Research article

Epidemiological Study of *Flavobacterium psychrophilum* in Ayu *Plecoglossus altivelis* Caught in Lake Biwa and the Inflowing Rivers from 1998 to 2011

Michitaka Yamamoto^{1,2,3*}, Kazuhiro Sugahara¹, Makoto Endo¹, Katsuya Ishimaru²
and Keitaro Kato²

¹Shiga Prefectural Fisheries Experiment Station, Shiga 522-0057, Japan

²Fisheries Laboratory of Kinki University, Wakayama 649-2211, Japan

³Lake Biwa Museum, Shiga 525-0001, Japan

(Received February 2, 2015)

ABSTRACT—We studied the epidemiology of *Flavobacterium psychrophilum* in ayu from Lake Biwa for establishing control measures to bacterial cold water disease (BCWD). During 1998–2011, 12,743 wild ayu were collected from coastal (set net and gill net), offshore (offshore scoop net) and inflow river (fishing weir) areas of Lake Biwa. We employed a nested-PCR technique targeting 16S rRNA gene for the detection of *F. psychrophilum* in the gills and kidney of ayu. Prevalence of the bacterium was greatest when the water temperature of Lake Biwa was 18–21°C, which coincides with the optimum temperature for multiplication of *F. psychrophilum*. The prevalence of *F. psychrophilum* in the gills was higher in fish from fishing weirs and set nets maintained in net cages than in fish just after catch with the other fishing techniques. This suggests that rearing ayu in net cages at high densities following catch by fishing weirs and set nets can advance infection with the bacterium among the captured fish which will be used for seedlings in aquaculture or releasing in rivers. Control measures for the captured ayu must be conducted in reliable ways to prevent spread of the disease after release and in aquaculture.

Key words: *Flavobacterium psychrophilum*, ayu, Lake Biwa, bacterial cold water disease, BCWD, epidemiology, prevalence

Ayu *Plecoglossus altivelis* is one of the most important species in the freshwater fisheries of Japan. The catch of ayu has decreased nationwide since the 1990s, and the main cause is considered to be a reduction in the survival of released ayu, as well as the degradation of natural resources (Uchida, 2010). Ayu has a 1-year life cycle. Usually, after hatching in rivers during autumn, ayu larvae flow into the sea where they remain until the juvenile stage. As juveniles, they return to rivers and swim upstream, where they grow to be sexual maturity. However, an exception to this pattern occurs among Lake Biwa's landlocked stock of ayu, which spend their whole life in freshwater, migrating up into rivers around Lake Biwa. To provide juveniles to fish farms for ayu aquaculture, landlocked ayu juveniles caught in Lake Biwa during early winter to late spring are distributed throughout Japan. Some seedlings are also cultured in ponds around Lake Biwa and released into many rivers in Japan for game fishing.

Bacterial cold water disease (BCWD), which is

caused by *Flavobacterium psychrophilum*, has been reported in various fish species and is known as one of the most serious diseases in salmonid cultures. BCWD causes high mortality of reared rainbow trout fry in Europe and the United States. In Japan, BCWD seriously damages freshwater aquaculture (Wakabayashi, 2004), especially for landlocked ayu in Lake Biwa. BCWD causes high mortality in ayu and occurs in fish farms as well as in natural waters (Iida and Mizokami, 1996; Amita, 2000). It has been noted that the occurrence of BCWD in many rivers principally results from the release of *F. psychrophilum*-infected ayu (Inouye, 2000). Recently, release of captured wild seedlings of ayu from Lake Biwa has been avoided throughout Japan because *F. psychrophilum* was detected in the stocks (Hara, 2007). Captured wild seedlings from Lake Biwa comprised about 70% of released ayu in rivers across Japan before 1990 (Otake and Uchida, 1998); however, this figure decreased to 23% in 2011 (National Federation of Inland Water Fisheries Cooperatives, 2014).

