et al., 2000).

Dibothriocephalus latus has a complex life cycle that involves two intermediate hosts (crustaceans and fish) and a definitive host (fish-eating mammals including humans; Von Bonsdorff, 1977). Adult *D. latus* live in the intestines of mammalians where they produce eggs that are excreted with the stool and released into water bodies (Jenkins et al., 2011). In the aquatic environment, the eggs hatch and the first larval stage (coracidium) enter the water column; if eaten by a crustacean copepod (first intermediate host), the coracidium further develops into procercoid larva. Development into infectious procercoid larva takes several weeks (Von Bonsdorff, 1977). Predatory fish (i.e. *P. fluviatilis)* acting as a second intermediate host ingest an infected copepod, and the parasite migrates from the gut to the flesh of the fish where it develops further into the plerocercoid (Menconi et al., 2020).

The plerocercoid may be transmitted to other paratenic hosts (i.e. larger *P. fluviatilis*, *Lota lota*, *Esox* sp.), until consumed by a definitive host (human or carnivore mammal) (Radačovská et al., 2019). Because the reproductive potential of *D. latus* is very high (up to 1 million eggs/day), even sporadic human cases can give rise to a high prevalence of plerocercoid larvae in a fish population (Bylund, 2003). The resistance of *D. latus* eggs to external factors is low, and they cannot hatch unless they reach a suitable water body quickly (Bylund, 2003). The discharge from faulty sewage treatment plants can lead to faecal contamination of a water body, thus facilitating the parasite's life cycle (Menconi et al., 2020). The water body may be contaminated and continuously re-contaminated (Perdek et al., 2003).

Aquatic ecosystems are under increasing anthropic pressure: pollution, changes in flora and fauna and physicochemical characteristics of the environment (Mallin et al., 2000; Pastorino, Elia, et al., 2020; Pastorino, Polazzo, et al., 2020; Vitousek et al.,1997). Sources of contamination of surface water include municipal wastewater discharge, septic leachate, agricultural or storm runoff, wildlife populations, or nonpoint sources of human and animal waste (An et al., 2002). A standard method for microbial examination of water (drinking and bathing) is isolation and quantification of indicator organisms related to faecal contamination (Acharya et al., 2019).

Escherichia coli is a member of the faecal coliform group, and it is considered a specific indicator of faecal pollution than other faecal coliforms (Odonkor & Ampofo, 2013). Indeed, *E. coli* is more stable in environmental water during the course of a year, whereas faecal and total coliforms are more dependent on seasonal variations (Briancesco, 2005; Odonkor & Ampofo, 2013). Furthermore, *E. coli* does not multiply appreciably in the environment (Edberg et al., 2000; Paruch & Paruch, 2018). The World Health Organisation (WHO) recommends the use of *Escherichia coli* as faecal indicator bacteria for drinking water (Cotruvo, 2017). The presence of enteric bacteria in aquatic environments can reveal the presence of pathogenic organisms (Cabral, 2010; Dadswell, 1993). Direct contact with faecal polluted water and the consumption of fish and shellfish from contaminated environments may result in human illness in severe cases, since the seafood can harbour zoonotic agents such as *D. latus* (North et al., 2014). The risk associated with the consumption of wild fish is well documented, while the long-neglected association between farmed fish and fish-borne parasitic zoonoses has only recently gained greater attention (dos Santos & Howgate, 2011).

Lake Iseo is located within the endemic area of *D. latus* (Borroni & Grimaldi, 1973; Gustinelli et al., 2016; Parona, 1887). In their recent study, Menconi et al. (2020) reported on the distribution of *D. latus* in *P. fluviatilis* from Lake Iseo and found a decreasing gradient from north (10.2%) to centre (7.3%) to south (1.5%). The prevalence was highest in the northern area, which is closest to a wastewater treatment plant (WWTP) on the lake. In this study, we wanted to explore the hypothesis for a link between *D. latus* prevalence and faecal contamination. To do this, we identified sampling sites of Lake Iseo for collecting water samples (*E. coli* load) and fish (*D. latus* prevalence). An in-depth study of the factors involved in the distribution of *D. latus* will provide useful information for local health authorities and for future studies.

