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Original Research

Nitrogen Cycle Bacteria in the Waters of the River Drwęca

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Abstract

In 2004-05 counts of the bacteria proteolytic, ammonifying, AOB and NOB, NO_3-N to NO_2-N reducing, denitrifying, and atmospheric nitrogen fixing (*Azotobacter* sp. and *Clostridium pasteurianum*) were analyzed along with temperature, pH, dissolved oxygen saturation, ammonia, nitrite and nitrate nitrogen in waters of the River Drwęca. The counts of the above groups of bacteria ranged within a few orders of magnitude depending on the physiological group and sampling site. The smallest were the counts of autotrophic nitrifying and atmospheric nitrogen fixing bacteria *Azotobacter* sp. (10^2 cfu·1 cm^{-3}) and *Clostridium pasteurianum* (10^2 MPN·100 cm^{-3}). The most numerous were ammonifying bacteria (10^3 – 10^6 MPN·100 cm^{-3}). The quantitative occurrence of the bacteria in question varied in relation to the physical and chemical parameters of the water, which was evidenced by Spearman's statistical analysis.

Keywords: river, water, nitrogen cycle bacteria, temperature, pH, oxygen saturation, ammonia nitrogen, nitrite nitrogen, nitrate nitrogen

Introduction

A river is a system comprising both the main course and the tributaries, carrying in one-way flow a significant load of matter in dissolved and particulate phases from both natural and anthropogenic sources. This matter moves downstream and is subject to intensive chemical and biological transformations [1]. Nitrogen cycle processes are particularly important as the amounts and character of nitrogen compounds condition water productivity. In addition, the nitrogen uptake (by plants) and the resulting effects on aquatic organisms are different from one nitrogen species to another [2]. The rate of ammonification and nitrification play a key role in the nitrogen cycle by making nitrogen available for plants and microbes, and by making nitrogen susceptible to leaching and denitrification losses [1-4]. N_2 gas leakage out of the various natural environments (e.g. lakes, rivers, soil, sediments) can limit biological nitrogen fixation. It is the biochemical process that is confined to numbers of specific groups of prokaryotes. It plays an indispensable role in the global nitrogen cycle by providing fixed nitrogen. *Azotobacter* species, belonging to diazotrophs, performing this function are broadly dispersed in virtually all ecosystems [5, 6]. Assemblages of bacteria which participate in nitrogen metabolism are physiologically diverse but together they make up the natural microflo-

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ra of surface waters [7, 8]. Transformations of nitrogen compounds are widespread in the natural environment. The actual course, intensity as well as the final products of those processes depend on the surrounding physical and chemical conditions [7], which changed under the influence of various human activities (such as fertilization, cultivation, aquaculturing, deforestation, etc.). The anthropogenic disturbances of aquatic environments self-regulation ability may result in chemical and/or microbiological pollutions of reservoirs on a regional to global scale [9]. This is especially important in water reservoirs like the River Drwęca, which constitute water reserves or which are used as a source of drinking or production water. Given the importance of nitrogen cycle bacteria in the self-purifying process of waters, the aim of the present research has been to determine changes in numbers of proteolytic, ammonifying, AOB, NOB, NO_3-N to NO_2-N reducing, denitrifying, atmospheric nitrogen fixing (*Azotobacter* sp. and *Clostridium pasteurianum*) bacteria, their mutual correlations in the quantitative occurrences, as well as to establish any relationship between the occurrence of those bacteria and water temperature, pH, dissolved oxygen saturation, ammonia, nitrite and nitrate nitrogen in the River Drwęca in 2004-05.

Material and Methods

Study Area

The River Drwęca, which is a right tributary of the Vistula River, flows through a lake district. The river is 207.2 km long and drains a catchment basin of 5343.5 km^2 in surface area. The section of the river flowing within the boundaries of the Province of Warmia and Mazury is about 95 km long [10]. In its upper course the river flows through a small lake known as Ostrowin and a typical ribbon lake called Drwęckie [11] (Fig. 1).

