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Consequences of fish introduction in fishless Alpine lakes: preliminary notes from a sanitary point of view

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Abstract

The introduction of fish for stoking purpose is a common operation to manage fisheries. This may cause ecological and sanitary risks to native aquatic biodiversity especially in fishless Alpine lakes. In these ecosystems, fish have been introduced by anglers for ages. Europe imposes strictly controls on aquaculture facilities that breed fish for stoking purpose only for viral disease but not for bacterial or parasitic ones. Moreover, the role of alien fish as carriers of pathogens is poorly studied. For these reasons, we performed two fish sampling campaigns in two Italian Alpine lakes (Dimon Lake and Balma Lake) to have qualitative information about alien fish populations and to perform a bacteriological survey on captured fish. In Dimon Lake we captured individuals of *Cottus gobio* and of *Phoxinus phoxinus*, while only individuals of *Salvelinus fontinalis* were sampled in Balma Lake. We isolated *Aeromonas sobria* and *Plesiomonas shigelloides* in Dimon Lake, which are bacteria widespread in aquatic environment. In Balma Lake, one fish was positive for *Yersinia ruckeri*, a primary pathogen present in aquaculture facilities that reared salmonids. Our study show how alien species may introduce bacteria that could be more virulent for native aquatic fauna or even pathogenic bacteria.

Introduction

Knowledge about diseases in freshwater wild fish are very limited and often focus on study about the interaction between wild and farmed fish (Johansen et al., 2011) or regarding specific fish parasites (Costello, 2006; Finstad, Boxaspen, Asplin & Skaala, 2007; Heuch, Øines, Knutsen & Schram, 2007; Helland et al., 2015; Krkošek, Ford, Morton, Lele & Lewis, 2007; Krkošek, Lewis, Volpe & Morton, 2006), viral (Brudeseth & Evesen 2002; Godoy et al., 2008; Wallace, Gregory, Murray, Munro & Raynard, 2008) or bacterial pathogens (Chambers, Gardiner & Peeler, 2008; Esteve, Merchán, & Alcaide, 2017; Johnsen & Jensen, 1994). There is controversy about whether or not disease in farmed fish may be transferred to wild fish population, or *vice versa*, since some pathogens as *Renibacterium salmoninarum* were recognized initially among wild fish (Mackie et al., 1933) suggesting the diffusion from wild to reared fish (Austin & Austin, 1987). On the other hand, pathogens as *Aeromonas salmonicida* was initially discovered in farmed fish (Emmerich & Weibel, 1894). The introduction of fish fauna for stoking purpose is a common operation to manage fisheries in inland freshwater. This operation may cause ecological and sanitary risks to aquatic biodiversity. This is particularly true in fishless mountain lakes as Alpine lakes which are particularly sensitive to anthropogenic impacts. In these ecosystems, fish fauna is non-native, introduced by anglers for ages from aquaculture facilities (Tiberti, von Hardenberg & Bogliani, 2014). The most common negative effects that an alien fish may cause in lentic and lotic freshwater are: 1) predation; 2) competition with native species; 3) changes in the habitat; 4) hybridization with indigenous species and 5) spreading of new pathogenic agents (parasites, bacteria, virus, fungi) (Gozlan, Britton, Cowx & Copp, 2010) that may be more virulent to new hosts due to the lack of innate immunity in the native species (Sheath, Williams, Readingm & Britton, 2015). In addition, certain pathogens accidentally introduced with fish stoking may be potentially dangerous for humans including some indirect life cycle parasites present as intermediate hosts in freshwater fish: these parasites can pass from fish to human (zoonosis) and can cause direct effects on human health. Indeed, humans can be the final host for some parasites. Stoking with infected fish could allow the parasite to complete its cycle life (depending on the presence of suitable host species and appropriate environmental conditions) with possible repercussion on human health. An example is *Diphyllobothrium*, a genus of tapeworms which can cause diphyllobothriasis in humans through consumption of raw or undercooked fish (Hall, Hewitt, Tuffrey & De Silva, 2008; Prearo et al., 2013). The main species that cause diphyllobothriosis is *D. latum*. This tapeworm is native to Scandinavia, western Russia, and the Baltics, though it is now also present in North America, especially the Pacific Northwest (Scholz, Garcia, Kuchta & Wicht, 2009) but it also presents in some subalpine lakes in the North of Italy (Gustinelli et al., 2016). Nowadays, Europe imposes strictly controls on aquaculture activities that breed fish for stoking purpose only for certain viral diseases (Council Directive 2006/88/EC) but not for bacterial or parasitic ones (Pastorino et al., 2017). Unfortunately, the real health status of the introduced fish is not well investigated. The lack of an appropriate health monitoring can lead to an introduction of bacterial or parasitic diseases in public waters and can treat native aquatic fauna as endangered amphibians or aquaculture facilities located in proximity of the fish introduction, with possible negative consequences on public health. On this basis, we performed a bacteriological survey on wild fish population from two Alpine lakes in Northern Italy to underline the human pressure on pristine ecosystems through the introduction of fish.

