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EVALUATION OF ANTHROPOGENIC POLLUTION
IN RIVER WATER BASED ON THE GENETIC DIVERSITY
OF *AEROMONAS HYDROPHILA*

KAROL KORZEKWA*, IWONA GOŁAŚ, MONIKA HARNISZ

Department of Environmental Microbiology, Faculty of Environmental Sciences
M. Oczapowskiego 5; 10-719 Olsztyn, Poland
University of Warmia and Mazury in Olsztyn
*Corresponding author's e-mail: p2p2@gmx.us

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Abstract: *Aeromonas hydrophila* is a valuable indicator of the quality of water polluted by sewage and pathogens that pose a risk for humans and cold-blooded animals, including fish. The main aim of this research was to evaluate anthropogenic pollution of river water based on genetic diversity of 82 *A. hydrophila* strains by means of RAPD, semi-random AP-PCR (ISJ) and the rep-BOX conservative repeats test. Genetic diversity of *A. hydrophila* was $HT = 0.28$ ($SD = 0.02$) for all DNA markers (RAPD, semi random and rep-BOX). None of the analyzed electrophoretic patterns was identical, implying that there were many sources of strain transmission. The presence of genes for aerolysin (*aerA*), hemolysin (*ahh1*) and the cytotoxic enzyme complex (AHCYTOGEN) was verified for all tested strains, and drug resistance patterns for tetracycline, enrofloxacin and erythromycin were determined. The most diverse *A. hydrophila* strains isolated from river water were susceptible to enrofloxacin ($HS = 0.27$), whereas less diverse strains were susceptible to erythromycin ($HS = 0.24$). The presence of the multidrug resistance marker (ISJ4-25; 1100 bp locus) in the examined strains (resistant to three analyzed drugs) indicates that intensive fish cultivation affects the microbiological quality of river water.

INTRODUCTION

Aeromonas hydrophila Gram-negative heterotrophic and nonsporulating bacterium is an important etiological factor of disease in fish and humans [2, 11], and together with *Escherichia coli*, it is an indicator of water quality [29]. *A. hydrophila* as well as *Pseudomonas* spp. and *Vibrio* spp. are the predominant microorganisms in surface waters such as rivers, lakes and ponds [16]. The ecology, biochemistry and the genome of *A. hydrophila* has been very well researched [7, 24]. In their efforts to analyze the genetic diversity of *A. hydrophila*, most researchers have focused on taxonomic aspects of identification [10], mutations in drug resistance genes [12], selected sequences responsible for virulence [5], transposable genetic elements [6] and repeated sequences [19, 25, 30]. There is a general scarcity of information

about the diversity of the *A. hydrophila* genome, with the exception of selected, non-representative and well-researched regions [26]. Although the structure of the discussed genome has been well documented, little is known about the genetic variability of the entire *A. hydrophila* genome. The use of randomly amplified and semi-random DNA markers could support a comparison of genetic diversity between *A. hydrophila* and *E. coli*. A higher level of genetic diversity points to various sources of origin (pollution) and high genetic potential for multidirectional phenotypic differentiation, in particular varied resistance to drugs (single drug, multi-drug and extended resistance) [15]. The aim of this paper was to evaluate the anthropogenic pollution of river water based on genetic diversity and similarity of drug-resistant and virulent strains of *A. hydrophila*.

MATERIALS AND METHODS

Genetic variation in arbitrary regions of the *A. hydrophila* genome was analyzed based on 82 strains that had been isolated during a previous study of the upper section of the Drwęca River. The process of phenotypic and molecular identification of *A. hydrophila* has been described in detail by Gołaś *et al.* [2009]. The selected strains were Gram-negative, motile, oxidase and catalase positive and β -hemolytic positive with confirmed presence of *aer* and *ahh1* genes. Their resistance to tetracycline (TE), enrofloxacin (ENR) and erythromycin (E) was tested on Oxoid discs. The tested strains were described as resistant (R), moderately resistant (MR), susceptible (S) as well as multidrug resistant to TE+ENR+E (MDR). Only moderately resistant and susceptible strains were tested to determine the enrofloxacin resistance pattern.