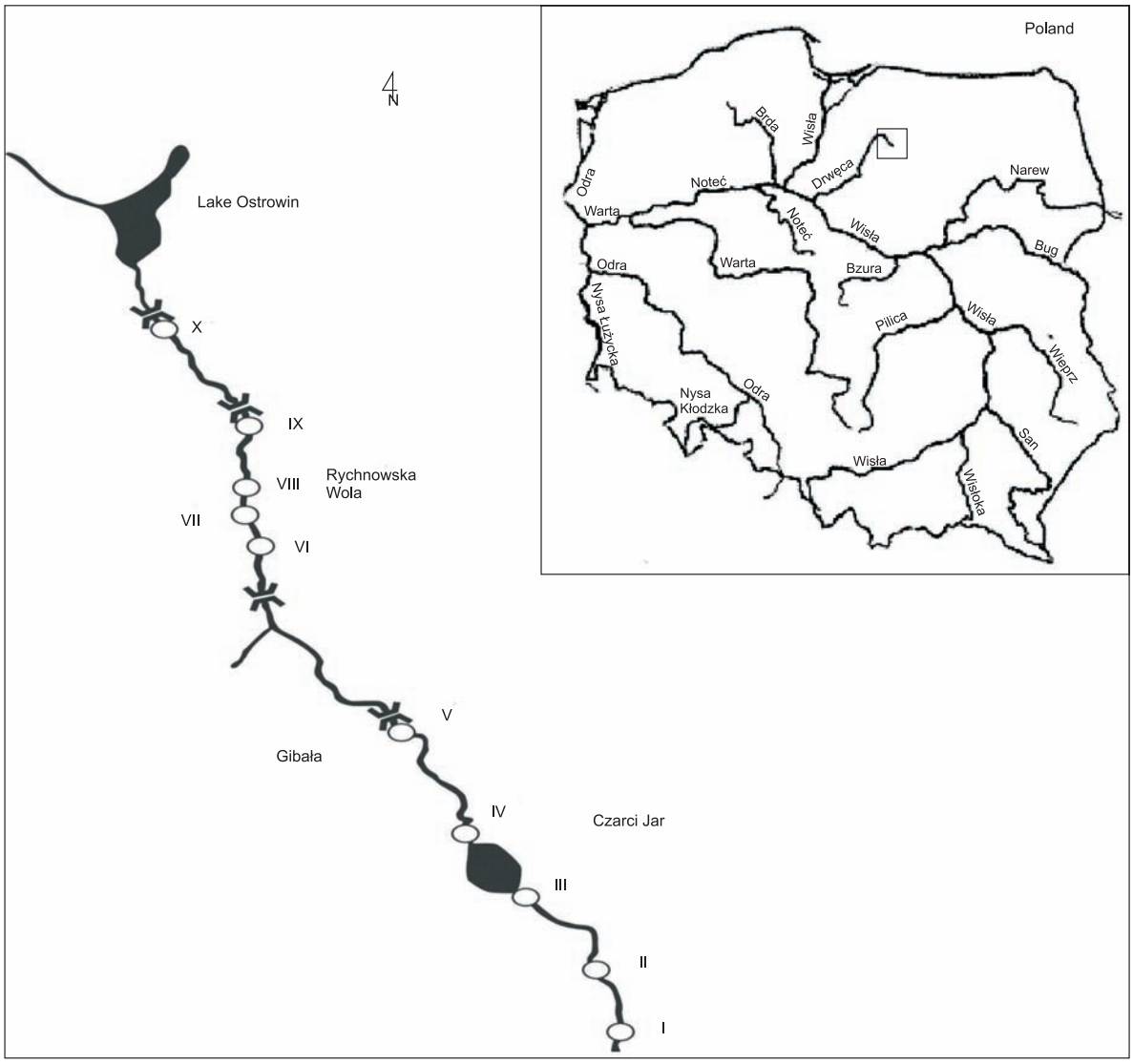

Fig. 1. Map of Poland showing the studied River Drwęca. Location sketch of sampling sites (I, II…X) in River Drwęca.

In 1961 the whole length of the River Drwęca was turned into a nature reserve. This aquatic nature reserve covers 1888.4 ha from the river sources to its outflow to the Vistula. The reserve, called the "River Drwęca Nature Reserve", was established to protect the river's water habitats as well as the fish living in the Drwęca such as trout, salmon, brown trout and vimba. The River Drwęca Nature Reserve is the longest ichthyological reserve in Poland, comprising 444.38 ha of protected area. Owing to large differences in elevation between the Drwęca and its tributaries, at several sections the river appears submontane in character. This favours the occurrence of rare fish and lamprey species that prefer waters high in oxygen saturation [12-14].

In its upper course, the valley of the River Drwęca forms a gorge 20-30 m deep and 8 km long. Known as Czarci Jar (Devil's Gorge), the gorge comprises a Polish Angling Association fish hatchery.

The major sources of point pollution reaching the Drwęca include household and industrial sewage and wastewater, as well as post-production water from two fish farms (in the villages of Czarci Jar and Rychnowska Wola). Besides, pollutants are also carried by the tributary rivers: the Gizela, Iławka, Sandela and Wel, which collect wastewater from the villages and towns of Bałcyny, Iława, Lubawa and Lidzbark Welski [10].

Sampling Sites

The microbiological assays covered a 15-km long section of the upper course of the Drwęca. Water samples were collected at 10 sampling sites designated in certain characteristic places along the river from its sources to the outflow into Lake Ostrowin (Fig. 1).