Materials and methods

Study areas

In this study, we focused attention on two Alpine lakes: one is Dimon Lake in Carnic Alps (Friuli-Venezia Giulia, northeast Italy) and the other is Balma Lake in Cottian Alps (Piedmont, Northwest Italy). Both are typical glacial-origin lakes, classified as Sites of Community Interest (SCIs), and originally were fishless lakes affected by the introduction of alien fish species for ages.

Dimon Lake is placed at 1857 m above sea level, in a small valley on the slopes of the Mount Dimon. The Lake is localized in the Municipality of Ligosullo, a small mountain village far about 70 Km from the main urban centre of Udine. Carnic Alps are one of the most remote areas in Italy, and the anthropic impacts are very limited, except for the pasture activity. This lake is quite far away from direct industrial sources of pollution, and the principal pressure is the long-distance transport of pollutants coming from the lowland of Friuli-Venezia Giulia and Veneto Regions.

Balma Lake is situated at 2100 m a.s.l. in the municipality of Coazze, a small urban centre about 40 kilometres far from the country's main city of Turin. This lake is quite far away from direct industrial sources of pollution and the principal pressures are the long-distance transport of pollutants coming from the lowland, pasture and fishing activities.

Fish sampling campaigns

During 2017, two sampling campaigns (July and October) were performed for each lake to have qualitative information about fish populations. We received the authorizations by competent authorities (Città Metropolitana of Torino and Friuli-Venezia Giulia Region) to retain the maximum of 40 specimens of fish for each sampling campaigns. In Dimon Lake fish were sampled using an electrofishing boat in the littoral and in the deep zones. By contrast, in Balma Lake, since can only be reached on foot, fish were captured using two multimesh gillnets (36 x 1.8 m) divided into 6 panels with different mesh size able to capture indiscriminately all the size classes except for the smallest fish (young of the year). The gill nets were placed in two zones (littoral and deep) for about three hours and then recovered.

Bacteriological analysis

Captured fish (bullhead and brook trout) were weighed and measured for their total length. Subsequently, the individuals were suppressed by deep anaesthesia with MS-222 dissolved in water in a lethal concentration after the granting of an authorization released by the competent authorities. The drug used is the MS-222 (Tricaine methanesulfonate, Sigma Aldrich), an anaesthetic approved by Food and Drug Administration and registered in Italy. The subjects were necropsied under aseptic conditions and evaluated for the presence of lesions such as wounds, bleeding or other pathological alterations. The bacteriological exam was performed from kidney and the inoculum was directly plated out on first isolation media as Columbia Blood Agar or Tryptic Soy Agar. The colonies, eventually grown after 24-72 hours of incubation at 22 ± 2°C, have been cloned and identified by phenotypical and biochemical tests (API System 20E, NE, bioMérieux, France). Furthermore, agglutination test kit (BIONOR

Mono-Yr, Bionor Laboratories, Skien, Norway) was also used to identify *Yersinia ruckeri*. Phenotypic bacteria identification was confirmed by Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) technology on VITEK MS system (bioMérieux, France).

