

Distribution of *Pseudomonas fluorescens* and *Aeromonas hydrophila* Bacteria in a Recirculating Aquaculture System during Farming of European Grayling (*Thymallus thymallus* L.) Broodstock

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Abstract: *Pseudomonas fluorescens* and *Aeromonas hydrophila* bacteria are opportunistic pathogens that occur naturally in the aquatic environment and in the gut flora of healthy fish. Both species can pose a serious threat for fish that are highly sensitive to water pollution. The aim of this study was to determine the extent to which the amount of administered fish feed and fish biomass affect the distribution and abundance of *Ps. fluorescens* and *A. hydrophila* bacteria in a recirculating aquaculture system (RAS) during farming of European grayling (*Thymallus thymallus* L.) broodstock. A total of 68 water samples from the inflow, two rearing tanks and the outflow as well as 17 feed samples were collected and analyzed separately. Bacterial populations were analyzed by the culture-dependent method and a molecular method (fluorescence in situ hybridization, FISH) to detect culturable strains and viable but non-culturable strains, respectively. Fish biomass, feed and 16 water quality parameters (temperature, pH, concentration of dissolved oxygen, oxygen saturation, five-day biochemical oxygen demand (BOD₅), total phosphorus, total organic phosphorus and nitrogen, orthophosphates, total nitrogen, nitrite and nitrate nitrogen, ammonia nitrogen, ammonium nitrogen, total suspended solids, and total organic carbon) were the explanatory factors. Statistically significant differences (RM-ANOVA, $p \leq 0.05$) were stated in bacterial abundance in samples from the inflow, rearing tanks and the outflow. Water samples from the RAS were abundantly colonized by non-culturable *Ps. fluorescens* and *A. hydrophila* bacteria. Feed was not a source of bacteria, but a redundancy analysis (RDA) revealed that the amount of feed, fish biomass, BOD₅, and total suspended solids and total organic carbon were positively correlated in both *Ps. fluorescens* and *A. hydrophila*. These parameters also influenced the distribution of both potentially pathogenic bacterial populations and contributed to the bacterial contamination of water in the RAS. Our results are particularly valuable for aquacultures that help to replenish wild stocks and rebuild populations of threatened species in natural aquatic environments.

Keywords: RAS; European grayling; fish rearing; bacteria; *Ps. fluorescens*; *A. hydrophila*

1. Introduction

In most commercial RAS, the safety of the production process and the well-being of fish and other aquatic organisms is controlled based on the physicochemical parameters of water (temperature, pH, and oxygen and ammonia content) and the growth performance of fish (body weight gain, relative growth rate, relative biomass, and changes in total body length). The significance of microbiological analyses of the aquatic environment, aquatic organisms, and feeds is often under-appreciated. In some cases, microbiological assays are not performed even when the first worrying physical or behavioral symptoms are observed in aquatic organisms. This negligence often stems from the lack of awareness that aquatic microbiota can have a greater influence on the health and growth of fish than feed microbiota [1–3]. Water microbiota can decrease production results or even cause massive production losses. From the environmental and epidemiological point of view, the increase in the abundance and activity of heterotrophic and potentially pathogenic microbiota in aquatic environments can pose a serious threat for human and animal health [4–6].

Recirculating aquaculture systems (RAS) with intensively farmed fish are specific ecosystems where the continuous recycling of water contributes to microbial heterotrophy. The physicochemical parameters of water (e.g., temperature, pH, concentration of dissolved oxygen, and $\text{NH}_4\text{-N}$, $\text{NO}_2\text{-N}$), the type and quantity of the administered feed, stock size, and the growth stages of aquatic organisms induce rapid periodic changes in the structure of dominant physiological groups, genera and species of heterotrophic bacteria [7–13]. These processes should be monitored to evaluate the quality of aquatic environments [14]. The above changes cannot be determined based on chemical assays alone. Microorganisms are among the most sensitive [15]. In intensive fish farming, the above applies particularly to changes in the populations of *Pseudomonas fluorescens* and *Aeromonas hydrophila* which constitute natural microbiota in both the aquatic environment and fish, but are also one of the most frequently diagnosed epizootic risk factors affecting fish around the world [6,13,16,17]. The above bacterial species belong to the class *Gammaproteobacteria*. They are ubiquitous in aquatic environments, and they are capable of decomposing various organic compounds due to a broad range of enzymatic activities [13,14,18,19]. Pathogenic strains of *Ps. fluorescens* and *A. hydrophila* have hemolytic and proteolytic properties, and they rapidly acquire resistance against popular antibiotics [4–6,16,20–23]. However, the activity and abundance of these bacteria in aquatic environments depend on physicochemical parameters (water temperature, pH, content of mineral substances, and organic compounds) and biological factors (predation and interspecific competition).

Ps. fluorescens and *A. hydrophila* can be easily cultured on selective media where they form characteristic colorful colonies [24,25]. These bacteria can also be identified with the use of molecular techniques such as fluorescent in situ hybridization (FISH). In the FISH method, specific oligonucleotide probes are used to identify bacterial species under an epifluorescence microscope [26]. This method supports rapid detection (6–8 h) and identification of viable but non-culturable (VBNC) *Ps. fluorescens* and *A. hydrophila* bacteria which lose the ability to proliferate on standard growth media but remain viable in the environment [19,27]. Culturable and non-culturable potentially pathogenic *Ps. fluorescens* or *A. hydrophila* can spread from aquacultures and induce changes in the total populations of bacteria in aquatic ecosystems. For this reason, conventional (culture-dependent) and molecular (culture-independent, such as FISH) methods can be combined to detect the bacteria that colonize those ecosystems, and observe changes in the quantitative and qualitative composition of microbiota in aquatic ecosystems [28–30].

The complete life cycle or selected growth stages (e.g., until the achievement of commercial value) of aquatic organisms can be monitored in experimental RAS. Experimental farms are often established to strengthen or reinstate aquatic species whose natural populations have declined due to anthropogenic pressure. In RAS, farming conditions can be optimized and controlled to produce organisms capable of adapting to degraded aquatic environments. Such experiments are often performed on the European grayling (*Thymallus thymallus* L.) which is highly popular among anglers and is the most economically valuable salmonid species [31]. However, this rheophilic

species has high oxygen requirements (7–10 mg O₂·L⁻¹), and it is highly sensitive to water contamination [31]. For this reason, the European grayling is regarded as a valuable bioindicator of anthropogenic pollution. Anthropogenic pressure [32–34], including strong pressure from recreational fishing [35], progressing eutrophication and pollution of rivers [36–38], have led to a dramatic decline in European grayling populations throughout Europe. These observations are particularly important for European graylings aged 3 to 5 years, which are characterized by the highest reproductive potential, as well as individuals older than 5 years which account for up to 4% of the grayling population in many European regions [39,40]. For these reasons, the European grayling was classified as a conservation dependent (CD) species on the 2009 Red List of Fish and Lamprey Species [41]. This implies that active and effective protection is required to preserve the natural populations of the European grayling.