- site I – 2 km away from the river sources, as the control station (the least of all the sampling stations exposed to contamination);
- site II – before fish farm No. 1 (which produced 6 tons of trout fry in 2004 and 4 tons in 2005) located the village of Czarci Jar;
- site III – the outflow from the 'trout section' of the fish farm no. 1 in Czarci Jar;
- site IV – the outflow from the ground fish farming ponds at the fish farm No. 1 in Czarci Jar;
- site V – 2 km away from fish farm No. 1, in the village of Gibała;
- site VI – in Rychnowska Wola, before fish farms Nos. 2 and 3;
- site VII – the outflow from fish farm No. 2 (which produced 50 tons of commercial trout in 2004 and 54 tons of the same fish in 2005) located in Rychnowska Wola;
- site VIII – the outflow from fish farm No. 3 (which produced 25 tons of commercial trout in 2004 and 40 tons of the same fish in 2005) located in Rychnowska Wola;
- site IX – 2.5 km from fish farms Nos. 2 and 3 in Rychnowska Wola;
- site X – the bridge on the river before its outflow into Lake Ostrowin.

Sampling

Water samples were taken from the River Drwęca at 0.3-0.5 m depth every 6 weeks from January 2004 to December 2005. Water was collected directly into sterile bottles according to the Standard Methods [15] and Polish Standards [16]. The samples were transported to a laboratory for microbiological assays. The time which elapsed between each sampling event and assays never exceeded 6 hours.

In 2004-05, 30 samples of water were collected and analyzed per station. Generally, the microbiological and physico-chemical tests were performed on 300 samples of water from the River Drwęca.

Microbiological Studies

Microbiological tests were performed on the river water for:

- number of proteolytic bacteria (cfu·1 cm^{-3}) on Frazier gelatine medium [17] after 6 days of incubation at 20°C [18];
- number of ammonifying bacteria (MPN·100 cm^{-3}) on broth medium with 3% peptone addition (pH 7.2) after 7 days of incubation at 26°C [19];
- number of NH_4-oxidizers bacteria (*Nitrosomonas*) – as AOB (MPN·100 cm^{-3}) on Meiklejohn medium with the addition of $(NH_4)_2SO_4$ (pH 7.8) after 14 days of incubation at 28°C, and a number of NO_2^-oxidizer bacteria (*Nitrobacter*) – as NOB (MPN·100 cm^{-3}) on the same medium but with the addition of $NaNO_2$ (pH 7.7) after 21 days of incubation at 28°C [20];
- number of bacteria reducing NO_3-N to NO_2-N (MPN·100 cm^{-3}) on Giltay medium with Durham test tubes [17], at pH 7.0 after 7 days of incubation at 25°C, and a number of denitrifying bacteria (MPN·100 cm^{-3}) on the same medium with Durham test tubes after 14 days of incubation at 25°C [17];
- number of bacteria *Azotobacter* sp. fixing atmospheric nitrogen under aerobic conditions (cfu·1 cm^{-3}) on Fiodorow's medium with 2% mannitol after 7 days of incubation at 25°C [17];
- number of bacteria *Clostridium pasteurianum* fixing atmospheric nitrogen under anaerobic conditions (MPN·100 cm^{-3}) on Winogradsky's medium after 7 days of incubation at 25°C [17];

The composition of media used in the microbiological analyses has been described in Table 1.

Tests of proteolytic bacteria were carried out with the flooded dishes technique. For quantitative identification of proteolytic bacteria after 6 days of incubation, dishes with a gelatine agar medium were flooded with a Frazier solution [17]. After 15–20 minutes of incubation at 20°C, colonies surrounded with transparent spheres were counted [18].

In order to determine the MPN·100 cm^{-3} of ammonifying bacteria their growth, reflected in water turbidity,

Table 1. The composition of media and reagents used in the microbiological analyses of nitrogen bacteria in the waters of River Drwęca.