Results

Fish populations

In Dimon Lake we captured a total of 35 individuals of bullhead (*Cottus gobio*) (15 in the first campaigns, 20 in the second one) and some individuals of minnow (*Phoxinus phoxinus*), not retained for bacteriological analysis. In Balma Lake, a total of 40 individuals of brook trout (*Salvelinus fontinalis*) (20 specimens for each campaign) were sampled. The average total length for *C. gobio* captured during the two sampling campaigns was 13.52 ±1.29 cm, with an average weight of 28.63±7.42 g. Instead, the average total length for *S. fontinalis* captured during the two sampling campaigns was 18.81±4.57 cm, with an average weight of 79.20±50.75 g.

Bacteriological analysis

All fish captured in our lakes during the two-sampling campaign did not show lesions or clinical signs. Results of bacteriological analysis and relative biometrics of specimens of bullhead sampled in Dimon Lake during the first sampling campaign are reported in Table 1. Bacteriological analysis was positive in 5 fish (33.33%) from the first sampling campaign. In specimen number 9 we isolated not significant germs (NSG), that are environmental bacteria ubiquitously in nature or wide spread in aquatic environment, which are not significant for the aim of the study. Samples 6, 8, 13 and 15 instead were positive for *Aeromonas sobria*. The bacteriological analysis of fish collected during the second campaign was negative for all the fish sampled. In Balma Lake instead, the results are shown in Table 2. During the first campaign, only fish no. 1 and 2 were positive (10%). Fish no. 1 was infected by *Yersinia ruckeri*, while fish no. 2 was infected by *Plesiomonas shigelloides*. During the second sampling campaign, tree captured fish (15%) showed only NSG bacteria.

Discussion

In this study we want to underline the effects of fish stoking in remote ecosystems, not so much from an ecological point of view (in fact, we did not perform a quantitative analysis of fish populations) since that issue have been already dealt in other papers (Pastorino et al., 2016; Tiberti, von Hardenberg & Bogliani, 2014), but from a sanitary point of view, due to the lack of data about introduction of pathogens through alien fish. We know that in Dimon Lake individuals of brook trout (*Salvelinus fontinalis*) have been introduced in the 80s for fishing purpose (Ente Tutela Pesca FVG, personal communication). Nowadays, *S. fontinalis* is no longer present in the lake since we have not caught it during the two sampling campaigns. This forced us to think about how brook trout have disappeared from Dimon Lake, knowing that no eradication events have been occurred over the years. Nevertheless, we found that in this lake is present a small population of minnow (*Phoxinus phoxinus*). The minnow is usually used as live bait by fisherman, for this reason, it was probably introduced in Dimon Lake in this way or accidentally with fish stocking. Mirò and Ventura (2013, 2015) reported that in lakes in which minnow and brook trout were introduced together, *S. fontinalis* starts to decrease, and in some cases, completely disappears. Instead, in lakes in which only brook trout are present, the population remains stable. They showed that this phenomenon is due to the ecological strategy of minnow that feed on brook trout's eggs, reducing or eradicating the population of brook trout in absence of further introductions. In fact, we hypothesized that in Dimon Lake a sum of effects led to a not direct human-mediated eradication of *S. fontinalis*, among them: the absence of new introductions in recent years, the presence of fishing pressure and the minnow that feed on brook trout's eggs. However, both lakes under study were altered with non-native fish introduction. The implications of stocking into high mountain lakes derive primarily from the fact that fish occupy a higher trophic level that was previously inexistent, leading to some severe ecological changes to native aquatic communities. Non-native fish introduction in original fishless lakes is commonly associated with the reduction of native biodiversity (as invertebrate and amphibian) and can have indirect effects on the whole aquatic ecosystem (Eby, Roach, Crowder, & Stanford, 2006). The majority of the studies concerning the ecological effects of fish introduction in high mountain lakes mostly focus the attention on salmonid fish as *S. fontinalis*. Despite this, *P. phoxinus*, *C. gobio* and others small fish are also a great threat to biodiversity conservation too. These species are autochthonous in Padano-Veneto district (Friuli-Venezia Giulia included) (Forneris, Paradisi & Specchi, 1990) but introduced accidentally many years ago in Dimon Lake. For this reason, they are considered a fully-fledged alien species. In fact, in Dimon Lake (and in many other lakes among European mountains) these species are also at the top of the food chain and are responsible of the same cascade-effects on the whole ecosystem, including the terrestrial habitat. Salmonids are visual predators, and most of the direct impacts are attributable to their size selective predation strategy, affecting only larger non-fossorial taxa (Tiberti, Brighenti, Canedoli, Iacobuzio & Rolla, 2016a). In literature, published studies from different mountain regions of the world have focused on the effects of trout introductions that produce a drastic reduction or elimination of native species of bigger size. The effects on amphibians are of special concern, since they are one of the most threatened animal groups worldwide (Beebee & Griffiths, 2005). The effects of fish introduction are also related with the terrestrial habitat, especially on macroinvertebrate during their emergency phase when passing through the water column, which results in a substantial alteration of their emergency rates (Epanchin, Knapp & Lawler, 2010; Tiberti, Rolla, Brighenti & Iacobuzio, 2016b). This is also linked with indirect effects on surrounding habitats through resource depletion for terrestrial insectivores, including amphibians (Epanchin et al., 2010).