Nucleic acids of the studied *A. hydrophila* strains were extracted by the modified [17] CTAB method [4]. The quality and quantity of DNA were analyzed photometrically. Long RAPD and short semi-random primers were applied with the following respective sequences: OPB06-5'TGCTCTGCCC3', OPA11-5'CAATCGCCGT3', ISJ2-5'ACTTACCTGAGGCGC-CAC3', ISJ4-5'GTCGGCGGACAG-GTAAGT3'. Primer A1R-5'CTACGGCAAGGCGACGCTG-ACG3' was used to amplify the rep-boxA sequence for 15 randomly selected strains. The thermal profile and the chemical conditions for PCR were consistent with previous recommendations [8, 27, 28]. Each amplification was performed in three replications to compare results and test the reproducibility of electrophoretic patterns. Visible bands on gel were scored as loci (allele 1). The A1R primer indicated sites compatible with the region of the boxA repeat sequence. The absence of a compatibility site in the same locus was marked as allele 0 or a place of incompatibility (PI) in the boxA sequence. *A. hydrophila* is a haploid, therefore, it was assumed that the obtained molecular phenotypes corresponded to genotypes. Alleles "1" or "0" were observed in every locus. Allele frequencies were calculated using POPGENE 1.32 software [31]. Genetic diversity coefficients (H , h) were determined by Nei's method based on allele frequencies in a locus [22]. The genetic similarity coefficient (I) was calculated based on shareable bands between strains. *A. hydrophila* populations were grouped by minimum evolution (ME), neighbor joining (NJ) and unweighted pair grouping with arithmetic mean (UPGMA) [22]. Dendrograms were developed using POPGENE 1.32 and MEGA v 3.1 software [31]. Clustering results were validated by principal component analysis.

RESULTS

All primers revealed 82 different molecular phenotypes ($G = 1$ or $G = 100\%$). A total of 168 loci were scanned, and all of them were found to be polymorphic ($P = 100\%$). Short primers (10 nt) revealed 1 to 24 amplification products (amplicons), including 1–13 for OPB and 1–15 for OPA. In 42 strains, “allele 1” was observed predominantly in locus OPB-33 as a band with molecular weight of around 2.5 kbp. Long primers (18 nt) generated 1 to 31 amplicons. Allele 1 was most frequently determined in locus ISJ2-30 (46 strains) and ISJ4-23 (36 strains) as a band with molecular weight of 1.6 kbp and 1 kbp, respectively (Fig. 1).

An analysis of 0–1 patterns produced after amplification with RAPD and AP-PCR primers revealed that the strains were not grouped as resistant (R), moderately resistant (MR) or susceptible (S) in line with TE, ENR and E patterns. Minimum evolution (ME) changes in 82 strains grouped based on their resistance to tetracycline (TE) revealed two main clusters of 11 and 71 strains each (Fig. 2). Strain grouping by the neighbor joining (NJ) method revealed 9 clusters containing strains with different TE resistance patterns. UPGMA revealed 5 clusters, one of which contained 20 out of the 30 strains susceptible to TE.

The results were analyzed by the ME method in view of the enrofloxacin resistance pattern to reveal two clusters and a similar number of strains in each cluster (12 and 70). In cluster I, all strains were resistant with one exception, and in cluster II, nine different groups of strains were identified. The neighbor joining (NJ) method was used to divide the strains into 11 clusters of strains with various ENR resistance patterns. Cluster ENR contained 11 out of the 12 strains characterized by moderate resistance to enrofloxacin (Fig. 2). The UPGMA algorithm was used to divide strains into 5 main clusters, including one with 29 strains that were mostly resistant to ENR.

The clustering of 82 *A. hydrophila* strains by the ME method based on the erythromycin resistance pattern revealed two main clusters of 11 and 71 strains, respectively. The first cluster contained erythromycin resistant strains, whereas the second cluster was divided into 10 groups of mostly erythromycin-resistant strains (66 out of 71). Grouping by the NJ method produced 9 clusters without a clear E resistance pattern, with the exception of cluster E^R where 9 out of 11 strains were resistant to E. The UPGMA