Groups of microorganisms	Composition of media used to enumerate bacteria
Proteolytic bacteria	Medium: in 1000 cm^3 of distilled water dissolve: NaCl – 3 g, K_2HPO_4 – 1.5 g, KH_2PO 0.5 g, gelatin – 4.0 g, decstrose – 0.05 g, peptone – 0.1 g, beef extract – 5 cm^3, agar – 15 g and pH 7.0; Frazier's reagent (mercuric chloride): in 100 cm^3distilled water dissolve: $HgCl_2$ – 15 g, HCl – 20 cm^3
Ammonifying bacteria	Medium: in 1000 cm^3 of municipal water dissolve: peptone – 30 g, K_2HPO_4 – 1 g, KH_2PO_4 – 1 g, $MgSO_4 \cdot 7\ H_2O$ – 0.5g, NaCl – 0.001 g and pH 7.2; Nessler's reagent (K_2HgJ_4): dissolve 100 g of mercuric iodide and 70 g of potassium iodide in a small amount of water. Add this mixture slowly, with stirring, to a cooled solution of 160 g of NaOH in 500 cm^3 of water. Dilute the mixture to 1000 cm^3.
AOB	Medium: in 1000 cm^3 of distilled water dissolve: $(NH_4)_2SO_4$ – 0.66 g, NaCl – 0.3 g, KH_2PO_4 – 0.1 g, $MgSO_4 \cdot 7\ H_2O$ – 0.14g, $FeSO_4 \cdot 7\ H_2O$ – 0.03g, solution of microelements (in 1000 cm^3 of distilled water dissolve about 0.001 g of each compounds: Li_2SO_4, $CuSO_4$, $ZnSO_4$, H_3PO_4, $Al_2(SO_4)_3$, $SnCl_2$, $MnCl_2$, $NiCl_2$, $CoSO_4$, $TiCl_4$, KJ, KBr) – 1 cm^3, Ca CO_3 – 10g and pH 7.8; Griess dry reagent to detection of ammonia: 1 g α-naphthylamine, 10 g sulphuric acid and 89 g tartaric acid are mixed and ground in a mortar to a fine powder. This is kept in a dark glass bottle with a ground glass stopper.
NOB	Medium: in 1000 cm^3 of distilled water dissolve: $NaNO_2$ – 0.66 g, NaCl – 0.3 g, KH_2PO_4 – 0.1 g, $MgSO_4 \cdot 7\ H_2O$ – 0.14g, $FeSO_4 \cdot 7\ H_2O$ – 0.03g, solution of microelements (in 1000 cm^3 of distilled water dissolve about 0.001 g of each compounds: Li_2SO_4, $CuSO_4$, $ZnSO_4$, H_3PO_4, $Al_2(SO_4)_3$, $SnCl_2$, $MnCl_2$, $NiCl_2$, $CoSO_4$, $TiCl_4$, KJ, KBr) – 1 cm^3, Ca CO_3 – 10 g and pH 7.7; to detection of nitrate the 0.017% solution of diphenylamine in H_2SO_4
Bacteria reducing NO_3-N to NO_2-N	Medium: in 1000 cm^3 of distilled water dissolve: asparagine – 0.5 g, glucose – 10 g, KNO_3 – 2 g, KH_2PO_4 – 2 g, $MgSO_4 \cdot 7\ H_2O$ – 2 g, $CaCl_2 \cdot 6\ H_2O$ – 0.2 g, $FeCl_2 \cdot 4\ H_2O$ – 0.001 g, bromothymol blue (of 1.5% solution in ethanol) – 5 cm^3 and pH 7.0
Denitryfying bacteria	Medium: in 1000 cm^3 of distilled water dissolve: asparagine – 0.5 g, glucose – 10 g, KNO_3 – 2 g, KH_2PO_4 – 2 g, $MgSO_4 \cdot 7\ H_2O$ – 2 g, $CaCl_2 \cdot 6\ H_2O$ – 0.2 g, $FeCl_2 \cdot 4\ H_2O$ – 0.001 g, bromothymol blue (of 1.5% solution in ethanol) – 5 cm^3 and pH 7.0
Azotobacter sp.	Medium: in 1000 cm^3 of distilled water dissolve: mannitol – 20 g, NaCl – 0.5 g, $FeCl_3 \cdot 6\ H_2O$ – 0.1 g,K_2HPO_4 – 0.3 g, $CaHPO_4$ – 0.2 g, $MgSO_4 \cdot 7\ H_2O$ – 0.3 g, K_2SO_4 – 0.2 g, Ca CO_3 – 5 g, solution of microelements (in 1000 cm^3 of distilled water dissolve: H_3BO_3 – 5 g, $(NH_4)_2MoO_4$ – 5 g, KJ – 0.5 g, NaBr – 0.5 g, $ZnSO_4 \cdot 7\ H_2O$ – 0.2 g, $Al_2(SO_4)_3 \cdot 18\ H_2O$ – 0.3 g) – 1 cm^3 and pH 7.4.
Clostridium pasteurianum	Medium: in 1000 cm^3 of distilled water dissolve: glucose – 20 g, K_2HPO_4 – 1 g, $MgSO_4 \cdot 7\ H_2O$ – 0.5 g, NaCl – 0.001 g, $MnSO_4 \cdot 5\ H_2O$ – 0.001 g, $FeSO_4 \cdot 7\ H_2O$ – 0.001 g, Ca CO_3 – 40 g, and pH 7.8;

surface coating or bottom deposit, was observed in test tubes with a medium. The reaction of the medium was measured and the presence of ammonia was tested with Nessler's reagent. Positive samples were the ones with observable growth of bacteria, presence of ammonia and alkaline reaction to *ca* 8-9 pH, which indicated that ammonification processes had taken place [19].

In the case of nitrifying bacteria after incubation, the cultures were identified for the presence of NO_2-N (AOB) or NO_3-N (NOB). The NO_2-N was identified with the use of a Griess dry reagent [17], while NO_3-N with the use of diphenylamine in concentrated H_2SO_4 followed by decomposition of the remaining un-oxidized NO_2-N in the medium with the use of carbamide and sulfuric acid. The samples where NO_2-N or NO_3-N was present were additionally assayed microscopically for the presence of respective nitrifying bacteria.