Nowadays fish stocking activities are still a widespread practice worldwide also in high lakes as Alpine lakes. In the majority of the cases, anglers release fish for fishing. In order to restore the natural conditions, remediation action should be adopted. The most effective way seems to be the eradication of non-native fish. In this contest, anglers can have an active role in the process of eradication (Tiberti et al., 2016c). However, recreational anglers can have an effect only on adult population. Therefore, to obtain a complete eradication, other measures (gillnets and electrofishing) should be added in order to reestablish original conditions.

As regard bacteriological analysis, we isolated environmental bacteria and also pathogenic bacteria, demonstrating how alien species may introduce pathogens even in Alpine lakes, contributing to the deterioration of these pristine ecosystems. In particular, in Dimon Lake we isolated motile Aeromonads. *Aeromonas sobria* is gram-negative bacteria widespread in aquatic environment. It is an opportunistic bacteria, and usually is not pathogenic, but in certain stress condition can cause disease (Noga, 2010). Different species of Aeromonads are linked with a variety of diseases in different fish species and motile aeromonads are often involved in fish disease (Roberts, 1993). Among the motile Aeromonads, *Aeromonas hydrophila*, *A. sobria* and *A. caviae* are most commonly bacteria associated with fish (Austin, McIntosh & Austin, 1989; Toranzo, Baya, Romalde & Hetrick, 1989; Carnahan & Altwegg, 1996; Abbott, Cheung & Janda, 2003). The aeromonads can produce a range of potential virulence factors including extracellular haemolysins, cytotoxins and proteases (Chacòn, Figuras, Castro-Escarpulli, Soler, & Guarro, 2003; Wang et al., 2003; Xia, Ma, Rahman & Wu, 2004). In fish, *A. sobria* can act as primary pathogen, depending on the different strains existing and on the general health status of the population. In fact, there are virulent and avirulent strains of this bacteria (Wahli, Burr, Pugovkin, Mueller & Frey, 2005). The virulent strain has haemolytic and cytotoxic effects (Wahli et al., 2005). Fishes affected by virulent strain present skin lesions, fin erosion and haemorrhages at fin, gills, body and eyes level (Cipriano, Bullock & Pyle, 1984). In literature, wild fish mortality events caused by *A. sobria* are reported (Manfrin et al., 2004; Wahli et al., 2005; Fichi et al., 2013). Moreover, *A. sobria* is recognized as important causes of septicaemia in immunocompromised humans (Buchanan & Palumbo, 1985; Janda & Brenden, 1987). However, in all the fish sampled, no clinical signs were reported. On this basis, we deduce that the bullheads' population of Dimon Lake present the non-virulent strain of *A. sobria* or that the stress or scarce environmental conditions that can lead to the onset of the disease were not present. Moreover, the fact that no clinical signs are reported in pathological analysis is an indication of the good status of the fish population. Although in Dimon Lake we found only environmental bacteria, in Balma Lake we isolated a primary fish pathogen: *Yersinia ruckeri*, the etiological agent of the Enteric Red Mouth Disease (ERMD) in salmonids. It is a gram-negative bacteria belonging to the Enterobacteriaceae family. This disease causes significant economic loss every year in aquaculture facilities worldwide (Tobback, Decostere, Hermans, Haesebrouck & Chiers, 2007). ERMD can affects fish of all ages but is most acute in small fish. In larger fish, the disease appears as chronic condition. Changes in fish behaviour may be observed and often including lethargy and standing near the surface. Haemorrhages on the body surface are common, with reddening at the base of the fins and along the lateral line, as well as in the head region. The characteristic haemorrhages in and around the oral cavity have led to the name “red mouth” disease, although these reddened areas are not apparent in some affected fish and thus absence of classic “red mouth” does not rule out infection with *Y. ruckeri*. Petechial haemorrhages on the surface of the liver, pyloric caeca, swim bladder and in the lateral musculature can be evident (Austin & Austin, 2007). The spleen is often enlarged. The intestine is inflamed and filled with a thick, opaque and purulent fluid. The abdomen is distended as a result of fluid accumulation. Exophthalmia occurs and is commonly accompanied by orbital haemorrhages, sometimes as haemorrhagic rings around the eyes (Horne & Barnes, 1999; Avci & Birincioğlu, 2005). *Yersinia ruckeri* is usually absent in pristine alpine lakes. The presence of these primary fish pathogen in Alpine lakes is due to alien fish introduction. In fact, this bacteria is often present in aquaculture facilities that reared salmonids. Despite this, fish sampled didn't show any clinical signs. This phenomenon can be easily explained because the disease appears as a more chronic condition in older/larger fish (Kumar, Menanteau-Ledouble, Saleh & El-Matbouli, 2015).