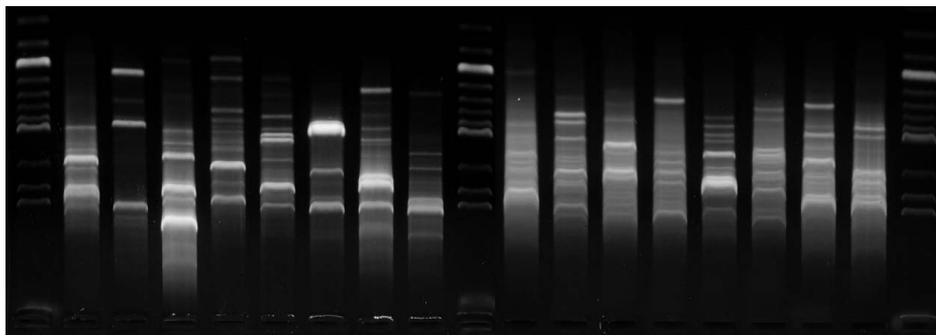


Fig. 1. Electrophoretic patterns of *A. hydrophila* strains obtained with the use of OPB (A) and ISJ4 (B) primers; M – perfect 100bp DNA ladder, AKOR Laboratories

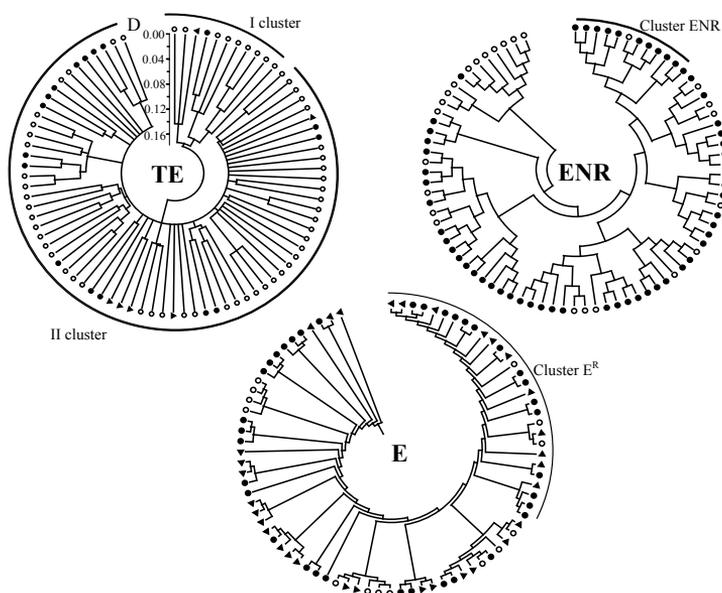


Fig. 2. Grouping of 82 strains after an analysis of arbitrarily primed regions of *A. hydrophila* genome in view of tetracycline (TE), enrofloxacin (ENR) and erythromycin (E) resistance patterns; D – genetic distance, ○ – susceptible strains, ● – moderately resistant strains, ▲ – resistant strains; clustering TE, ENR and E strains by ME, NJ and UPGMA methods, respectively

method was applied to group 82 strains into 5 main clusters, one of which contained 26 out of 29 erythromycin-resistant strains (Fig. 2).

The clustering of 28 multidrug resistant *A. hydrophila* strains revealed 6 groups of strains that were mostly resistant to three drugs. Clusters RI, RII and SI, SII contained only strains that were resistant and susceptible to three drugs, respectively (Fig. 3).

The frequency of allele 1 in locus (A) was determined in a wide range of 0.01 to 0.50, with an average of 0.19 (Table 1). The mean and effective number of alleles in locus (n_a and n_e) was 2 and 1.44, respectively. Mean genetic diversity over a representative locus within species (H_T) was 0.27 with Shannon's index of 0.43. Genetic diversity over locus (h) ranged from 0.02 to 0.50. Nei's coefficient of genetic identity (I_N) for strains was determined in the range of 0.56–0.93, and mean genetic distance (D_N) reached 0.33. The mean genetic distance between populations with similar TE patterns was 0.05 at $I_N = 0.95$ (Fig. 4). Genetic diversity and population structure of TE-susceptible strains were similar to the mean values representative of the *Aeromonas hydrophila* species. Genetic variation between three populations grouped in view of their TE patterns, measured by parameter G_{ST} , reached 0.08. The theoretical gene flow between those populations was estimated at $N_m(G_{ST}) = 5.6$. In strains with varied patterns of resistance to ENR, the mean genetic distance was 0.02 and the genetic identity coefficient was determined at $I_N = 0.98$. Similarly to TE-susceptible strains, genetic diversity and population structure of ENR-sensitive strains approximated the average values for the entire *Aeromonas hydrophila* species.