Epidemiological information such as the distribution of the causative pathogen is essential for the establish-

* Corresponding author

E-mail: yamamoto-michitaka@pref.shiga.lg.jp

ment of control measures for the disease. No systematic epidemiological studies on BCWD among ayu populations have been conducted. Here, we report an epidemiological investigation using PCR to detect *F. psychrophilum* in wild ayu caught in Lake Biwa and the inflowing rivers with different fishing methods, namely set net, gill net, offshore scoop net and fishing weir.

Materials and Methods

Ayu sample collection and preparation

During 1998–2011, 12,743 wild landlocked ayu (219 batches) were captured from coastal areas (set nets and gill nets) and offshore (offshore scoop nets) in Lake Biwa, and from the lower most reaches of inflow rivers (fishing weirs). Live fish caught at set nets or fishing weirs, and fish captured at fishing weirs were transferred into holding ponds. Approximately 60 (20–100) individuals were randomly selected from the catch to comprise each batch. The selected fish were frozen at -80°C until being processed for examination using the polymerase chain reaction (PCR).

Body weight of each fish was measured after thawing. The fish were then dissected to collect the gills and kidney. Thirty milligrams of gill filaments were removed from each fish sample and vigorously shaken with 1 mL of phosphate-buffered saline (PBS) for 30 s in a 1.5-mL microtube. Then 0.95 mL of the gill washing was transferred into another tube, followed by centrifugation at $10,000 \times g$ for 10 min. The supernatant was discarded, and the pellet was resuspended in 50 μL of PBS and immediately used for DNA preparation. For the analysis of kidney, samples from 1,935 fish (40 batches) randomly selected among the fish weighing at least one gram in body weight were used. The dissected 10 mg kidney tissue from each fish were homogenized with 200 μL of STE buffer (100 mM NaCl, 50 mM Tris-HCl, 100 mM EDTA, pH 8.0) for DNA preparation.

DNA extraction

Template DNA for PCR amplification was prepared using a chelating resin (Izumi and Wakabayashi, 1997). Ten μL of gill washing or kidney homogenate was mixed with 300 μL of 5% Chelex100 (Bio-Rad Laboratories). These mixtures were incubated for 30 min at 55°C , vortexed at high speed for 10 s, incubated for 20 min at 100°C , and centrifuged for 10 min at $10,000 \times g$. A 2- μL aliquot of the supernatant containing DNAs was directly used for PCR amplification as a template.

Nested PCR amplification and gel electrophoresis

The universal primer pair of 20F and 1500R were used as external primers, with sequences of 5'-AGAGTTTGATC(AC)TGGCTCAG-3' and 5'-GGTTACCTTGTTACGACTT-3', respectively (Weisburg

Fig. 1. Sampling locations in Lake Biwa. Wild ayu were mainly caught from set nets in the lake (solid circles) and fishing weirs in rivers (open triangles). Dotted ovals show the gill-net sampling grounds. Offshore scoop nets were used throughout the gray area. Sites monitoring water temperature were indicated by open circles.

et al., 1991). The sequences of the two internal primers, PSY1 and PSY2, were 5'-GTTGGCATCAACACACT-3' and 5'-CGATCCTACTTGCCTAG-3', respectively (Toyama *et al.*, 1994). After the first amplification by the external primers had finished, each PCR product was diluted 20 times with TE buffer (Izumi and Wakabayashi, 1997). These diluted PCR products were then used as the template for the second amplification with the internal primers. First PCR and nested-PCR were performed under the same PCR temperature conditions, and PCR amplifications were performed in a total reaction volume of 10 μL . The reaction mixture contained 2 μL of template, 0.1 nmol of each dNTP, 10 pmol of each primer, and 0.5 units of TaKaRa Taq (Takara Bio Inc.). The PCR protocol was modified from the methods of Toyama *et al.* (1994). Samples were subjected to 30 cycles (first PCR) or 40 cycles (nested-PCR) of amplification in a DNA thermal cycler (Gene Amp PCR system 9700, Applied Biosystems). A preheating cycle at 94°C for 5 min was included. The amplification cycles used for denaturation, primer annealing to the template, and primer extension were as follows: 94°C for 30 s, 51°C for 90 s, 72°C for 120 s, and in the final cycle samples were incubated at 72°C for 5 min. Four microliters of the PCR product was analyzed by 1% agarose gel electrophoresis for 40 min