In Balma Lake we also isolated *Plesiomonas shigelloides*. This bacteria is oxidase-positive, gram-negative, motile that has been implicated as an agent of human gastroenteritis (Miller & Koburger, 1985) and can be found in freshwater and marine ecosystems in tropical and temperate climates (Levin, 2008). In fish, is not a primary pathogen and the occurrence of disease is often correlated with the increase of water temperature, especially in summer. Moreover, there is a pronounced increase of the prevalence in water with high organic matter (Cruz, Saraiva, Eiras, Branco & Sousa, 1986). Furthermore, *P. shigelloides* may be normally resident in the gastro-intestinal tract of fish (Austin & Austin, 2007), from where it could serve as a reservoir of infection. The low water temperature throughout the year and the absence of nutrients pollution in water of Balma Lake, explains the absence of clinical signs.

A. sobria and *P. shigelloides* are widely present in aquatic environment but during fish stoking, especially with non-native species, introduced fish could act as carriers of these bacteria which could be more virulent or with an antibiotic resistance different from native aquatic bacteria and could affect native aquatic fauna. For example, fish from aquaculture facilities could harbour bacteria which could be dangerous for other species as amphibian. In fact, Alpine lakes are suitable breeding sites of the European common frog (*Rana temporaria*) (Tiberti, 2011). *R. temporaria* can be infected by *Aeromonas hydrophila*, one of the pathogenic agents of Red-leg disease and one of the most isolated bacteria in fish (Saikot, Zaman & Khalequzzaman, 2013). Fish stoking with individuals infected by *A. hydrophila* could introduce virulent strain that could affect and lead to death endangered native amphibian with implications on their biodiversity conservation.

It is possible to affirm that the isolation of *Y. ruckeri* in remote ecosystems is quite unexpected, especially because it is a primary pathogen present in aquaculture facilities. The probable absence of stress in fish, the good environmental condition as demonstrated in hydrochemistry and nutrients concentration (data not reported in this paper) and the possible role of fish as chronic carriers of this pathogen can explain the absence of symptoms in sampled fish. In any case, the spread of pathogens as a consequence of alien species introduction is a problem of major concern in Alpine lakes, and in many other pristine habitats. In fact, *Y. ruckeri* is common in aquaculture facilities, and our study demonstrates as bacteria isolated in farmed fish may be transferred to wild by human intervention.