In the case of bacteria-reducing NO_3-N to NO_2-N, as well as denitrifying bacteria-reducing NO_2-N to N_2O or N_2, the samples which changed their colour from blue-green into green-yellow, blue or yellow were assayed for: the presence of nitrites – with the use of a Griess dry reagent [17], the lack of ammonia – with the use of Nessler reagent, the lack of nitrates – with the use of diphenylamine in concentrated sulfuric acid [20], and the presence or lack of gas in Durham test tubes.

Characteristic large cream and white colonies of bacteria were assumed to be *Azotobacter* sp. bacteria fixing nitrogen under aerobic conditions [17].

Regarding *Clostridium pasteurianum* bacteria, which fix nitrogen under anaerobic conditions, samples containing turbid substrata, smelling of butyric acid and containing gas in Durham's tubes, were assumed to be the positive ones [17].

The results obtained for proteolytic and atmospheric nitrogen bacteria *Azotobacter* sp. under aerobic conditions were recalculated and reported in colony forming units (cfu) per 1 cm^3 of water according to the methodology described by Alef and Nannipieri [21] and Rodina [17]. The most probable number (MPN): of ammonifying bacteria, bacteria of AOB and NOB, bacteria reducing NO_3-N to NO_2-N, denitrifying bacteria and atmospheric nitrogen bacteria *Clostridium pasteurianum* under anaerobic conditions was determined with the method of increasing 10-fold dilutions in three parallel repetitions of the same water sample. The MPN results (converted into 100 cm^3 of water) of these bacteria were drawn from McCrady's tables [22].

Physico-Chemical Tests

In the experimental period, the river water was additionally subjected to physico-chemical determinations of the following parameters: temperature, pH, oxygen saturation (mg $O_2 \cdot dm^{-3}$), ammonia nitrogen (mg NH_4-N$\cdot dm^{-3}$), nitrite nitrogen (mg NO_2-N$\cdot dm^{-3}$), nitrate nitrogen (mg NO_3-N$\cdot dm^{-3}$). All microbiological and physico-chemical determinations were carried out on the same (common) water samples. The to physico-chemical determinations of temperature, pH, and oxygen saturation (mg $O_2 \cdot dm^{-3}$), were used by multimeasurement aparatus Hydrolab Multi 12 (Schott) with the precision of measurements: ± 0.1°C, ± 0.01 pH, ± 0.01 mg $O_2 \cdot dm^{-3}$.

However, determinations of ammonia nitrogen (NH_4-N), nitrite nitrogen (NO_2-N), and nitrate nitrogen (NO_3-N) were performed following the recommendations of Hermanowicz et al. [23] and the obtained results were calculated and quoted in the units commonly adopted in hydrochemistry: $mg \cdot dm^{-3}$.

Statistical Evaluation

The results of microbiological and physico-chemical tests were analyzed statistically. In order to estimate the significance of the differences in the counts of all the physiological groups of bacteria active in nitrogen cycling compounds in the water of the River Drwęca and the sampling sites, a single factor analysis of variance (ANOVA) was conducted, verifying the hypothesis of the equality of means (H_0: $x_1 = x_2 = \ldots = x_n$) at the level of significance $\alpha = 0.05$, assuming that the variance for the counts of the bacteria groups under study are uniform. The uniformity of variance was tested using Levene's test. If the test results proved significant, the hypothesis was rejected. Next, the Kruskal-Wallis test was applied, which is a non-parametric equivalent of the analysis of variance [24]. The correlations between the numbers of the all analyzed physiological groups of nitrogen cycle bacteria and water temperature, pH, oxygen saturation (mg $O_2 \cdot dm^{-3}$), ammonia nitrogen (mg NH_4-N$\cdot dm^{-3}$), nitrite nitrogen (mg NO_2-N$\cdot dm^{-3}$), and nitrate nitrogen (mg NO_3-N$\cdot dm^{-3}$) were realized by the non-parametric Spearman's rank correlation test [24]. The method allowed determination of nonlinear dependencies between dependent and independent variables. In order to determine correlations between the groups of nitrogen cycle bacteria under study, counts of the particular groups of microorganisms were adopted as dependent and independent variables. In the analysis of the relationship between the bacteria under study and the physico-chemical parameters of the waters of the River Drwęca, numbers of the assayed groups of microorganisms were adopted as the dependent variables. The independent variables, however, were the values of the physico-chemical parameters. The Spearman's rank correlation coefficient in both statistical analyses was calculated with the use of the STATISTICA PL 7.0 computer software.

Results

Microbiological Studies

The mean numbers, standard errors and relationship between the numbers of all the physiological groups of bacteria active in nitrogen cycling compounds and sampling sites collected from the River Drwęca in 2004-05 are contained in Table 2.