and stained with 0.5 mg/mL ethidium bromide for visualization of the products (1,089 bp). In this nested-PCR, we analyzed DNA sequence of the positive products amplified from the gills of 61 fish and the kidney of 19 fish collected in set nets or fishing weirs by sequencing with the CEQ DTCS Quick Start Kit (Beckman Coulter) and CEQ 8000 genetic analysis system (Beckman Coulter).

Water temperature

Water temperature data for Lake Biwa included data obtained by the Lake Biwa Environmental Research Institute (LBERI) from an automatic monitoring station off Imazu (10 m water depth) during 1998–2004, as well as estimates made by LBERI by linear approximation from water temperature measured at Hikone by the Shiga Prefecture Fisheries Experiment Station (SPFES) from 2005–2011. Water temperature data measured at a monitoring station in the Yasu River during 1998–2007 was obtained from the Ministry of Land, Infrastructure, Transport and Tourism, Japan (<http://www1.river.go.jp/>). The average monthly water temperatures during 1999–2011 are shown in Table 1.

Data analysis

The data were analyzed using the Statcel statistical software package (OMS, Inc.). The prevalence of *F. psychrophilum* was determined as the percentage of positive fish among all fish. Differences in prevalence of *F. psychrophilum* among fishing methods (219 batches), water temperatures (164 batches), fish body weights (219 batches) and fish catch (120 batches) were tested by the Tukey-Kramer multiple comparisons

test. With regard to fishing methods, samples from April to August were chosen to minimize the effect of seasonal fluctuations: we used spring-summer samples because the number of cases of the infection was higher in these seasons than in the rest of the year. Correlations between the annual commercial catch of ayu in Lake Biwa and the prevalence of *F. psychrophilum* in batches of ten or more ayu caught with set nets during 2000–2007 were analyzed by Spearman's correlation coefficient.

Results

Prevalence of *F. psychrophilum* during 1998–2011

The nested-PCR products of *F. psychrophilum*-positive samples from the gills of 61 fish and the kidney of 19 fish were sequenced, and we confirmed to include homologous sequences of *F. psychrophilum* in all samples (data not shown). The prevalence of *F. psychrophilum* in the gills and kidney of ayu caught in Lake Biwa and three inflowing rivers (Chinai River, Ado River and Ane River) during 1998–2011 is shown in Table 1. The DNA fragment of *F. psychrophilum* was detected in 18.5% of the gills (2,359 of 12,743 individuals) and 8.9% of the kidney (172 of 1,935 individuals) of the sampled fish. Most of the examined ayu were asymptotically infected with *F. psychrophilum*. Symptoms associated with BCWD such as anemia of gills and ulcers on the body were rarely observed in ayu caught in set nets or fishing weirs in June–July (total 40 fish).

Effect of fishing methods

The prevalence of *F. psychrophilum* in the gills and

Table 1. Prevalence of *Flavobacterium psychrophilum* as detected in the gills and kidney of wild ayu collected with set net, gill net, scoop net and fishing weir in Lake Biwa and the inflowing rivers in 1998–2011

Month	Percentage of prevalence of <i>F. psychrophilum</i> (number of positive fish/ number of examined)		WT (°C)*	
	Gills	Kidney	Lake Biwa	Yasu River
Nov.	3.0 (24/800)	7.4 (6/81)	13.8	
Dec.	2.5 (30/1,202)		12.1	
Jan.	0.8 (1/120)		7.8	
Feb.	1.2 (13/1,105)	0.0 (0/123)	7.6	12.0
Mar.	6.8 (56/821)	2.7 (5/186)	8.0	11.0
Apr.	23.3 (373/1,600)	27.4 (96/350)	10.3	14.7
May	17.9 (370/2,070)	0.7 (1/150)	14.5	18.2
June	32.4 (882/2,725)	5.1 (26/505)	18.5	22.4
July	30.1 (548/1,820)	7.0 (38/540)	22.5	22.7
Aug.	12.9 (62/480)		23.8	23.9
Total	18.5 (2,359/12,743)	8.9 (172/1,935)		

On each sampling, approximately 60 (20–100) individuals in one batch were randomly selected from the captured population.