Impacts

- With this study, the hypothesis for a relationship between the prevalence of *Dibothriocephalus latus* in *Perca fluviatilis* and the *Escherichia coli* load in water of Lake Iseo (north Italy) was explored.
- There was a decreasing gradient of *E. coli* load and prevalence of *D. latus* infestation from north to south of the lake. Linear regression showed a significant relationship between *E. coli* load and prevalence of infestation.
- The potential factors implicated could be the efficiency of the local wastewater treatment plant, the bathymetric profile of the lake and the life cycle of *D. latus*.

2 | MATERIALS AND METHODS

2.1 | Sampling and parasitological analysis

During the summer of 2020 (from June to July), a total of 550 specimens of *Perca fluviatilis* were captured from 11 sampling sites (4 north; 3 centre; 4 south) along the shore of Lake Iseo. The sampling

TABLE 1 Geographical coordinates (latitude and longitude) of the sampling sites

Area	Site	Geographical coordinates
North	Costa Volpino	45.818244; 10.083798
	Lovere	45.809104; 10.074262
	Castro	45.800721; 10.068409
	Pisogne	45.803217; 10.103681
Centre	Riva di Solto	45.771817; 10.041573
	Marone	45.739263; 10.086518
	Tavernola Bergamasca	45.710581; 10.051087
South	Pilzone	45.675849; 10.069694
	Predore	45.677815; 10.018563
	Clusane	45.664600; 10.007846
	Sarnico	45.666057; 9.966530

sites were georeferenced and selected to cover the largest possible surface area of the littoral zone (Table 1; Figure 1). Fifty fish were sampled at each site.

The fish were provided by commercial fishermen who used mesopelagic gill nets (mesh size 2.5 cm). The fish were kept refrigerated (4°C) until arrival at the Fish Diseases Laboratory, Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta (Turin, Italy), where they were measured for total length (cm) and total weight (g), sexed, and subjected to parasitological examination of musculature and visceral organs to detect plerocercoid larvae of *D. latus*. The fish were then filleted in thin slices (approximately 5 mm) and inspected using a white light transilluminator (UVP White Light Transilluminators, TW-43, Analytik Jena). Plerocercoid larvae were isolated with dissecting needles, counted and fixed in 70% ethanol solution (Molecular Biology Grade, Fisher BioReagents™, Thermo Fischer Scientific). Morphological identification of plerocercoid larvae was performed according to Andersen and Gibson (1989).

2.2 | Molecular analysis

The whole body of each isolated plerocercoid larva was subjected to DNA extraction on silica columns using a commercial kit (Extractme Genomic DNA kit, Blirt S.A.). The DNA underwent multiplex PCR of the cytochrome *c* oxidase 1 (COI mtDNA) following the protocol in Wicht et al. (2010), which allows for the simultaneous amplification of *D. latus*, *A. pacificus*, *D. nihonkaiensis* and *D. dendriticus*. The PCR products were visualized by electrophoresis on 2% agarose gel (UltraPure™Agarose, Thermo Fisher Scientific).

2.3 | Water sample analysis

In July 2020, water samples were collected along the shore of Lake Iseo at the same sites ($n = 11$) as the fish sampling (Table 1; Figure 1). The sampling sites were all located in the shoreline area, since it is the primary discharge site. Water samples ($n = 3$ per site) were collected in sterile 1 L glass bottles at a depth of 50 cm. The lid of the sterile bottle was opened and closed aseptically. Each bottle was rinsed with water from the same source before sample collection. The water samples were collected in triplicate at each site to determine the *Escherichia coli* load. The samples were immediately stored in cold boxes containing ice packs (4°C), brought to the laboratory within a few hours and processed the same day.