The means of the quantitative occurrence of the bacteria varied within a few orders of magnitude depending on the physiological group and sampling site.

The mean numbers of proteolytic bacteria varied from 201 cfu$\cdot$1 cm^{-3} at site III to 3,325 cfu$\cdot$1 cm^{-3} at site VIII. The quantitative differences in bacteria counts between the particular sampling sites were confirmed by the Kruskal-Wallis statistical test (level of significance α=0.05).

The smallest mean numbers of ammonifying bacteria were observed at site VI, where they reached 128,800 MPN$\cdot$100 cm^{-3} of water. The highest numbers of those bacteria were noticed at sites VII and VIII, where they went up to 266,600 and 265,300 MPN$\cdot$100 cm^{-3}, respectively. Their counts in all the sampling sites were usually similar and stood at 10^6 MPN$\cdot$100 cm^{-3}. This was confirmed by a statistical analysis which did not show significant differences in the counts of these bacteria between the sampling sites.

The smallest mean counts of AOB in the water samples from the River Drwęca were detected at sites I and III, where their nominal values were 15 and 14 MPN$\cdot$100 cm^{-3}. The highest mean counts of those bacteria were found at sites VII and VIII, where they reached 107 and 103 MPN$\cdot$100 cm^{-3}, respectively. The means of NOB in the River Drwęca waters were several fold lower than AOB at the analogous sampling sites. The quantitative differences in the counts of AOB and NOB between the particular sampling sites were statistically significant and respectively stood at: 0.001 and 0.000.

In 2004-05 the minimum mean numbers of bacteria-reducing NO_3-N to NO_2-N (12,264 MPN$\cdot$100 cm^{-3}) were noticed at site IV, whereas the maximum ones (86,528 MPN$\cdot$100 cm^{-3}) occurred at site II. No statistically significant quantitative differences in the presence of the bacteria were determined between the particular sampling sites.

The differences in the mean numbers of the denitrifying bacteria in the water samples drawn from the River Drwęca varied within a few orders of magnitude, depending on the sampling site. The lowest mean numbers of denitrifying bacteria, i.e. 429 MPN$\cdot$100 cm^{-3}, were observed at site V. On the other hand, the highest mean numbers of the denitrifying bacteria were noticed at two sampling sites (III and X) where they equalled 9,426 and 9,768 MPN$\cdot$100 cm^{-3} of water. The quantitative differences in bacteria counts between the particular sampling

Table 2. The mean numbers, standard errors and relationship between of the numbers of the physiological groups of bacteria active in nitrogen cycling compounds and sampling sites based on the Kruskal-Wallis test assayed in samples of water of the River Drwęca.

Sampling sites (N)	Groups of microorganisms							
	Proteolytic bacteria (cfu·1 cm^{-3})	Ammonifying bacteria	AOB	NOB	Bacteria reducing NO_3-N to NO_2-N	Denitryfying bacteria	Bacteria fixing atmospheric nitrogen	
		(MPN·100 cm^{-3})					*Azotobacter* sp. (cfu·1 cm^{-3})	*Clostridium pasteurianum* (MPN·100 cm^{-3})
I (30)	240[1] ±2128.173[2]	141 800 ±334.897	15 ±4.524	2 ±1.416	20 051 ±6342.399	6 805 ±5346.787	52 ±9.006	6 ±3611.095
II (30)	297 ±58.070	256 700 ±880.150	23 ±5.593	3 ±1.383	86 528 ±40198.770	3 166 ±40434.650	95 ±18.178	3 ±7935.889
III (30)	201 ±29.841	224 800 ±29.840	14 ±4.490	3 ±1.674	14 829 ±5160.788	9 426 ±5159.349	55 ±10.887	8 ±4968.717
IV (30)	267 ±34.755	154 000 ±34.755	29 ±9.045	7 ±3.513	12 264 ±5127.387	3 292 ±1881.805	61 ±14.508	11 ±1599.309
V (30)	317 ±62.159	168 700 ±1199.03	89 ±38.230	6 ±8.676	21 835 ±6339.510	429 ±1834.652	68 ±11.345	9 ±356.7692
VI (30)	637 ±203.068	128 800 ±449.314	24 ±48.644	6 ±13.325	13 599 ±5167.407	3 055 ±2050.162	71 ±14.781	15 ±1609.771
VII (30)	771 ±144.938	266 600 ±395.083	107 ±38.370	21 ±8.350	20 551 ±6363.990	7 340 ±4236.756	67 ±15.849	5 ±3911.350
VIII (30)	3 325 ±130.548	265 300 ±434.387	103 ±5.625	22 ±4.672	15 950 ±11848.940	3 131 ±2945.425	84 ±19.903	3 ±1651.513
IX (30)	675 ±1831.478	142 400 ±1441.155	37 ±9.718	15 ±12.687	31 095 ±5306.511	7 481 ±4133.077	60 ±24.550	13 ±3904.457
X (30)	587 ±159.343	138 700 ±291.750	53 ±12.156	8 ±9.259	13 337 ±5128.250	9 768 ±5118.715	105 ±28.438	19 ±4959.737
Kruskal –Wallis Test								
Probability (p)	**0.004***	**0.066**	**0.001***	**0.000***	**0.969**	**0.003***	**0.867**	**0.000***

(N) –Number of samples, [1] – mean number, [2] – *standard error,* ***-** statistically significant differences (at α = 0.005)

sites were confirmed by the Kruskal-Wallis statistical test (level of significance α=0.05).