Control actions should be avoided, and remediation measures should be applied in contaminated habitat in order to re-establish the sanitary and ecological conditions altered by anthropogenic activity. In Balma Lake in particular, fishing activity is permitted in certain periods of the year (from June to October) and involves the presence of anglers which introduce large fish, fry or eyed-eggs from aquaculture facilities without a proper sanitary monitoring.

The introduction of pathogens in these remote ecosystems could be also through transhumance. In fact, in our sites cows and sheep which graze on around the lakes' shoreline could harbor bacteria that once entered in water by manure could affect aquatic susceptible species.

Nowadays fish stocking activities is still a widespread practice worldwide also in mountain lakes. In the majority of the cases, anglers release fish in Alpine lakes for fishing. As we know, these introductions have huge ecological and sanitary impacts on these ecosystems. In order to restore the natural conditions, remediation actions should be adopted. The most effective way seems to be the eradication of non-native fish. In this contest, anglers can have an active role in the process of eradication. Therefore, to obtain a complete eradication, the use of gillnets and electrofishing should be used to reestablish original conditions as adopted in projects funded by European Commission (LIFE+ Bioaquae and LIFE+ LimnoPirineus). Furthermore, the monitoring of the real health status of freshwater fish fauna in public waters should be a fundamental tool to environmental preservation, species conservation and to guarantee public health protection. For this reason, fish sampling campaigns are necessary to map fish pathogens distribution and to better understand how stocking activities in freshwater can led to native aquatic communities, introducing new pathogens. Finally, further studies are necessary to deepen the knowledge about the interaction between farmed and wild fish in terms of exchange of pathogens.

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Table 1. Biometric measures and bacteriological test results of *Cottus gobio* sampled in Dimon Lake (July 2017)

Specimen	Length (cm)	Weight (g)	Test	Isolated bacteria	Gram	API code	MALDI-TOF Confidence value
1	14.5	31	(-)				
2	15.5	33	(-)				
3	15.0	32	(-)				
4	15.5	39	(-)				
5	13.5	23	(-)				
6	14.5	34	(+)	*Aeromonas sobria*	(-)	API 20NE 5076755 (98.8%)	99.8
7	13.0	26	(-)				
8	13.5	28	(+)	*Aeromonas sobria*	(-)	API 20NE 5076755 (98.8%)	99.8
9	11.0	20	(+)	NSG		-	-
10	12.5	24	(-)				
11	13.5	27	(-)				
12	11.8	19	(-)				
13	12.0	20	(+)	*Aeromonas sobria*	(-)	API 20NE 3176754 (97.9%)	98.9
14	11.7	19	(-)				
15	12.0	23	(+)	*Aeromonas sobria*	(-)	API 20NE 5076755 (98.8%)	99.8

Table 2. Biometric measures and bacteriological test results of *Salvelinus fontinalis* in Balma Lake (July 2017)

Specimen	Length (cm)	Weight (g)	Test	Isolated bacteria	Gram	API code	MALDI-TOF Confidence value
1	22.0	104	(+)	*Yersinia ruckeri*	(-)	API 20E 5105113 (99.9%)	99.9
2	19.5	67	(+)	*Plesiomonas shigelloides*	(-)	API 20NE 7162744 (99.9%)	99.9
3	18.5	61	(-)				
4	22.0	115	(-)				
5	17.0	56	(-)				
6	13.0	20	(-)				
7	19.0	69	(-)				
8	13.0	38	(-)				
9	11.5	18	(-)				
10	21.0	116	(-)				
11	24.0	146	(-)				
12	21.0	119	(-)				
13	18.5	61	(-)				
14	16.5	50	(-)				
15	22.0	123	(-)				
16	20.5	101	(-)				
17	12.0	17	(-)				
18	11.5	15	(-)				
19	16.0	43	(-)				
20	17.0	59	(-)				