2.4 | *Escherichia coli* enumeration and confirmation tests

Each water sample (1 L) was initially filtered through a 0.45 μm size nitrocellulose membrane. The membrane filter was placed on 10 ml of sterile saline water and then submitted to 10-fold dilution to 10^{-6}. For each dilution, 100 μl was streaked twice onto a plate of TBX agar (tryptone bile x-glucuronide agar; Liofilchem®) and incubated at 42°C for 24 hr; the number of positive (blue green) colonies on the plate was counted.

Presumptive *E. coli*-positive colonies were submitted to confirmation tests: Gram staining, growth on MacConkey Agar (Microbiol) at 42°C, lactose fermentation ability and indole production. Phenotypic bacteria identification was confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) on a VITEK MS system (bioMérieux).

The membrane filtration method is based on the assumption that each colony on the plate is formed by only one *E. coli* bacterium. The results are expressed as colony-forming units per millilitre (CFU/ml), according to the proportion between the dilution and the number of colonies encountered in each TBX plate as reported by UNI-EN ISO 9308–1:2017 (ISO, 2017).

2.5 | Statistical analysis

The D'Agostino–Pearson test was used to verify normal data distribution. The prevalence of infestation was calculated for each site. Differences in the prevalence of infestation between the sampling areas were tested using the chi-square (χ^2) test. Prevalence of infestation was calculated according to Bush et al. (1997); 95% confidence intervals (95% CI) are given for prevalence values. Since the null hypothesis for normal distribution could not be rejected, the