In the water samples taken from the River Drwęca the lowest mean numbers of *Azotobacter* sp. bacteria fixing nitrogen under aerobic conditions were determined at sites I and III (52 and 55 cfu·1 cm^{-3}, respectively). The highest mean numbers of those bacteria occurred at site X (105 cfu·1 cm^{-3}). No statistically significant quantitative differences in the presence of the bacteria were determined between the particular sampling sites.

In the years 2004-05 the water samples collected from the River Drwęca contained only a few MPN·100 cm^{-3} of *Clostridium pasteurianum* bacteria fixing nitrogen under anaerobic conditions. The lowest mean numbers of those bacteria (not exceeding 3 MPN·100 cm^{-3}) occurred at sites II and VII, whereas the highest mean numbers reaching 15 and 19 MPN·100 cm^{-3} were observed at sites VI and X, respectively. The quantitative differences in the counts of AOB and NOB between the particular sampling sites were statistically significant and respectively stood at 0.000.

Physico-chemical Tests

The ranges of the physicochemical parameters (temperature, pH, oxygen, ammonia nitrogen, nitrite nitrogen and nitrate nitrogen) measured in the waters of the River Drwęca in 2004-05 are presented in Table 3. Their values changed within a few orders of magnitude, depending on the sampling site and parameter.

The temperature of the water varied from 0°C at site I to 20.8°C at site IV. The smallest differences in this parameter were noticed at site II (1.0–11.9°C) and the largest ones occurred at site IV (2.5–20.8°C). Nonetheless, in the whole period of the experiment, the value of this index did not exceed the border value of 22°C recommended for water purity class I [25].

The value of water reaction (pH) measured for the water samples taken from the River Drwęca varied from 7.14 (site X) to 8.35 (site II), never exceeding the range of 6.5 to 8.5 set for purity class I waters [25]. The smallest differences in the water reaction value (7.30–7.71) were observed at site IX and the largest ones appeared at sites

Table 3. The ranges of same physico-chemical parameters of the waters of River Drwęca.

Sampling sites	Psysico-chemical parameters					
	Temperature (°C)	pH	Oxygen (mg O_2·dm^{-3})	Ammonia nitrogen (mg NH_4-N·dm^{-3})	Nitrite nitrogen (mg NO_2-N·dm^{-3})	Nitrate nitrogen (mg NO_3-N·dm^{-3})
I	0.0 – 11.6	7.50 – 8.10	8.00 – 11.68	0.00 – 0.07	0.000 – 0.017	0.21 – 0.45
II	1.0 – 11.9	7.50 – 8.35	7.40 – 11.06	0.00 – 0.09	0.000 – 0.018	0.16 – 0.42
III	2.4 – 18.3	7.37 – 8.20	6.40 – 12.50	0.00 – 0.14	0.000 – 0.026	0.09 – 0.43
IV	2.5 – 20.8	7.56 – 8.32	8.80 – 12.60	0.01 – 0.13	0.000 – 0.059	0.20 – 0.90
V	3.3 – 15.2	7.50 – 8.10	8.50 – 11.50	0.00 – 0.15	0.000 – 0.058	0.15 – 0.87
VI	3.0 – 14.9	7.65 – 8.10	8.50 – 12.50	0.00 – 0.08	0.000 – 0.036	0.17 – 0.71
VII	3.0 – 14.9	7.20 – 8.04	7.40 – 12.50	0.03 – 0.36	0.000 – 0.040	0.24 – 0.81
VIII	2.9 – 15.4	7.34 – 7.79	5.90 – 13.10	0.03 – 0.27	0.000 – 0.038	0.24 – 0.91
IX	2.9 – 16.1	7.30 – 7.71	4.00 – 10.24	0.02 – 0.33	0.000 – 0.032	0.23 – 0.72
X	3.1 – 16.1	7.14 – 7.90	5.90 – 12.80	0.02 – 0.26	0.000 – 0.062	0.16 – 0.70

II, III and VII (7.50–8.35, 7.37–8.20 and 7.20–8.04, respectively).