* Mean water temperature were calculated using data at depth of 10 m in the north basin of Lake Biwa (Imazu and Hikone) and at a monitoring station in Yasu River as shown in Fig. 1.

kidney of ayu caught by using four fishing methods in Lake Biwa and three inflowing rivers are shown in Fig. 2 and Table 2. Prevalence in the gills varied considerably, and the greatest fluctuation in prevalence was found in fish from fishing weirs, up to 85% fluctuated within a week in the period from June to August in same fishing weir, resulting in large standard error as shown in Fig. 2(a). In fish from set nets, the prevalence of the bacterium was less than 3.0% from November to February and increased gradually from March, reaching a peak (35.2%) in July and then decreasing in August. In fish from fishing weirs, the prevalence increased rapidly in April, decreased in May, increased again in June, and decreased in August. In fish taken with offshore scoop nets and gill nets, the prevalence was less than 10% throughout the fishing season. Among the four fishing methods, the prevalence of *F. psychrophilum* during April–August was the highest in fish from fishing weirs (40.6%), followed by set nets (21.0%), gill nets (6.3%) and offshore scoop nets (4.6%). A high prevalence in kidneys was observed only in April for fish caught at fishing weirs and only in July for fish taken by set nets.

Fig. 2. Monthly changes during 1998–2011 in prevalence of *Flavobacterium psychrophilum* in Lake Biwa ayu in the gill tissue (a) and kidney tissue (b). Marks and bars indicate means and standard error, respectively.

Table 2. Prevalence of *Flavobacterium psychrophilum* in the gills of ayu caught between April and August in 1998–2011 using different fishing methods

Fishing gear	No. of batches studied	Prevalence (%)	
		Mean (Range)	SD
Set net	67	21.0 ^b (0–91.7)	0.272
Fishing weir	59	40.6 ^a (0–100)	0.360
Offshore scoop net	12	4.6 ^b (0–26.7)	0.082
Gill net	14	6.3 ^b (0–26.7)	0.082
Total	152	26.0 (0–100.0)	0.316

Different superscripted letters (a or b) show a significant difference by the Tukey multiple comparison test ($P < 0.01$).

Effects of water temperature, fish catch, and body size

The relationship between water temperature and *F. psychrophilum* prevalence in ayu is shown in Fig. 3. For set nets, the highest prevalence (34.5%) occurred when the water temperature was 18–21°C, and this prevalence was significantly higher than at temperatures of less than 15°C ($P < 0.05$). In fishing weirs, the highest prevalence (55.6%) was observed at 18–21°C. From February to June, water temperatures in the inflowing Yasu River were 3.0–4.4°C higher than those in Lake Biwa (Table 1). The relationship between amount of fish catch (by set nets and fishing weirs) and prevalence is shown in Fig. 4. Although the prevalence tended to increase with increasing fish catch, this trend was not significant ($P > 0.05$). In set nets, the prevalence in fish of greater than 2 g in body weight

Fig. 3. Relationship between water temperature and prevalence of *Flavobacterium psychrophilum* in ayu caught by set nets during 1998–2011(a) and fishing weirs during 1998–2007 (b). Bars indicate standard error. An asterisk indicates a significant difference by the Tukey multiple comparison test ($*P < 0.05$). n; the number of batches.

Fig. 4. Relationship between fish catch and prevalence of *Flavobacterium psychrophilum* in ayu caught by set nets (a) and fishing weirs (b) during 1998–2011. Bars indicate standard error. n; the number of batches.