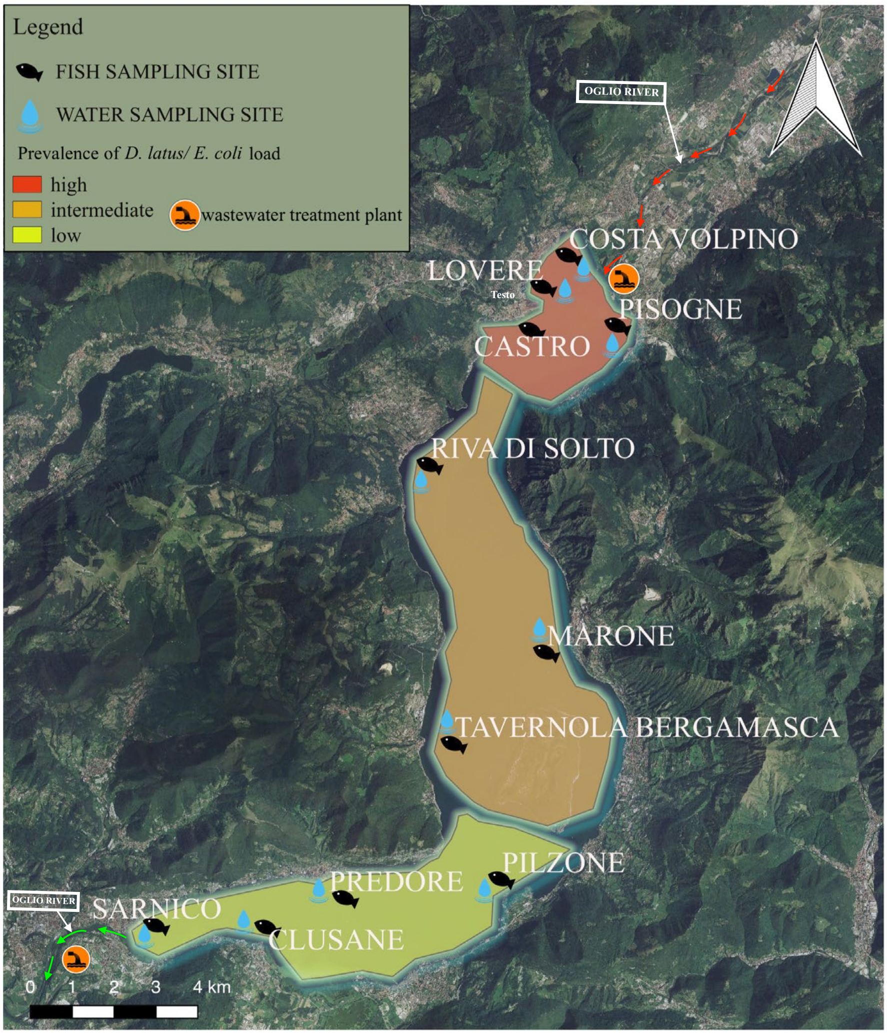

FIGURE 1 Decreasing gradient of *Dibothriocephalus latus* prevalence and *Escherichia coli* load from north (red) to south (light green). The symbol indicates the two main wastewater treatment plants on the lake (Pisogne, north; Paratico, south)

TABLE 2 Biometric measures (mean ± standard deviation) and sex ratio (*M* = male; *F* = female; in percentage) of *Perca fluviatilis* from the 11 sampling sites in the three areas

Area	Site	Total length (cm)	Total weight (g)	Sex (%)	
				M	F
North	Costa Volpino	17.42 ± 1.51	72.23 ± 12.32	48	52
	Lovere	16.71 ± 2.12	69.12 ± 10.34	52	48
	Castro	17.20 ± 2.41	70.01 ± 14.54	46	54
	Pisogne	16.88 ± 1.23	69.15 ± 10.67	42	58
Centre	Riva di Solto	17.13 ± 0.79	71.56 ± 9.77	40	60
	Marone	17.98 ± 1.78	72.87 ± 10.65	45	56
	Tavernola Bergamasca	16.32 ± 2.43	67.78 ± 8.41	52	48
South	Pilzone	17.56 ± 2.48	68.40 ± 9.35	40	60
	Predore	17.27 ± 1.56	69.71 ± 11.81	44	56
	Clusane	16.96 ± 2.21	66.87 ± 12.57	46	54
	Sarnico	17.02 ± 1.20	73.02 ± 11.03	42	58

non-parametric Kruskal–Wallis test was used to verify the differences in biometric measures (total length and total weight) of the fish and the *E. coli* load for the 11 sampling sites. Pearson's correlation matrix was applied to determine the correlation between the prevalence of infestation and *E. coli* load after data normalization. Simple linear regression was used to check the strength of the correlation (R^2) between the prevalence at each site (dependent variable) and the *E. coli* load (independent variable). In this analysis, R^2 describes the proportion or percentage of variance in the dependent variable explained by the variance in the independent variable. The criterion for significance was set at *p*-value <.05. Statistical analysis was performed using RStudio® version 1.1.463 (RStudio, Inc.).

3 | RESULTS

A total of 550 specimens of *P. fluviatilis* were caught from the 11 sites (*n* = 50 per site) and examined for the presence of *D. latus*. Total length, total weight and sex of fish are presented in Table 2. In the North area, the mean total length of fish ranged from 16.71 cm in Lovere to 17.42 cm in Costa Volpino, in the centre area from 16.32 cm in Tavernola Bergamasca to 17.98 cm in Marone, in the South area from 16.96 cm in Clusane to 17.56 cm in Predore. As regard the mean total weight of fish, it ranged from 69.12 g in Lovere to 72.23 g in Costa Volpino (North area), from 67.78 g in Tavernola Bergamasca to 72.87 g in Marone (Centre area) and from 66.87 g in Clusane to 73.02 g in Sarnico (South area). The biometric measures did not differ significantly between the sampling sites (Kruskal–Wallis test; *p*-value >.05), indicating that the fish were similar in size.

All the plerocercoid larvae tested on biomolecular analysis showed an amplicon in electrophoresis. The size of the electrophoresis band was found to be compatible (~440 bp) with that expected for *D. latus* (437 bp). The prevalence of the plerocercoid larvae of *D. latus* and the 95% confidence intervals (95% CI) are presented in Table 3. Prevalence of infestation ranged from 0% (0.71–0.0) in Sarnico (southern area) to 20% (95% CI: 33.0–11.2) in Pisogne (northern area). The chi-square test showed significant differences in prevalence between the 11 sites ($\chi^2 = 31.12$; *p*-value = .0006).