In order to produce good health fry for stocking the European grayling (*Thymallus thymallus* L.) broodstock (between 3 to 5 years old), was cultivated in RAS at the Department of Fish Biology and Pisciculture of the Faculty of Environmental Sciences at the University of Warmia and Mazury in Olsztyn (Poland) [42,43]. During experimental farming in RAS, the microbiological and physicochemical parameters of water, fish growth parameters (increase in fish biomass) and the amounts of feed were monitored in characteristic sites. The studied microorganisms were *Ps. fluorescens* and *A. hydrophila* which: (i) constitute the natural microbiota of water and fish [4,5,10–12], (ii) can be potentially pathogenic for fish and humans [6,21,22,44], (iii) are characterized by high levels of cross-resistance to popular antibiotics [4,5] and (iv) are detected in growing concentrations in water evacuated from various aquaculture systems in Poland [4,10–12,44] and in the world [13,14,45].

Very little is known about the factors that affect the distribution of potentially pathogenic *Ps. fluorescens* and *A. hydrophila* bacteria during intensive fish rearing in RAS. This study tested the hypothesis postulating that the amount of fish feed and fish biomass influence the distribution and abundance of *Ps. fluorescens* and *A. hydrophila* in a recirculating aquaculture system (RAS) during farming of European grayling (*Thymallus thymallus* L.) broodstock.

Standardised culture-dependent and culture-independent methods (FISH) were used to determine changes in the populations of *Ps. fluorescens* and *A. hydrophila* during intensive fish rearing in RAS. Bacterial contamination is a very important consideration in freshwater ecology because potentially pathogenic *Ps. fluorescens* and *A. hydrophila* bacteria colonizing water and intensively farmed fish can spread from aquacultures and contaminate the natural environment with non-specific microbiota.

2. Materials and Methods

2.1. Study Area and Sampling Sites

European grayling (*Thymallus thymallus* L.) broodstock was experimentally bred in a recirculating aquaculture system in the Department of Fish Biology and Pisciculture of the Faculty of Environmental Sciences at the University of Warmia and Mazury in Olsztyn (Poland). Total bacterial counts, the counts of *Ps. fluorescens* and *A. hydrophila* bacteria, and the physicochemical parameters of water, including temperature, pH, concentration of dissolved oxygen (DO), oxygen saturation (OS), 5-day biochemical oxygen demand (BOD₅), orthophosphates (PO₄-P), total phosphorus (TP), NH₄-N, NH₃-N, NO₂-N, NO₃-N, total suspended solids (TSS), total organic carbon (TOC), total nitrogen (TN), total organic nitrogen (TON), and total organic phosphorus (TOP) were monitored over a period of 17 months between December 2013 and April 2015.

European grayling (*Thymallus thymallus* L.) was experimentally bred in nine fish rearing tanks (Figure 1), including six tanks with a volume of 0.32 m³ each and three tanks with a volume of 0.98 m³ each. Tank water was filtered by a purification system composed of a microstrainer (60 µm pore size, processing capacity of 20 m³·h⁻¹), a trickling filter (with a volume of 1.5 m³ and an active surface area of 1100 m²) and a UV lamp (105 W). Water was cooled to the optimal temperature in a glycol chiller. Daily water loss of around 3% (200 L·day⁻¹) was supplemented. During 17 months of

the experiment, total fish biomass increased from 9.27 kg to 54.49 kg. The fish were fed commercial feed with 45–55% protein content and 15–20% fat content, depending on the age, growth stage and body weight of fish. The feed ration was adjusted to feed intake. A total of 87.44 kg of feed was used during the experiment, and $5.5 \pm 1.3 \text{ kg} \cdot \text{m}^{-1}$ of feed was administered monthly on average, subject to the nutrient requirements and the growth stages of fish. The average total monthly increase in fish biomass was $2.7 \pm 0.8 \text{ kg} \cdot \text{m}^{-1}$.

The experimental materials were water samples collected from four sites and fish feed. Water was sampled from the inflow, two rearing tanks and the outflow from rearing tanks (Figure 1). The triplicate subsamples were combined in equal proportions, and the pooled sample was subjected to microbiological and physicochemical analyses. Pooled water samples were collected from each site at monthly intervals. A total of 17, 17, 17, and 17 water samples were acquired from: the inflow, two rearing tanks (no. 1 and no. 2) and the outflow, respectively. Feed samples, fish biomass and the amount of feed were monitored. A total of 68 water samples and 17 feed samples were collected and analyzed separately. All water samples were placed in sterile bottles at 4 °C, transported to a laboratory and assayed within 12 h of collection. Fish feed was sampled at the experimental site and placed in sterile measuring vessels.

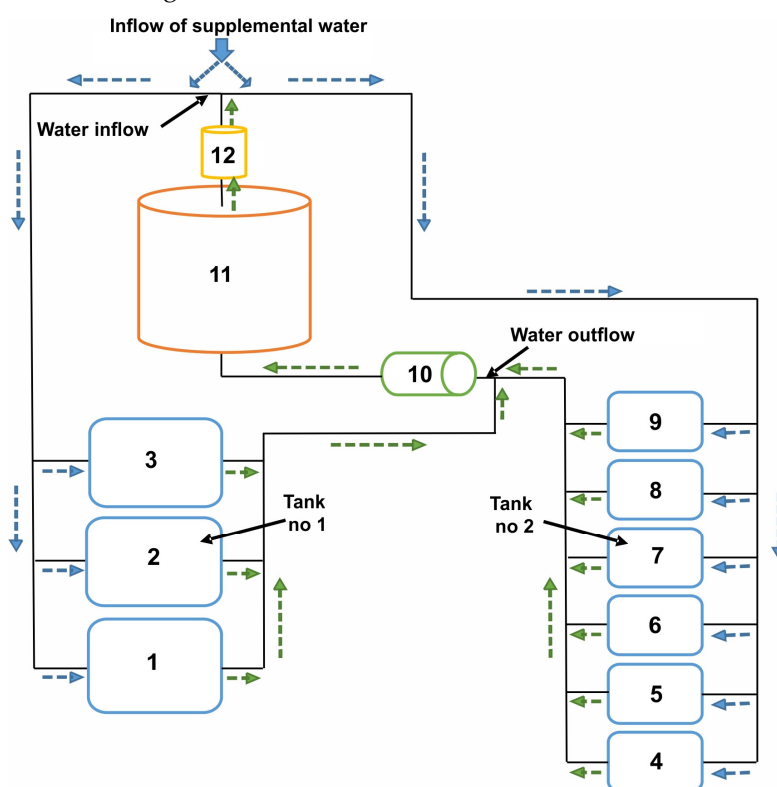


Figure 1. Diagram of the recirculating aquaculture system (RAS) and water sampling sites. Denotations: 1, 2, 3—fish rearing tanks (0.98 m³); 4, 5, 6, 7, 8, 9—fish rearing tanks (0.32 m³); 10—microstrainer; 11—trickling filter; 12—UV lamp.