Fig. 5. Relationship between fish body weight and prevalence of *Flavobacterium psychrophilum* in ayu caught by set nets during 1998–2011. Bars indicate standard error. Values of bars with different letters (a or b) are significantly different by the Tukey multiple comparison test ($P < 0.05$).

was significantly higher than those in fish of less than 2 g (Fig. 5, $P < 0.05$). The other three fishing methods produced no ayu of less than 2 g, and no significant correlation between prevalence and body weight was detected.

Yearly fluctuation in prevalence of *F. psychrophilum*

Year-to-year changes in the monthly prevalence of *F. psychrophilum* in gills are shown in Fig. 6. Except for 2005 and 2006, when markedly lower catches of

Fig. 6. Monthly prevalence of *Flavobacterium psychrophilum* in the gills of ayu caught by set nets and fishing weirs each year during 2000–2007. n; the number of batches.

Fig. 7. Correlation between annual commercial catch of ayu in Lake Biwa and prevalence of *Flavobacterium psychrophilum* in the gills of ayu caught by set nets each year during 2000–2007. A significant positive correlation was confirmed by Spearman's correlation coefficient.

ayu were recorded, the prevalence was higher in April, June, and July than in the other months. Prevalence was also extremely low in 2005 and 2006, and a significant positive correlation was observed between annual fish catch and prevalence in the gills of fish caught in set nets. To illustrate this, the relationship between annual volume of the commercial catches of ayu in Lake Biwa and *F. psychrophilum* prevalence in the gills

of fish caught in set nets, which is the most important component of the ayu fishery in the lake, is shown in Fig. 7.

Discussion

At the beginning of this study, a nested-PCR targeting 16S rRNA gene described Toyama *et al.* (1994) appeared to be a suitable method for detection of *F. psychrophilum*. As some researchers pointed out in later years (Urdaci *et al.*, 1998; Izumi *et al.*, 2005; Suzuki *et al.*, 2008), we had considered it was difficult to apply this nested-PCR to environmental samples such as gill washings, because we often detected ambiguous faint band(s) after the nested-PCR amplification leading to false-positive as pointed out by Izumi *et al.* (2005) and Suzuki *et al.* (2008). Therefore, we improved the number of thermal cycles, and then inspected by DNA sequencing whether the PCR products detected in ayu were truly amplified from *F. psychrophilum*. The sequences of all 80 positive samples (gills of 61 fish and kidneys of 19 fish) accorded with that of 16S rRNA of *F. psychrophilum*. The improvement of PCR protocol could increase specificity for the detection of the bacterium and consequently minimize the risk of false-positive result in this study.

The prevalence of *F. psychrophilum* in the gills of fish caught at fishing weirs and in set nets was higher than that in fish caught using the other two methods. In contrast to dead fish caught with gill nets or offshore scoop nets, live fish caught at fishing weirs or set nets were kept at high density in net cages for several days before being transferred or released as seedlings. In addition, fish captured at fishing weirs were transferred from net cages into holding ponds before their release into rivers or culture in ponds.

The prevalence of *F. psychrophilum* may have been higher at fishing weirs than in set nets, because the water temperature in rivers where fishing weirs were installed was more suitable for the multiplication of *F. psychrophilum*. High fish density may promote growth of the bacterium and consequently horizontal infection among the fish reared. Owing to the higher than natural fish density conditions, spread of infection might occur more readily within net cages and preservation ponds. In this study, the water temperature of the Yasu River was higher approximately 4°C higher than that of Lake Biwa in spring, as in an earlier study (Endo *et al.*, 2007), and nearer to the optimum temperature for multiplication of approximately 20 °C for *F. psychrophilum* (Holt *et al.*, 1989; Uddin and Wakabayashi, 1997). In the present study, bacterial prevalence was low when water temperature deviated from 20°C. The prevalence in set nets and at fishing weirs was greatest when water temperature was 18–21°C, and this value in set nets was significantly higher than that at temperatures

of less than 15°C. In Lake Biwa and the inflowing rivers, the water temperature in ayu habitat is approximately 20°C in June–July (Endo *et al.*, 2007). Thus, the increased bacterial prevalence during early summer could be due to the water temperature in ayu habitat coinciding with the optimum temperature for multiplication of *F. psychrophilum*.