The load of *Escherichia coli* (CFU/ml) ranged from 727 CFU/ml in Costa Volpino to 4 CFU/ml in Sarnico (Table 3). There was a significant difference in CFU/ml across the sampling sites (Kruskal–Wallis test; *p*-value = .0005). We noticed a decreasing gradient of *E. coli* load and prevalence of *D. latus* infestation from north to south, with high, intermediate and low prevalence/*E. coli* load (Figure 2).

TABLE 3 Load of *Escherichia coli* (CFU/ml) and prevalence (%) of *Dibothriocephalus latus* at the 11 sampling sites

Site	*Escherichia coli* (CFU/ml)	Prevalence (%; 95% CI)
Costa Volpino	727	18 (30.8–9.8)
Lovere	650	14 (26.2–7.0)
Castro	98	12 (23.8–5.6)
Pisogne	92	20 (33.0–11.2)
Riva di Solto	14	9 (18.8–3.2)
Marone	12	6 (16.2–1.6)
Tavernola Bergamasca	12	4 (13.5–0.7)
Pilzone	10	4 (13.5–0.7)
Predore	9	2 (10.5–0.1)
Clusane	9	2 (10.5–0.1)
Sarnico	4	0 (0.71–0.0)

A significant positive correlation (*r* = .881; *p*-value = .003) was found between *E. coli* load and prevalence of *D. latus*. Regression analysis showed a significant linear relationship between these two variables: *E. coli* load significantly explained 77.5% of the variability observed for prevalence at the 11 sampling sites.

4 | DISCUSSION

This study is the first to provide a spatial analysis of *D. latus* occurrence in *P. fluviatilis* and *E. coli* load in water. Our data show a significant relationship between these variables. The *D. latus* prevalence and the *E. coli* load can be explained by the location and efficiency of the local WWTPs. The municipalities on the northern shore of Lake Iseo are served by the WWTP in Pisogne (Figure 1) at the mouth of the Oglio River, the main tributary feeding the lake. When the capacity of a WWTP is exceeded, a combined sewer overflow can be released in a mixture of sanitary sewage and stormwater (McLellan et al., 2007, McLellan and Eren, 2014). Urban stormwater can contain not only pollutants (e.g. metals and pesticides) but also bacteria and pathogens adverse to public health (Bannerman et al., 1993; Gaffield et al., 2003; Haile et al., 1999). Also, faecal discharge and contaminants in soil can be washed into the drainage system by the flushing action of storm events. Furthermore, faecal waste from humans and animals (i.e. pets, farm animals and wild fauna) can enter a water body through the sewage from sanitary landfills or sewage effluents improperly processed by a WWTP and/or malfunctioning septic tanks. Local domestic animals, especially dogs, could represent a source of faecal contamination that maintains the *D. latus* life cycle (Chai et al., 2005; Sager et al., 2006; Torres et al., 2004).

Differently, the WWTP in the southern area (Paratico), which collects wastewater from both the centre and the southern municipalities on the lake, discharges wastewater into the Oglio River downstream from the lake. So also, in cases of overload it does not affect faecal contamination of the lake water or the occurrence of *D. latus*.

The difference in *E. coli* load and *D. latus* prevalence could also be related to the bathymetric profile of the lake. The maximum depth of the northern end of the lake, close to the Oglio River, is 85 m; proceeding southwards, the deepest point in the central part of the lake (Monte Isola) is 256 m, after which the depth decreases farther south (Bini et al., 2007). The sampling site close to the Pisogne WWTP was found to have the highest contamination level of *E. coli* and the highest prevalence of *D. latus*, whereas both contamination and prevalence were much lower in the central and the southern areas. This could be due to dilution by sewage drains when they reach maximum depth, which seems to create a sort of natural barrier to faecal contaminants and the life cycle of *D. latus*.