2.2. Microbiological Analysis

Microbiological analyses relied on culture-dependent and culture-independent methods. The culture-dependent method involved plate counts, isolation and biochemical identification to detect culturable *Ps. fluorescens* and *A. hydrophila* strains. Culture-independent methods relied on molecular techniques, DAPI staining (with 4',6-diamidino-2-phenylindole dihydrochloride) (Sigma-Aldrich, St. Louis, MO, USA) and fluorescence *in situ* hybridization (FISH) to determine the counts of visible but non-culturable *Ps. fluorescens* and *A. hydrophila* bacteria. The FISH method based on targeted with 16rRNA oligonucleotide probes, usually of the length of 15–30 nucleotides, allows to identify large phylogenetic groups, individual species of bacteria as well as to demonstrate the structure of microbial communities and their dynamics in the environments.

2.2.1. Determination of *Ps. fluorescens* and *A. hydrophila* Counts by the Culture-Dependent Method in Samples of RAS Water and Fish Feed

Aliquots of 1 mL of pooled samples of water collected from the inflow, two rearing tanks and the outflow were diluted in 9 mL of sterile phosphate-buffered saline (PBS). Feed samples of 10 g each were homogenized with 90 mL of PBS. Serial dilutions with PBS were prepared to reduce bacterial density. Subsequently, 0.1 mL of every homogenized solution was subjected to microbiological analysis. In quantitative microbiological analyses, water and feed samples were cultured on selective media, and the counts of *Ps. fluorescens* and *A. hydrophila* bacteria were determined by the pour plate method. *Ps. fluorescens* bacteria were cultured on King's B medium for 72 h at 28 °C according to Standard Methods for the Examination of Water and Wastewater (APHA) [46]. *Ps. fluorescens* colonies cultured on King's B medium were analyzed under Wood's UV lamp, and the number of fluorescein-producing colonies was counted. *A. hydrophila* were enumerated, incubated at 30 °C for 24 h, and isolated by plating on the Aeromonas Medium Base (Ryan). *A. hydrophila* colonies were counted based on the number of opaque green colonies with a dark centre formed in a color reaction with the Aeromonas Medium Base (Ryan). The number of colonies which were cultured on selective media and identified based on their morphology (cell shape, Gram stain reaction, motility in microscopic preparations) and enzymatic activity (ability to produce catalase and cytochrome oxidase, capacity for oxidative degradation, and fermentation of glucose on the Hugh-Leifson medium) was taken into account in the determination of *Ps. fluorescens* and *A. hydrophila* counts. *Ps. fluorescens* and *A. hydrophila* bacteria were further identified with the use of the API 20NE biochemical test strips (BioMérieux®, France) for Gram negative non-*Enterobacteriaceae*. The analyses were performed according to the Standard Methods for the Examination of Water and Wastewater [46]. All determinations were performed in three replicates in the same sample of water or fish feed. Plate counts were expressed as colony-forming units (CFU) per 1 mL of water or per 1 g of fish feed.

2.2.2. Determination of Total Bacterial Counts in RAS Water and Fish Feed Samples by DAPI Staining and in Situ Hybridization FISH

The protocols for the fixation, hybridization and microscopic quantification of bacterial communities by the FISH method and DAPI staining were similar to those described in a previous study [28,47]. After preliminary analyses, selected hybridization conditions were modified to optimize the results. This approach increased the efficiency of FISH analysis to 75–90 % of total cell counts.

Total bacterial counts were determined in 1 mL samples of RAS water (collected from different sites with different suspension concentrations) and 1 g of fish feed. Triplicate subsamples of water collected from the inflow, two rearing tanks and the outflow and fish feed were fixed with neutralized formaldehyde (pH 7.4, final concentration of 4%) and stained with 4',6'-diamidino-2-phenylindole (DAPI, showing affinity for cellular DNA) (Sigma-Aldrich, St. Louis, MO, USA) with a final concentration of 0.01 ng·mL⁻¹, for 15 min in the dark [48]. Fixed samples were gently passed through black Nuclepore filters with 0.2 µm pore size (GTTP, Millipore). Filters were rinsed with 10 mL of ultrapure water (MQ Millipore) to remove excess paraformaldehyde and dried for several minutes at room temperature. Bacteria were counted under an Olympus epifluorescence microscope. More than 1000 bacterial cells were counted in 20 fields of view.

The number of *Ps. fluorescens* and *A. hydrophila* bacteria was determined in 10–20 mL samples of water collected from the inflow, two rearing tanks and the outflow and in 1 g of fish feed. Triplicate subsamples were fixed with the same method that was used in the determination of total bacterial counts. Fixed samples were passed through white polycarbonate filters (0.2 µm pore size, 47 mm in diameter) (GTTP, Millipore) under low negative pressure with the use of base filters (0.45 µm pore size, Sartorius) to distribute cells evenly on filters. The samples fixed on filters were subjected to the FISH procedure with the use of specific oligonucleotide probes. *Ps. fluorescens* was identified with the Pseudo120 probe (Thermo Fisher Scientific GmbH, Ulm, Baden-Württemberg, Germany) [19] *A. hydrophila*—with the KO 229 probe (Thermo Fisher Scientific GmbH, Ulm, Baden-

Württemberg, Germany) [27]. The GAM42a competitor probe (Thermo Fisher Scientific GmbH, Ulm, Baden-Württemberg, Germany) [49] and the probe specific for domain Bacteria (EUB338) (Thermo Fisher Scientific GmbH, Ulm, Baden-Württemberg, Germany) were applied to identify all bacterial species [50]. All samples were simultaneously screened with the NON338 probe (Thermo Fisher Scientific GmbH, Ulm, Baden-Württemberg, Germany) [51] to control autofluorescence and the number of non-specifically stained cells which did not exceed 5% on average [52]. The probe sequences, hybridization conditions and references are given in Table 1. The composition of bacterial communities was investigated by fluorescent in situ hybridization (FISH) with the use of Cy3-labeled oligonucleotide probes (Thermo Fisher Scientific GmbH, Ulm, Baden-Württemberg, Germany) in accordance with the hybridization procedure for aquatic microorganisms proposed by Pernthaler et al. [47]. After the FISH procedure, the specimens were viewed under a fluorescence microscope (Olympus BX61) equipped with a $\times 100$ oil immersion lens (Olympus Poland, Warsaw, Masovia, Poland), UV lamp (Olympus Poland, Warsaw, Masovia, Poland), DAPI and CY3 filters (Olympus Poland, Warsaw, Masovia, Poland) and CCD camera (Olympus) (Olympus Poland, Warsaw, Masovia, Poland). Images were analyzed in the Cell F application (Olympus). More than 100 hybridized bacterial cells (FISH) and 1000 to 10,000 DAPI-stained bacterial cells were identified per every evaluated sample. Mean values, range and standard deviation were calculated from 20 random fields of view in every filter section and expressed in terms of 1 mL of water and 1g of fish feed. Pseudo120 and KO 229 probe-specific cell counts (of *Ps. fluorescens* and *A. hydrophila*, respectively) were expressed as the number and percentage of cells visualized by DAPI staining.