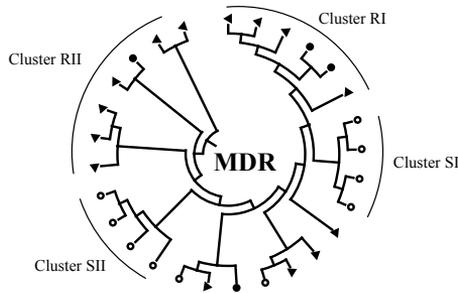


Fig. 3. Grouping of 28 multidrug resistant (MDR) strains based on an analysis of arbitrarily primed regions of *A. hydrophila*. ○ – susceptible strains, ● – moderately resistant strains, ▲ – resistant strains, SI, SII – susceptible clusters, RI, RII – resistant clusters; clustering by the UPGMA method

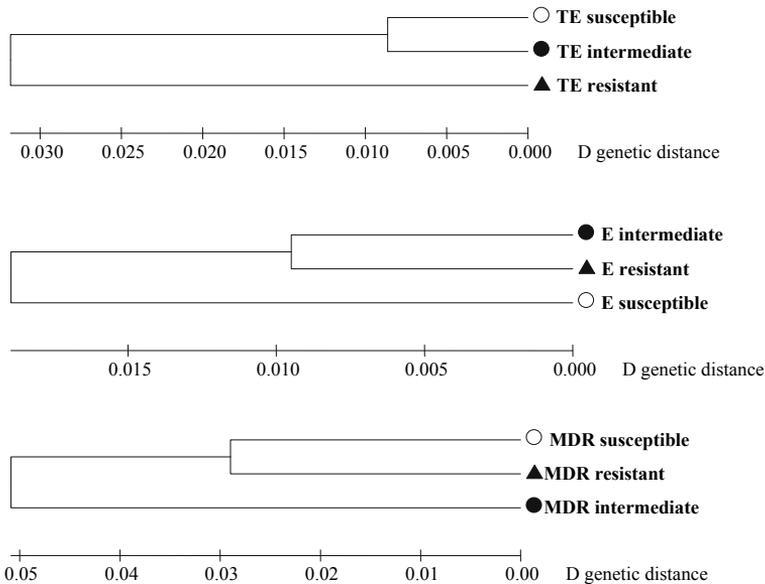


Fig. 4. Genetic distance between *A. hydrophila* strains with various TE, E and MDR resistance patterns; ○ – susceptible strains, ● – moderately resistant strains, ▲ – resistant strains

Genetic diversity between two populations with varied ENR patterns (G_{ST}) was 0.02, and theoretical gene flow was observed at $N_m(G_{ST}) = 21.7$. The mean genetic distance between strains with different erythromycin resistance patterns was 0.03, and genetic identity was determined in the range of 0.96 to 0.98 with an average of 0.97 (Fig. 4). Similarly to TE and ENR patterns, the values indicative of genetic diversity and population structure of the erythromycin-susceptible strains approximated the average values representative of the *Aeromonas hydrophila* species. Genetic diversity between three populations with varied

E patterns (G_{ST}) was 0.02, and theoretical gene flow was determined at $N_m(G_{ST}) = 8.4$. For multidrug-resistant *A. hydrophila* strains (MDR), the mean genetic distance D was 0.09, and the genetic identity coefficient I_N reached 0.92 (Fig 4). G_{ST} and gene flow parameter were determined at 0.16 and 2.6, respectively.

The amplification of regions characterized by structural similarities with boxA conserved repeat sequences revealed 15 different phenotypes ($G = 100\%$) and 39 compatible amplification sites (CAS) with $P = 100\%$. In a given CAS, the amplification product was observed with the frequency of 0.21. Genetic diversity for the entire species (H_T) was determined in the range of 0.1 and 0.5 with an average of 0.28. In reference to boxA repeats, genetic identity between 15 strains was 0.77 within the 0.43 and 0.95 range of values. A preliminary clustering analysis revealed two main clusters and a branched pseudocluster (data not shown) of seven strains.