There have been many reports of BCWD outbreaks occurring in rivers across Japan just after the start of the “tomozuri” angling season, which uses ayu as a decoy. BCWD outbreaks during this period are thought to be caused by *F. psychrophilum* being introduced into these rivers from released ayu (Inouye, 2000; Hara, 2007), decoy ayu (Izumi *et al.*, 2007; Arai *et al.*, 2007) and contaminated anglers (Hara, 2007). The river water temperature at this time is 15–20°C (Ministry of Agriculture, Forestry and Fisheries, 2008), which includes the optimum temperature for multiplication of *F. psychrophilum*. This evidence can support the critical importance of water temperature on increasing of prevalence of *F. psychrophilum*.

We consider the abrupt changes in prevalence in Fig. 2 to depend on the water temperature and the density of ayu. The prevalence of *F. psychrophilum* in the gills of fish caught in set nets gradually increased until July and decreased in August. The reason may be that the water temperature in Lake Biwa is at the optimum temperature for multiplication of *F. psychrophilum* in June and July but too high (24 °C) in August. The relatively early increase in prevalence in gills in fish from fishing weirs in June may be a consequence of water temperature, but also of the higher fish density in comparison with set nets. Curiously, the prevalence of *F. psychrophilum* in both the gills and kidney of fish caught from fishing weirs increased in April when water temperature was low. These increase had nothing to do neither with fish density in the rivers as indicated by fish catch nor with fish body size (data not shown). There is something special, perhaps physiological or behavioral, about ayu caught at fishing weirs in April, the characterization of which will require further investigation.

For set nets, the prevalence of *F. psychrophilum* in ayu of more than 2 g in body weight was significantly higher than in ayu of less than 2 g. Ayu caught by set nets grew to 2 g in March or April, and there was no dramatic increase in prevalence before this period (Student's *t*-test, *P* = 0.31). Prevalence might have risen in association with the increasing density of ayu in net cages at the period because prevalence changed with daily fish catch. Further studies are needed to clarify the reason for low prevalence of *F. psychrophilum* in ayu of less than 2 g.

In set nets we found a significant positive correlation between *F. psychrophilum* prevalence and annual ayu catch. Higher fish catches result from higher densi-

ties of ayu in set nets and fishing weirs, and this will likely promote increased rates of infection during the period when fish are kept in net cages. In our study, we found that the prevalence of the bacterium tended to be high when many fish were caught on the same day in set nets.

In culture ponds, low survival rates of ayu caught in Lake Biwa in April–July are common. This is due to difficulty in treating BCWD outbreaks that occur just after fish are transferred to ponds. Because the prevalence in gills during this period was 17.9–32.4% (Table 1), BCWD outbreaks and low survival rates of ayu in culture ponds could be the result of the increased bacterial prevalence. Moreover, the high densities experienced by ayu kept in net cages caused both fish chafing and high stress. Another important factor is water temperature as mentioned above, since culture ponds is usually 15–20°C at the period. To sum up, the results of this study suggest that an important source of BCWD infection in fish farms can be ayu seedlings captured under high-density conditions in Lake Biwa. Suppliers of ayu seedlings must therefore, consider applying countermeasures to prevent the spread of *F. psychrophilum* when seedlings are transferred into their rearing facilities. Because the highest prevalence of *F. psychrophilum* in ayu we observed occurred under artificial conditions during captivity in net cages and ponds for temporally fish stocking, it is necessary to develop effective concrete measures to prevent infection in the aquaculture industry. In addition, releasing untreated landlocked ayu seedlings into rivers should be avoided. Although practical treatments for BCWD have been developed, such as chemotherapy (Ninomiya and Yamamoto, 2001) and temperature control (Endo *et al.*, 2001; Sakai *et al.*, 2001; Yamamoto *et al.*, 2001), more effective and safe control methods to reduce the risk of BCWD outbreaks in rivers after the releasing are still needed.