Table 1. Oligonucleotide probes used for bacteria determination by hybridization in situ (FISH) method during farming of European grayling (*Thymallus thymallus* L.) broodstock in RAS.

Probe	Target Species	Target ¹ Site (rRNA Positions)	% FA ² in situ	TF ³	Reference
EUB338	Bacteria	16S (338–355)	35	48	[50]
NON338	Control probe complementary to EUB338	16S (338–355)	35	48	[51]
GAM42a	<i>Gammaproteobacteria</i>	23S (1024–1043)	35	47	[49]
GAM42a'	Gamma competitor	23S (1024–1043)	35	47	[49]
Pseudo120	<i>Ps. fluorescens</i>	16 S (120–142)	30	57	[19]
KO 229	<i>A. hydrophila</i>	16S (454–474)	30	49	[27]

¹—*Escherichia coli* numbering [53], ²—percentage of formamide (FA) in in situ hybridization buffer, ³—temperature of hybridization.

2.3. Physicochemical Parameters of RAS Water

Selected physicochemical parameters of RAS water were determined during the collection of microbiological samples. Microbiological and physicochemical assays were carried out in the same water samples from inflow, two rearing tanks and the outflow. Temperature (°C), dissolved oxygen concentration (DO mg O₂·L⁻¹), oxygen saturation (OS) and pH were analyzed in situ with the WTW Multiline P4 multi-parameter sensor (Xylem Analytics Germany Sales GmbH & Co. KG WTW, Weilheim, Bavaria, Germany) and YSI PRO-ODO optical oxygen sensor (YSI Incorporated, Yellow Springs, OH, USA) to the nearest ± 0.1 °C, ± 0.01 mg O₂·L⁻¹, $\pm 0.1\%$ O₂ and ± 0.01 pH, respectively. The values of five-day biochemical oxygen demand (BOD₅ mg O₅·L⁻¹) and the concentrations of orthophosphate (PO₄-P mg P·L⁻¹), total phosphorus (TP mg P·L⁻¹), ammonium nitrogen (NH₄-N mg N·L⁻¹), nitrogen-ammonia (NH₃-N mg·L⁻¹), nitrite nitrogen (NO₂-N mg N·L⁻¹), nitrate nitrogen (NO₃-N mg N·L⁻¹) and total suspended solids (TSS mg·L⁻¹) were determined according to the Standard

Methods [46]. All spectrophotometric analyses were performed with the Merck SQ118 photometer (Merck KGaA, Darmstadt, Hesse, Germany). Total organic carbon (TOC mg C·L⁻¹) was determined with the HACH IL 550 TOC-TN analyzer (Hach Lange, Dusseldorf, North-Rhine Westphalia, Germany). Total nitrogen (TN mg N·L⁻¹) was determined as nitrogen oxides after mineralization (850 °C) in a chemiluminescence detector (HACH IL 550 TOC-TN analyzer). Total organic phosphorus (TOP mg P·L⁻¹) and total organic nitrogen (TON mg N·L⁻¹) were determined as the difference between total phosphorus and nitrogen and mineral phosphorus and nitrogen, respectively.

2.4. Statistical Analysis

Statistical analyses were carried out using the XLSTAT ver. 2018.3 software (2018.3 version, Addinsoft, New York, NY, USA) provided by Microsoft Excel® by Addinsoft. To assess the influence of recirculated water on the bacteria counts and chemical composition of water throughout the experiment, one-way Repeated Measure ANOVA (RM-ANOVA) was carried out with “site” (three levels: inflow, tank, outflow) regarded as a fixed factor, with 17 repeated measurements over the period of 17 months. Homogeneity of variance was tested using Levene’s F-test. For RM-ANOVA Mauchly’s test was used to check the assumption of sphericity. When the assumption of sphericity was violated for the within-subject effect, the degrees of freedom and thereby the F-value were corrected using the Greenhouse–Geisser correction. Significant differences among means were identified using a Tukey’s multiple comparison test (HSD). Statistical significance was set at 0.05 [54].

The differences in the abundance of the studied bacterial populations determined by culture-dependent and culture-independent methods, and the relationships between bacterial populations and environmental variables (fish biomass, amount of feed, and the physicochemical parameters of water) were determined by linear redundancy analysis (RDA). This method was applied because a preliminary detrended correspondence analysis (DCA) had demonstrated that the data were characterized by a linear response (gradient length <2.0 SD units) to the analyzed environmental parameters [55]. In our analysis, the calculated gradient length was 0.4 SD units; therefore, redundancy analysis (RDA) was applied to determine the relationships between bacterial counts and fish biomass, amount of feed and water quality parameters. Full sets of environmental variables were screened by automatic forward selection in the Monte Carlo randomization test (999 permutations) [56], and only variables that were significantly ($p \leq 0.05$) related to assemblage structure were retained to further analysis. In the final RDA model, the appropriate subset of explaining variables were selected by the Bonferroni correction to reduce the factors that were not significant for the model. Following the Bonferroni correction, the collinearity between explanatory variables was reduced by eliminating selected variables with a variation inflation factor (VIF) greater than 10 [55]. For this reason twelve physicochemical variables (temperature, pH, DO, OS, PO₄-P, TP, NH₄-N, NH₃-N, NO₃-N, NO₂-N, TON, TN) were eliminated as redundant in the final RDA model. The multivariate analyses were carried out in CANOCO ver. 5.0 (Microcomputer Power, Ithaca, NY, USA) [57].

3. Results and Discussion

3.1. *Ps. fluorescens* and *A. hydrophila* Counts in Samples of Water and Fish Feed

The counts of *Ps. fluorescens* and *A. hydrophila* determined by culture-dependent and culture-independent methods were highly similar in water samples collected from both water rearing tanks (no. 1 and no. 2). In water samples from rearing tanks no. 1 and no. 2, *Ps. fluorescens* counts were determined at 140 ± 60 and 170 ± 75 cfu·mL⁻¹ by the culture-dependent method, respectively, and at $3.2 \times 10^6 \pm 1.3 \times 10^6$ and $2.9 \times 10^6 \pm 1.7 \times 10^6$ cells·mL⁻¹ by the culture-independent method, respectively. *A. hydrophila* counts in water samples from tank no.1 and 2 were determined at 240 ± 75 and 220 ± 85 cfu·mL⁻¹, respectively, by the culture-dependent method, and at $4.7 \times 10^6 \pm 1.6 \times 10^6$ and $4.3 \times 10^6 \pm 1.9 \times 10^6$ cells·mL⁻¹, respectively, by the culture-independent method. No significant

differences ($p \leq 0.05$) between the counts of *Ps. fluorescens* and *A. hydrophila* bacteria determined by both methods were found in water samples collected from tank no. 1 and 2 (t-test, $p > 0.05$). For this reason, bacterial counts in two fish tanks were pooled presented as the average values for both tanks.