The selection of loci with various drug resistance patterns revealed 7 marker bands of resistance and susceptibility to TE, ENR and E and 6 bands specific for MDR strains (Table 1).

DISCUSSION

In the literature [1, 3], the values of genetic diversity among enzymatic loci (HS) of *Aeromonas* spp. vary extensively between 0.09 and 0.88. In our study, *A. hydrophila* strains sampled from the Drwęca River were characterized by moderate genetic diversity of $H_T = 0.27$. Both types of primers were easy to use, and they produced repeatable, high resolution results in genetic analyses of the studied populations. The grouping of 28 MDR

Table 1. Nei's genetic identity coefficients for *A. hydrophila* strains with various TE, ENR and E resistance patterns

Drug	Pattern	A_{\max} (frequency of allele 1)	Resistance marker	Susceptibility marker	Mass [bp]
TE	S	0.56	OPA11-15; ISJ2-29; ISJ4-25; ISJ4-30	none	800; 1500; 1100; 1450
	I	0.59			
	R	0.83			
ENR	S	0.63	none	IS4-21	900
	I	0.56			
E	S	0.79	OPA11-29	ISJ2-33	1600 / 1900
	I	0.64			
	R	0.59			
MDR	S	0.80	OPB6-20	OPB6-25; OPA11-14; ISJ2-33; ISJ4-23	1500; 750; 1900; 1000
	I	1.00			
	R	0.79			

S – susceptible; I – intermediate; R – resistant;

strains revealed clusters of strains resistant to three drugs. A preliminary grouping analysis, performed separately for TE, ENR and E patterns, did not classify the strains according to their drug resistance profiles. In view of the above, all loci were reviewed to identify the resistance marker among RAPD and ISJ amplification products. This is an interesting consideration because there are few simple methods for screening bacterial resistance that can effectively determine a given strain's resistance to a specific drug and the degree of that resistance. In addition, the presented genetic diversity parameters should be correlated with resistance markers (relevant band on gel), and the resulting information should be combined with the degree of anthropogenic pollution in intensive fish farms. The values illustrating *A. hydrophila*'s qualitative and quantitative impacts on the environment can be used as bioindicators of anthropogenic pollution in fish farms.

In 2005, DNA markers were used for the first time to determine the genetic variation of *A. hydrophila*. Most papers do not present or vaguely discuss the parameters describing the genetic diversity of *A. hydrophila*, including the number of loci and amplification sites, types of polymorphism within loci, allele frequency, h parameters, genetic identity and distance. Enzymatic markers and DNA markers obtained in the bacterial population analyses are difficult to compare because very little is known about the dissonance between those molecular markers in bacterial domains. The value of HS reported by Feizabadi *et al.* [9] in an enzymatic analysis of the *M. tuberculosis* complex was lower than that noted by other researchers who relied on DNA markers [17, 26]. Many enzymatic analyses of bacteria, plants and animals produced genetic diversity values that were two- to three-fold lower in comparison with DNA marker tests [13, 21]. Based on the level of genetic diversity reported in this paper, *A. hydrophila* can be classified near the tubercle bacillus (*M. tuberculosis*) [17, 26]. In *A. hydrophila*, the value of H was less than half that reported in *E. coli* (0.85) and other clonal species. Some authors [17] argue that the level of genetic diversity produced by DNA markers should be regarded as acceptable when it is up to 3–4-fold higher than genetic variation values obtained in enzymatic analyses.

The genetic diversity analysis of *A. hydrophila* based on the boxA sequence revealed that this marker is an effective molecular tool in population studies. A box element is dispersed in several copies throughout the *Escherichia coli* genome as a complex of three sectors (boxA, boxB, boxC) [18]. Eight scored bands per strain may be equal to the number of boxA sequences with the length of 59 nt. From among 39 sites compatible with boxA, BOX-6 (450 bp) can be regarded as a selection marker capable of identifying MDR strains of *A. hydrophila* which are potentially harmful for the environment.

The majority of TE-susceptible *A. hydrophila* strains were closely related to moderately resistant strains (Fig. 3). At the same time, the degree of genetic correlation between TE-susceptible strains and highly resistant strains was higher than that between strains characterized by high resistance and moderate resistance to TE (Fig. 3). The above could point to intense selective antibiotic pressure on bacterial cells. Lower genetic similarity between TE-sensitive and moderately TE-resistant bacteria in comparison with TE-resistant strains could result from a higher number of genetic changes in genomic regions complementary to the applied DNA markers. Further studies investigating variations in *tet* and *otr* genes that condition TE resistance in *A. hydrophila* strains are needed to verify the above hypothesis [5, 20].