The reduction of BCWD outbreaks in rivers in ayu from Lake Biwa released for game fishing was notable in recent years (Ministry of Agriculture, Forestry and Fisheries, 2008). Most of the effective explanation on it is of using seedlings of amphidromonas ayu for releasing in rivers, which are relatively resistant to *F. psychrophilum* infection. But as indicated in Fig. 7, there is significant positive correlation between prevalence of the bacterium in the gills of ayu caught in set nets and the annual commercial catch of ayu. These may indicate that the reduction of BCWD outbreaks in rivers can be due to synergistic effect of the reduction of annual commercial catch in lake Biwa and the inflowing rivers and the reduction of prevalence of the bacterium in ayu after treatment (Ninomiya and Yamamoto, 2001; Endo *et al.*, 2001; Sakai *et al.*, 2001; Yamamoto *et al.*, 2001) or immunization (Kintsuji *et al.*, 2007) before releasing.

Acknowledgments

We sincerely thank Dr. Hisatsugu Wakabayashi, professor emeritus of The University of Tokyo and Dr. Shotaro Izumi, from Tokai University for helpful advice. We thank Dr. Mark J. Grygier of the Lake Biwa Museum for his critical reading of the manuscript. We are grateful to Akemi Kandori for her technical assistance. The fish catch data of ayu were provided by Shiga-Prefecture Federation of Fishermen's Co-operative Associations.

References

- Amita, K., M. Hoshino, T. Honma and H. Wakabayashi (2000): An investigation on the distribution of *Flavobacterium psychrophilum* in the Umikawa River. *Fish Pathol.*, **35**, 193–197. (In Japanese with English abstract)
- Arai, H., Y. Morita, S. Izumi, T. Katagiri and H. Kimura (2007): Molecular typing by pulsed-field gel electrophoresis of *Flavobacterium psychrophilum* isolates derived from Japanese fish. *J. Fish Dis.*, **30**, 345–355.
- Endo, M., K. Kouhashi, S. Takahashi and H. Iwasaki (2001): Prevention effect of water heating treatment against the bacterial coldwater disease in ayu *Plecoglossus altivelis* I. *Bull. Shiga Pref. Fish. Exp. Sta.*, **48**, 1–4. (In Japanese with English abstract)
- Endo, S., Y. Okumura, M. Kawashima, N. Fukuyama, Y. Onishi, N. Nakamura, R. Bamba and S. Tanaka (2007): Dispersion of Yasu River water in Lake Biwa. *Jpn. J. Limnol.*, **68**, 15–27. (In Japanese with English abstract)
- Hara, H. (2007): Current status of and countermeasures against cold-water diseases of ayu (*Plecoglossus altivelis*). *J. Antibact. Antifung. Agents*, **35**, 56–63. (In Japanese)
- Holt, R. A., A. Amandi, J. S. Rohovec and J. L. Fryer (1989): Relation of water temperature to bacterial cold-water disease in coho salmon, chinook salmon, and rainbow trout. *J. Aquat. Anim. Health*, **1**, 94–101.
- Iida, Y. and A. Mizokami (1996): Outbreaks of coldwater disease in wild ayu and pale chub. *Fish Pathol.*, **31**, 157–164.
- Inouye, K. (2000): Coldwater disease of ayu (*Plecoglossus altivelis*). *Aquabiology*, **126**, 35–38. (In Japanese with English abstract)
- Izumi, S. and H. Wakabayashi (1997): Use of PCR to detect *Cytophaga psychrophila* from apparently healthy juvenile ayu and coho salmon eggs. *Fish Pathol.*, **32**, 169–173.
- Izumi, S., H. Fujii and F. Aranishi (2005): Detection and identification of *Flavobacterium psychrophilum* from gill washings and benthic diatoms by PCR-based sequencing analysis. *J. Fish Dis.*, **28**, 559–564.
- Izumi, S., S. Ouchi, T. Kuge, H. Arai, T. Mito, H. Fujii, F. Aranishi and A. Shimizu (2007): PCR-RFLP genotypes associated with quinolone resistance in isolates of *Flavobacterium psychrophilum*. *J. Fish Dis.*, **30**, 141–147.
- Kintsuji, H., M. Yamamoto and K. Ninomiya (2007): The resistance of ayu *Plecoglossus altivelis* recovered from bacterial coldwater disease against reinfection with *Flavobacterium psychrophilum*. *Fish Pathol.*, **42**, 159–161.
- Ministry of Agriculture, Forestry and Fisheries (2008): *Ayu reisuibyou taisakukyougikai torimatome*, 3pp (In Japanese), http://www.maff.go.jp/j/syouan/suisan/suisan_yobo/ayu_reisui/pdf/matome_2008.pdf