The mean values and the range of *Ps. fluorescens* and *A. hydrophila* counts determined by culture-dependent and culture-independent methods are presented in Table 2 and Figure 2. Bacterial counts were determined within a range spanning several orders of magnitude (10^1 – 10^8), depending on species, the applied analytical method and sampling site (inflow, tank and outflow). The RM-ANOVA revealed significant differences in *Ps. fluorescens* (Tukey test, $F = 62.32$, $p = 0.0001$) and *A. hydrophila* counts (Tukey test, $F = 25.63$, $p = 0.0001$) between the inflow, the tank and the outflow (Figures 2A–D).

In a previous study, Michaud et al. [45] frequently observed smaller counts of *Pseudomonas* species (35 clones and 12 isolates) isolated from water samples in a sea bass (*Dicentrarchus labrax*) farm. Other studies of RAS also revealed differences in the size of microbial communities colonizing biofilters and culture water, as well as the presence of bacterial communities characteristic of unique and complex environments [7,18,20,58,59].

In our study, *Ps. fluorescens* and *A. hydrophila* counts and their percentages in total bacteria counts were lowest in water sampled at the inflow and were several to several hundred times higher in water sampled from tanks and at the outflow, regardless of the applied analytical method (Table 2, Figure 2).

Table 2. The counts of *Ps. fluorescens* and *A. hydrophila* determined by a culture-dependent method, total DAPI cell counts and the relative percentage of cells hybridized with specific probes in the water of RAS during farming of European grayling (*Thymallus thymallus* L.) broodstock.

Bacteria	Inflow ¹		Tank ¹		Outflow ¹	
	Mean	Range	Mean	Range	Mean	Range
<i>Ps. fluorescens</i> ²	65	20–145	141	40–250	446	220–930
<i>A. hydrophila</i> ²	26	1–76	244	90–342	11,000	930–35,000
Total bacterial counts ³	39.4	12.3–87.2	85.6	42.4–202.1	303.4	152.3–559.1
EUB338 (%) ⁴	66.0	27.1–69.2	61.7	16.6–69.0	67.2	63.8–79.1
GAM42a (%) ⁴	8.2	6.3–18.5	10.3	9.8–21.2	15.1	9.4–28.6
Pseudo120 (%) ⁴	0.17	0.1–0.3	2.6	1.5–3.9	4.6	3.3–6.4
KO229 (%) ⁴	0.07	0.05–0.10	5.1	1.8–5.6	9.9	7.3–11.1

¹—sampling sites, ²—numbers as colony forming units (CFU mL^{−1}), ³—total bacterial counts of $\times 10^6$ cells·mL^{−1},

⁴—percent detection compared to DAPI (numbers have been corrected by subtracting NON338 counts).

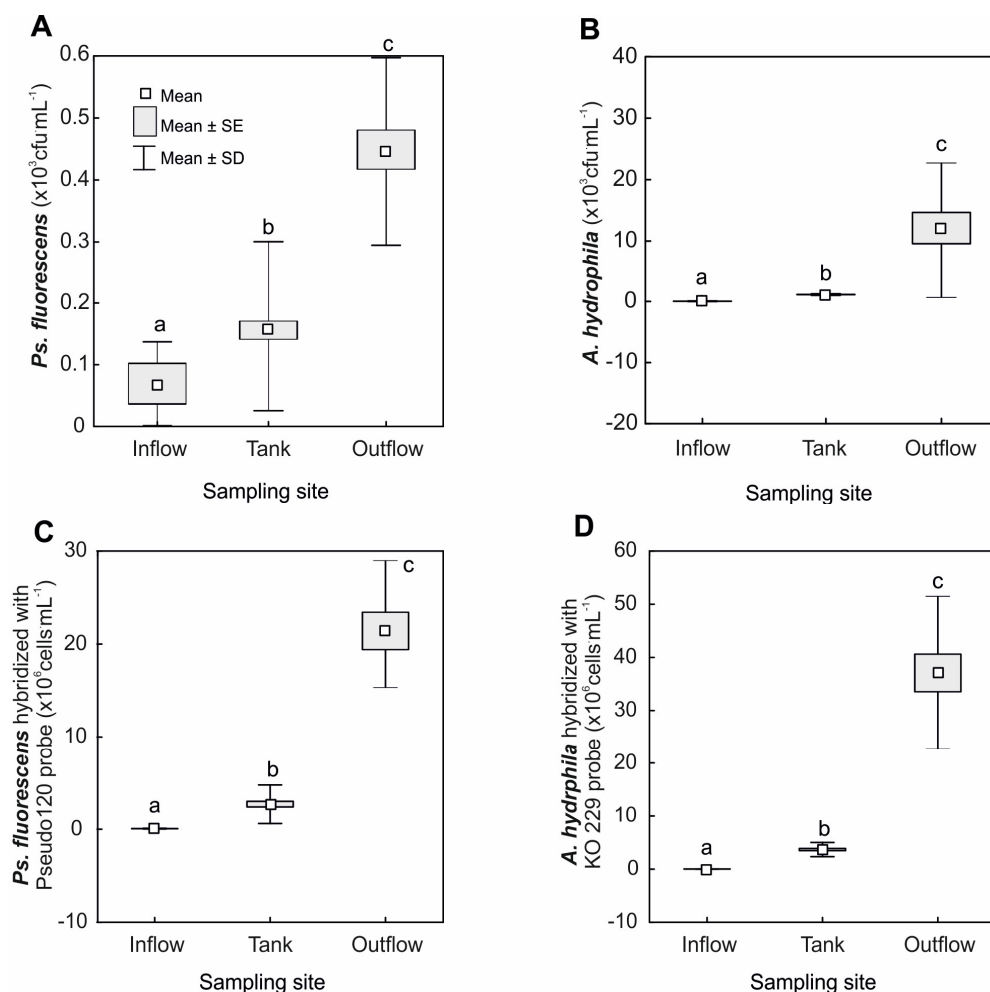


Figure 2. The counts of *Pseudomonas fluorescens* (A) and *Aeromonas hydrophila* bacteria (B) determined by a culture-dependent method and the culture-independent technique: bacteria hybridized with Pseudo120 (C) and KO 229 probes (D). The differences in the bacteria counts in water samples collected from the inflow (N = 17), tank (N = 34) and outflow (N = 17) were analyzed with the Tukey multiple comparison test (RM-ANOVA, $p < 0.05$). Statistically significant differences were depicted by different letters. Denotations: $\pm$ SE—standard error, $\pm$ SD—standard deviation.