Of note is that the persistence of the *D. latus* life cycle depends on other environmental factors as well. The best environmental conditions seem to be relatively shallow littoral water, where the temperature is relatively warm and the vegetation supports copepods and fish (Von Bonsdorff, 1977). Accordingly, we may assume

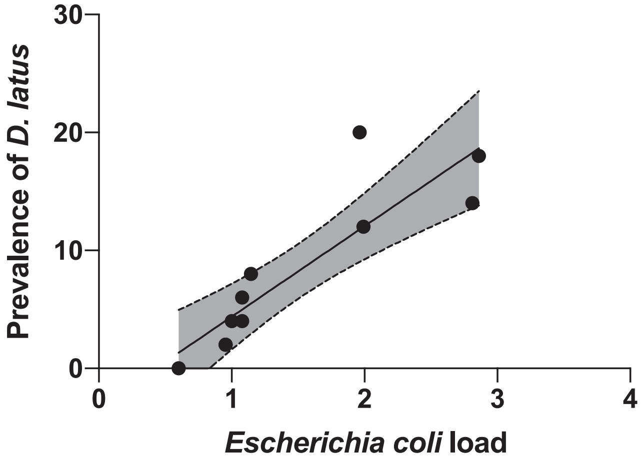

FIGURE 2 Simple linear regression between *Escherichia coli* load and prevalence of *Dibothriocephalus latus* at each sampling site. The solid line is the regression line and the dashed lines are the 95% confidence interval (grey zone)

that warmer seasons (spring and summer) are more favourable for the life cycle of *D. latus*. Furthermore, the sampling sites were located in the littoral zone, where environmental conditions (i.e. light and temperature) are more favourable to egg hatching and coracidium development (Von Bonsdorff, 1977). Since light activates the enzyme necessary to start egg hatching, hatching does not occur at depths of 15 m or more where light no longer arrives (Chubb, 1980; Von Bonsdorff, 1977). Overall, the prevalence gradient reflects a temporal and/or spatial overlap between the parasite and the host population: an increased contact between host and parasite, which is a vital factor for parasite occurrence (Henricson, 1978). Our findings provide a spatial analysis indicating a higher risk of diphyllobothriosis for fish consumers at the northern end of Lake Iseo.

5 | CONCLUSION

This study provides a comprehensive explanation for the *E. coli* load in water and its link to the prevalence of *D. latus* in Lake Iseo. The use of a geographical approach is fundamental for the identification of high-risk areas and to plan improvements in WWTP efficiency. The complex nature of fish-borne parasitic zoonoses and the lack of collaboration between policymakers in human and animal health result in underestimation of their impact on public health. Investigation into the dynamics of water contamination by *E. coli* and the survival and transmission strategies of *D. latus* require a multidisciplinary approach, which will be applied in future studies of other lakes.

ETHICAL APPROVAL STATEMENT

This research was not an experimental trial. Indeed, the fish exanimated for the presence of *D. latus* were sampled by professional fishermen of Lake Iseo. Fishermen have a fishing licence that allow them to caught fish from the three main areas of the lake.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Vasco Menconi involved in investigation; methodology; writing-original draft; Simona Zoppi involved in conceptualization; investigation; methodology; writing-reviewing and editing; Paolo Pastorino involved in conceptualization; data curation; investigation; writing-original draft; writing-reviewing and editing; Alessi Di Blasio involved in conceptualization; investigation; methodology; writing-reviewing and editing; Roberta Tedeschi involved in conceptualization; investigation; methodology; writing-reviewing and editing; Elisabetta Pizzul involved in conceptualization; investigation; methodology; writing-reviewing and editing; Davide Mugetti involved in conceptualization; investigation; methodology; writing-reviewing and editing; Mattia Tomasoni involved in conceptualization; investigation; methodology; writing-reviewing and editing; Alessandro Dondo involved in conceptualization; investigation; methodology; writing-reviewing and editing; Marino Prearo involved in funding acquisition; investigation; conceptualization, supervision; writing-reviewing and editing.

DATA AVAILABILITY STATEMENT

Data that support the findings of this study are available upon request.

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