- Ninomiya, K. and M. Yamamoto (2001): Therapeutic attempt to control of the coldwater disease in ayu, *Plecoglossus altivelis* by using Sulfisozole-Na. *Bull. Shiga Pref. Fish. Exp. Sta.*, **48**, 17–20. (In Japanese with English abstract)
- National Federation of Inland Water Fisheries Cooperatives (2014): *Kikanshi zennai*, **31**, 27pp (In Japanese), <http://www.naisuimen.or.jp/jigyou/zennai.html>
- Otake, T. and K. Uchida (1998): Application of otolith micro-chemistry for distinguishing between amphidromous and non-amphidromous stocked ayu, *Plecoglossus altivelis*. *Fisheries Sci.*, **64**, 517–521.
- Sakai, A., K. Ninomiya, S. Ota and Endo, M. (2001): Efficacy of elevated water temperature treatment against the bacterial coldwater disease in ayu. *Bull. Shiga Pref. Fish. Exp. Sta.*, **48**, 5–10. (In Japanese with English abstract)
- Suzuki, K., H. Arai, T. Kuge, T. Katagiri and S. Izumi (2008): Reliability of PCR methods for the detection of *Flavobacterium psychrophilum*. *Fish Pathol.*, **43**, 124–127.
- Toyama, T., K. Kita-Tsukamoto and H. Wakabayashi (1994): Identification of *Cytophaga psychrophila* by PCR targeted 16S ribosomal RNA. *Fish Pathol.*, **29**, 271–275.
- Uchida, K. (2010): Science of ayu fishing - Joint symposium by researches and anglers. Fisheries management for stock enhancement in ayu. *Nippon Suisan Gakkaishi*, **76**, 416. (In Japanese)
- Uddin, M. N. and H. Wakabayashi (1997): Effects of temperature on growth and protease production of *Cytophaga psychrophila*. *Fish Pathol.*, **32**, 225–226.
- Urdaci, M. C., C. Chakroun, D. Faure and J. F. Bernardet (1998): Development of a polymerase chain reaction assay for identification and detection of the fish pathogen *Flavobacterium psychrophilum*. *Res. Microbiol.*, **149**, 519–530.
- Wakabayashi, H. (2004): Bacterial cold-water disease. In “Infectious and parasitic diseases of fish and shellfish” (ed. by H. Wakabayashi and K. Muroga). Kouseisha-Kouseikaku, Tokyo, pp. 177–183. (In Japanese)
- Weisburg, W. G., S. M. Barns, D. A. Pelletier and D. J. Lane (1991): 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.*, **173**, 697–703.
- Yamamoto, M., K. Ninomiya and S. Takahashi (2001): Efficacy of the combined elevated water temperature treatment and chemotherapy for the bacterial coldwater disease in ayu. *Bull. Shiga Pref. Fish. Exp. Sta.*, **48**, 11–16. (In Japanese with English abstract)