In the FISH assay, the counts of *Ps. fluorescens* and *A. hydrophila* in inflow, tank and outflow water samples were approximately 10^2 – 10^5 higher than in the culture-dependent method (Figure 2). It indicates that studied closed water system was more colonized by analyzed non-culturable bacteria than culturable *Ps. fluorescens* and *A. hydrophila* strains. These microbial species, which rely on organic matter (e.g., digested and undigested feed, feces, and dead fish) as a source of nutrients and energy also colonize the skin and digestive tract of fish, proliferate readily in aquatic environments such as RAS [9–13]. However, it is possible that the studied species of culturable bacteria ceased to divide and proliferate under exposure to physicochemical environmental stressors in RAS (e.g., water temperature, pH, OS, concentrations of $\text{NH}_4\text{-N}$, $\text{NH}_3\text{-N}$, etc.). The culturable bacteria are more sensitive on unfavorable factors in water environments. Consequently bacterial cells could be damaged and lose the ability to proliferate on standard growth media during the laboratory cultivations. Early studies [7,8,13,60–62] focused on culturing techniques showed that very few bacteria species from natural populations (<1% in water) are able to grow under standard lab conditions. Therefore we used these two methods because they allowed to analyze the total *Ps. fluorescens* and *A. hydrophila* species richness in our studies of RAS than they would have been applied separately.

In the current study, the counts of both bacterial species in water samples collected from fish rearing tanks and the outflows from fish rearing tanks increased with a rise in feed dosage and an

increase in fish biomass regardless of the applied analytical method (Figure 3A,B). The greater variations in *A. hydrophila* counts than *Ps. fluorescens* counts were observed in water samples collected from both tank and outflow waters. Under aerobic conditions, microbial breakdown of organic matter leads to the production of new bacterial cells in amounts corresponding to 40–60% of metabolized organic matter [63].

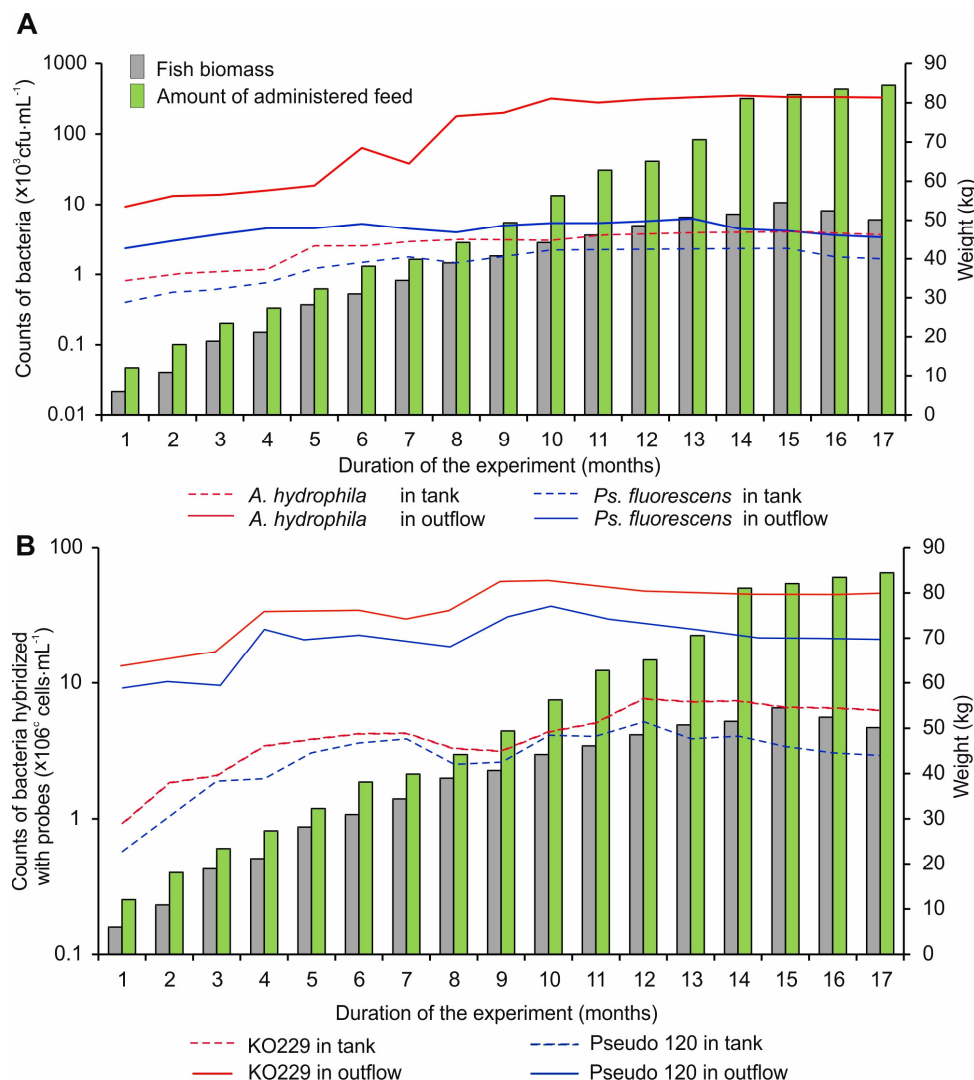


Figure 3. Changes in the counts of *Pseudomonas fluorescens* and *Aeromonas hydrophila* determined by a culture-dependent method (A) and a culture-independent method: Pseudo120, KO 229 (B) in water samples collected from the fish tank and the outflow relative to cumulative fish biomass and the total amount of feed administered during the intensive rearing of European grayling (*Thymallus thymallus* L.) broodstock in RAS. Dashed lines represent bacterial counts in the tank, and continuous lines denote *Ps. fluorescens* and *A. hydrophila* populations at the outflow.

In the culture-dependent method, the counts of *Ps. fluorescens* colonizing the feed administered to European graylings (*Thymallus thymallus* L.) did not exceed 5 cfu·g⁻¹ and these bacteria were not detected in 90% of feed samples. The presence of *A. hydrophila* was not observed in 17 feed samples determined this technique. During the experiment, the counts of *Ps. fluorescens* (Pseudo120) and *A. hydrophila* (KO 229) determined by the culture-independent method varied within a very narrow range. The abundance of *Ps. fluorescens* and *A. hydrophila* in the analyzed fish feed samples reached 80 to 120 cells·g⁻¹ and 20 to 60 cells·g⁻¹, respectively. No significant correlations between the counts of both bacterial species were observed between feed fish and water samples from the tank and the outflow ($r_{\text{tank}} = 0.200$, $r_{\text{outfl}} = 0.090$).