A high level of genetic identity ($I_N = 0.98$) was also observed between strains that were susceptible and moderately resistant to E. A change from a pattern of moderate

resistance to one of high resistance to E could result from a higher number of minor changes in the studied bacterial genome. Minor changes in DNA could be accumulated due to long-term chemical pressure on the Drwęca River resulting from erythromycin use. The patterns of erythromycin resistance in *A. hydrophila* strains sampled from the Drwęca River should be additionally validated by analyzing the diversity of *mel* and *mph* genes and other pRSB105 plasmid sequences [23]. A statistical analysis of multidrug resistance patterns revealed the presence of the closest genetic relations between strains that were susceptible and highly resistant to three drugs. These results could indicate a small number of genetic changes in the genome of highly MDR strains in comparison with moderately resistant and completely susceptible strains of *A. hydrophila*.

CONCLUSIONS

The evolution of *A. hydrophila* strains which were highly resistant to the tested drugs probably took place faster than the process of acquiring many mechanisms of moderate resistance. In the ecosystem of the Drwęca River, drugs exerted chemical selection pressure on bacterial strains. The regions of the *A. hydrophila* genome tested by arbitrary DNA markers revealed fewer genetic changes in highly resistant than in moderately resistant strains. The above could point to inadequate drug dosage during fish treatment. On the other hand, long-term administration of drugs poses an environmental threat by increasing the dilution of toxic chemicals in the river. The *A. hydrophila* genome was scanned with regard to different resistance patterns to reveal that genetic diversity was related to the tested strains' resistance or susceptibility to drugs. The above is validated by the presence of amplification products such as OPA11-15, ISJ2-29, ISJ4-25 or OPB6-20. Resistance marker ISJ4-25 could serve as an effective molecular tool for monitoring the environmental effects of multidrug (TE+ENR+E) resistant strains. The discussed marker supplies information about the effects of intensive fish farming on the microbiological quality of river water. The results of this study could support evaluations of aquatic habitats subjected to human pressure.

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OCENA ZANIECZYSZCZENIA ANTROPOGENICZNEGO WODY RZECZNEJ NA PODSTAWIE ZRÓŻNICOWANIA GENETYCZNEGO AEROMONAS HYDROPHILA

Aeromonas hydrophila jest cennym wskaźnikiem jakości wody w przypadku zanieczyszczeń ściekami oraz mikroorganizmami względnie patogennymi dla człowieka i zwierząt zimnokrwistych, w tym ryb. Celem niniejszych badań była ocena zanieczyszczenia antropogenicznego na podstawie zróżnicowania genetycznego 82 szczepów *A. hydrophila* poprzez analizy RAPD, pół-przypadkowo amplifikowanej klasy AP-PCR (ISJ) i konserwatywnego powtórzenia rep-BOX. Zróżnicowanie genetyczne *A. hydrophila* wyniosło $H_T = 0,28$ ($SD = 0,02$) dla wszystkich markerów DNA (RAPD, pół-przypadkowe i rep-BOX). Wszystkie szczepy dla wszystkich markerów ujawniły indywidualny wzór elektroforetyczny, nie ujawniono jednego źródła rozprzestrzeniania się szczepów. U szczepów potwierdzono obecność genów aerolizyny (*aerA*), hemolizyny (*ahh1*) i kompleksu enzymów cytotoksycznych (AHCYTOGEN), jak również określono wzorzec oporności na tetracyklinę, enrofloksacynę i erytromycynę. Najbardziej zróżnicowane okazały się szczepy *A. hydrophila* wrażliwe na enrofloksacynę ($H_S = 0,27$) a najmniej zróżnicowane były szczepy wrażliwe na erytromycynę ($H_S = 0,24$). Wyselekcjonowany marker wielolekooporności (locus ISJ4-25, 1100 pz) obecny u szczepów (opornych na 3 rozpatrywane leki) świadczy o wpływie intensywnej hodowli ryb na jakość mikrobiologiczną wody rzecznej.