These results suggest that the feed administered to European graylings was not a major source of *Ps. fluorescens* and *A. hydrophila* in RAS water. The results of both culture-dependent and culture-independent analyses indicate that both bacterial species were originated from autochthonous fish and water microbiota. Moreover, the administered feed contributed more to the growth of non-culturable than culturable strains of *Ps. fluorescens* and *A. hydrophila* bacteria in RAS water (Figure 3 A,B).

During intensive fish farming, 60–80% of the administered feed is not converted into bodily tissue [64] and acts as the main source of biogenic substances and the ideal medium for the development of heterotrophic bacteria in water or bacteria which are deposited in water with fish excrement [10,65]. For this reason, the gut microbiota is more likely to influence the composition of bacterial communities in RAS water than feed microbiota [1,2].

3.2. Physicochemical Parameters of Water Samples

All of the evaluated physicochemical parameters were characterized by minor fluctuations in water samples collected from the inflow, the tank and the outflow in successive months of the experiment (Table 3). The lowest values most parameters and the highest oxygen levels were noted in samples of water flowing into fish rearing tanks. The evaluated physicochemical parameters were highest in water flowing out of fish tanks. In most tank and outflow samples, an increase in $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$, BOD_5 , TSS, TOP, and TOC values was generally accompanied by a rise in *Ps. fluorescens* and *A. hydrophila* counts determined by both culture-dependent and culture-independent methods (Table 2).

According to Cytryn et al. [7] relatively high values of physicochemical parameters in water samples are indicative of a wide range of microbially-mediated transformation processes. In our study, significant differences ($p \leq 0.05$) in the values of $\text{NH}_4\text{-N}$, BOD_5 , TSS, TOP, and TOC were observed between sampling sites (Table 3). The physicochemical parameters of all water samples were within the limits recommended by the Global Aquaculture Alliance [66]. In line with the above guidelines, water parameters should be kept within the following limits to optimize fish culturing conditions in aquacultures and to minimize their adverse effects on the natural environment: pH of 6 to 9, $\text{TSS} \leq 50 \text{ mg}\cdot\text{L}^{-1}$, $\text{TP} \leq 0.3 \text{ mg}\cdot\text{L}^{-1}$, $\text{NH}_3\text{-N} \leq 3.0$, $\text{BOD}_5 \leq 30 \text{ mg}\cdot\text{L}^{-1}$ and $\text{DO} \geq 5.0 \text{ mg}\cdot\text{L}^{-1}$.

Table 3. Mean values and standard deviation ($\pm\text{SD}$) of the physicochemical parameters of water samples collected from the inflow to fish tanks, from fish tanks and from the outflow from tanks in RAS during farming of European grayling (*Thymallus thymallus* L.) broodstock. Statistically significant differences between water samples collected from the inflow ($N = 17$), tank ($N = 34$) and outflow ($N = 17$) were assessed by the Tukey multiple comparison test (RM-ANOVA, $p \leq 0.05$) and depicted by different letters: a—inflow and b—tank. Dissolved oxygen (DO), oxygen saturation (OS), 5-day biochemical oxygen demand (BOD_5), orthophosphates ($\text{PO}_4\text{-P}$), total phosphorus (TP), $\text{NH}_4\text{-N}$, $\text{NH}_3\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$, total suspended solids (TSS), total organic carbon (TOC), total nitrogen (TN), total organic nitrogen (TON), and total organic phosphorus (TOP).

Parameter (Unit)	Sampling Sites		
	Inflow	Tank	Outflow
T ($^{\circ}\text{C}$)	10.0 ± 2.5	10.6 ± 2.4	10.6 ± 2.3
DO ($\text{mg}\cdot\text{L}^{-1}$)	10.03 ± 0.53	9.9 ± 0.6	9.01 ± 0.61
OS (% O_2)	90.9 ± 3.7	84.6 ± 21.4	80.1 ± 3.2
pH	7.9 ± 0.14	8.0 ± 0.15	8.20 ± 0.15
$\text{PO}_4\text{-P}$ ($\text{mg P}\cdot\text{L}^{-1}$)	0.545 ± 0.152	0.554 ± 0.161	0.596 ± 0.121

TOP (mg P·L ⁻¹)	0.164 ± 0.051a	0.233 ± 0.059b	0.281 ± 0.064b
TP (mg P·L ⁻¹)	0.710 ± 0.164	0.784 ± 0.188	0.910 ± 0.191
NH ₄ -N (mg N·L ⁻¹)	0.038 ± 0.017	0.065 ± 0.026	0.075 ± 0.070
NH ₃ -N (mg N·L ⁻¹)	0.002 ± 0.001	0.003 ± 0.001	0.003 ± 0.001
NO ₂ -N (mg N·L ⁻¹)	0.004 ± 0.003	0.018 ± 0.009	0.011 ± 0.001
NO ₃ -N (mg N·L ⁻¹)	8.848 ± 2.385	8.455 ± 2.727	8.152 ± 2.837
TON (mg N·L ⁻¹)	3.041 ± 1.147	4.576 ± 1.203	4.733 ± 1.282
TN (mg N·L ⁻¹)	11.83 ± 2.93	13.10 ± 2.95	13.17 ± 2.95
BOD ₅ (mg O ₂ ·L ⁻¹)	1.95 ± 0.47a	2.64 ± 0.57b	3.82 ± 0.67b
TSS (mg·L ⁻¹)	1.94 ± 0.78a	4.29 ± 1.69b	4.86 ± 2.02b
TOC (mg C·L ⁻¹)	3.01 ± 0.33a	4.31 ± 0.62b	4.45 ± 0.71b

3.3. Relationships between Bacterial Counts and Environmental Variables

The RDA demonstrated that 6 out of 18 environmental factors markedly affected the total counts of *Ps. fluorescens* and *A. hydrophila* in the RAS, determined by culture-dependent and culture-independent methods (Figure 4). Regardless of the sampling site (inflow, tank or outflow), the two main factors (axes) explained 70.35% of total variance. After forward selection of environmental variables, the RDA model generated the following significant variables (λ_A) at $p \leq 0.05$: feed amount ($\lambda_A = 0.018$), fish biomass ($\lambda_A = 0.018$), TSS ($\lambda_A = 0.018$), TOP ($\lambda_A = 0.018$), BOD₅ ($\lambda_A = 0.018$) and TOC ($\lambda_A = 0.036$). After the Bonferroni correction, the first axis explained 68.5% of the variance, and the second axis explained 1.6% of the variance (70.35% of total variance) in the abundance of *Ps. fluorescens* and *A. hydrophila* determined by culture-dependent and culture independent methods, and all the canonical axes were significant (Monte Carlo test, $p = 0.002$). The first factor was very strongly linked with the amount of feed ($\lambda_A = 0.500$), fish biomass ($\lambda_A = 0.016$), TSS ($\lambda_A = 0.064$) and TOC ($\lambda_A = 0.032$), which explained 56.3% of total variance. In the RDA model, the strongest positive relationships were noted between *Ps. fluorescens* (Pseudo120) and *A. hydrophila* (KO229) counts determined by the culture-independent method vs. the amount of feed and total organic carbon (TOC) concentration. In contrast, fish biomass, TSS and BOD₅ were most closely correlated with *Ps. fluorescens* and *A. hydrophila* counts determined by the culture-dependent method.

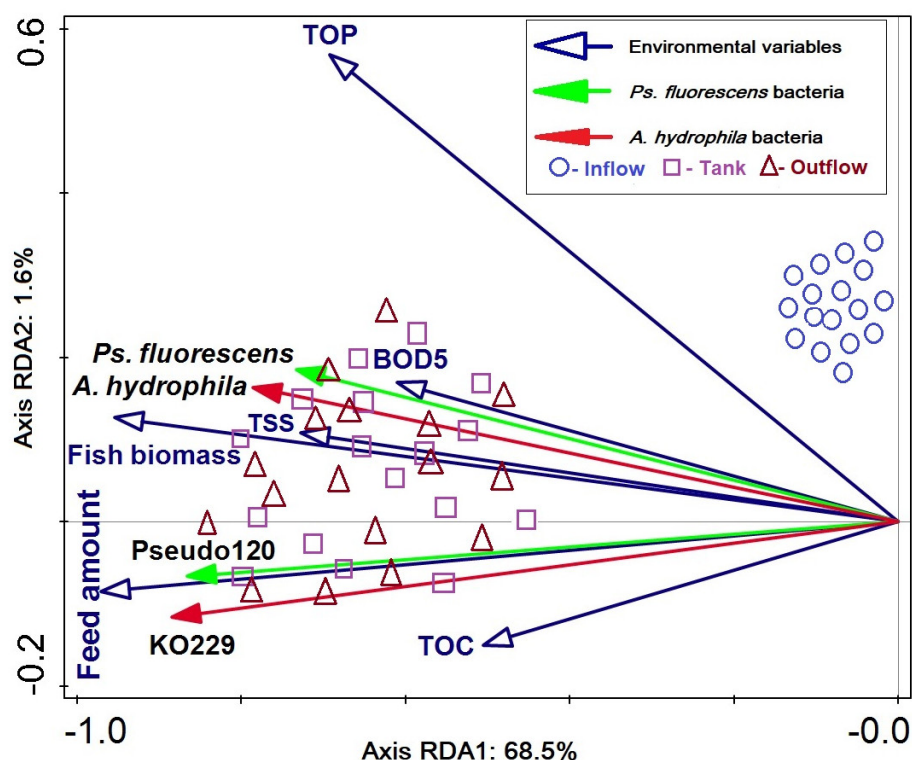


Figure 4. Redundancy analysis (RDA) biplot of the correlations ($p \leq 0.05$) between *Pseudomonas fluorescens* and *Aeromonas hydrophila* counts and environmental variables determined by culture-dependent and culture-independent methods (Pseudo120, KO 229) in samples of RAS water during intensive rearing of European grayling (*Thymallus thymallus* L.) broodstock.

The results of this study are consistent with the findings of other authors [2,7,8] who reported significant correlations ($p \leq 0.05$) between the physiochemical parameters of water (pH, conductivity, $\text{PO}_4\text{-P}$, $\text{NO}_3\text{-N}$) versus the diversity and distribution of bacteria in horizontal and vertical profiles in experiments performed in Nile tilapia larvae [2], a marine culture system [7], and a rainbow trout farm [8].

In our study, the positive correlations between *Ps. fluorescens* and *A. hydrophila* counts determined by culture-dependent and culture-independent methods versus the amount administered feed, fish biomass, and BOD_5 , TSS, and TOC concentrations indicate that some factors were influenced mainly by the size of *Ps. fluorescens* and *A. hydrophila* populations in the analyzed RAS. The studied bacterial species are capable of metabolising nitrogen and carbon from various sources, and they proliferate most rapidly in the presence of readily oxidizable organic matter from fish feed which is utilized with 80–90% efficiency [18,67]. These results indicate that intensively farmed European graylings increase the abundance of culturable and non-culturable potentially pathogenic *Ps. fluorescens* and *A. hydrophila* in RAS.

4. Conclusions

The counts of *Ps. fluorescens* and *A. hydrophila* bacteria determined with the use of culture-dependent and culture-independent methods had mainly relationships with the amount of feed administered to European graylings (*Thymallus thymallus* L.), fish biomass and increasing water pollution (BOD_5 , TSS, and TOC concentrations) in the RAS. Significant differences in the counts of *Ps. fluorescens* and *A. hydrophila* between sampling sites, and the fact that they were strongly correlated with feed dose and total fish biomass, indicate that these farming conditions and the nutrient requirements of European graylings affect the abundance of the analyzed potentially pathogenic bacteria. *Ps. fluorescens* and *A. hydrophila* constitute natural microbiota of water environments and aquatic organisms, but an increase in their populations and activity under

exposure to environmental stressors and pollution can induce adverse changes in biotopes and pose a risk for microorganisms, animals and humans.

The administration of commercial fish feed significantly increased the populations of all bacterial groups in water samples collected directly from fish rearing tanks and from water flowing out of fish tanks relative to purified water flowing into fish tanks. The counts of *Ps. fluorescens* and *A. hydrophila* bacteria in a European grayling (*Thymallus thymallus* L.) farm were significantly higher in analyses conducted with the use of a culture-independent method than a culture-dependent method. The acquired data support reliable evaluations of bacterial contamination in RAS water, which is an important consideration in ensuring microbiological safety, maintaining fish welfare and monitoring the farming process. The results of this study indicate that culture-dependent and culture-independent methods should be applied simultaneously to evaluate the bacteriological stability of aquacultures to minimize the risk of underestimating microbiological contamination levels. These findings can be used to control the growth of potentially pathogenic bacterial species and their transfer between natural environments and aquatic organisms. Our results are particularly valuable for aquacultures that help to replenish wild stocks and rebuild populations of threatened species in natural aquatic environments, which was the case in the present experiment. Potentially pathogenic microorganisms, such as *Ps. fluorescens* and *A. hydrophila*, can spread from aquacultures and contaminate the natural environment with non-specific microbiota. These bacteria are evacuated from various aquaculture systems in high concentrations in water. Furthermore, most of them can be potentially pathogenic for fish and humans as they are characterized by high levels of cross-resistance to different antibiotics. These allochthonous microorganisms can lead to changes in the populations of autochthonous bacteria in aquatic ecosystems and disrupt natural processes induced by indigenous microbial communities.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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