The concentrations of oxygen dissolved in water (oxygen saturation) in the River Drwęca ranged from 4.00 to 13.10 mg O_2·dm^{-3} at sites IX (the lowest value) and site VIII (the highest value). The smallest differences in the concentration of this index (8.50–11.50 mg O_2·dm^{-3}) were observed at site V and the largest ones (5.90–13.10 mg O_2·dm^{-3}) at site VIII. During the whole time covered by the experiment water values of oxygen saturation enabled us to classify the water samples as water purity class I (sites I, II, IV, V and VII), class I or II (site III), class I, II or III (sites VIII and IX) and to class I, II, III or IV (site X) [25].

The water samples taken from the River Drwęca contained between 0.00 mg NH_4-N·dm^{-3} at sites I, II, III, V and VI to 0.36 mg NH_4-N·dm^{-3} at site VII. The smallest variation in this parameter (0.00–0.07 mg NH_4-N·dm^{-3}) occurred at site I, whereas the largest difference was noticed at site VII (0.03–0.36 mg NH_4-N·dm^{-3}). According to the norms [25], all the water samples corresponded to water purity class I (<0.5 mg NH_4-N·dm^{-3}) in terms of ammonia nitrogen content.

The concentrations of nitrite nitrogen ranged from 0.000 mg NO_2-N·1dm^{-3} at all the sampling sites to 0.062 mg NO_2-N·dm^{-3} at site X. The minimum differences in the content of this compound (0.000–0.017 mg NO_2-N·dm^{-3}) were noticed at site I. The largest ones (0.000–0.062 mg NO_2-N·dm^{-3}) were determined at site X. During the whole time covered by the experiment the values of nitrite nitrogen concentrations implied that the water samples belonged to water purity class I (sites I, II, III) or to class I or II (the remaining sampling sites) [25].

The concentrations of nitrate nitrogen ranged from 0.09 mg NO_3-N·dm^{-3} at site III to 0.91 mg NO_3-N·dm^{-3} at site VIII. The smallest variation in this parameter (0.21–0.45 mg NO_3-N·dm^{-3}) was recorded at site I, whereas the largest one (0.15 – 0.87 mg NO_3-N·dm^{-3}) occurred at site V. According to the binding norms [25] all the water samples belonged to water purity class I (5 mg NO_3-N·dm^{-3}) in terms of nitrate nitrogen concentration.

Statistical Evaluation

The results of the statistical analysis of the correlation between the numbers of the studied nitrogen cycle bacteria: proteolytic, ammonifying, AOB, NOB, NO_3-N to NO_2-N reducing, denitrifying, atmospheric nitrogen fixing (*Azotobacter* sp. and *Clostridium pasteurianum*) recovered from the water of River Drwęca during the whole time of the study and the values of some physico-chemical compounds (temperature, pH, O_2 saturation, concentrations of ammonia nitrogen, nitrite nitrogen and nitrate nitrogen) in the analyzed water samples are shown in Table 4. Spearman's test proved that there were both positive and negative statistically significant ($p<0.05$) correlations:

- the numbers of proteolytic bacteria with some microorganisms like: ammonifying, AOB, NOB, reducing NO_3-N to NO_2-N and *Azotobacter* sp.;
- the quantitative occurrence of ammonifying bacteria with proteolytic and reducing NO_3-N to NO_2-N microorganisms, NOB and *Azotobacter* sp.;
- the numbers of AOB with some bacteria like: proteolytic, NOB, reducing NO_3-N to NO_2-N, denitrifying and *Azotobacter* sp.;
- the quantitative occurrence of NOB with proteolytic, ammonifying bacteria, AOB and *Azotobacter* sp.;
- the number of bacteria reducing NO_3-N to NO_2-N with proteolytic, ammonifying, AOB, denitryfying bacteria, *Azotobacter* sp. and *Clostridium pasteurianum*;

Table 4. Statistic estimation by Spearman's correlation between the numbers (cfu·1 cm^{-3}, MPN·100 cm^{-3}) of studied nitrogen cycle microorganisms recovered from the water of River Drwęca during whole study time and some physico-chemical compounds in water. BD eliminated in couple.

Groups of bacteria	Groups of bacteria							Physico-chemical parameters					
	Proteolytic	Ammonifying	AOB	NOB	Reducing NO_3-N to NO_2-N	Denitryfying	*Azotobacter* sp.	*Clostridium pasteurianum*	Temperature	pH	Oxygen	NH_4-N	NO_2-N	NO_3-N
Proteolytic	1.000	**0.369***	**0.229***	**0.388***	**– 0.129***	– 0.064	**0.153***	– 0.018	**0.137***	**0.188***	**0.236***	**0.266***	0.028	**0.390***
Ammonifying	**0.369***	1.000	0.089	**0.124***	**– 0.128***	– 0.014	**0.321***	– 0.093	**0.074***	0.019	**0.134***	**0.251***	– 0.089	**0.185***
AOB	**0.229***	0.089	1.000	**0.186***	**– 0.208***	**0.138***	**0.126***	0.042	**0.188***	**0.130***	**0.284***	**0.224***	0.071	**0.409***
NOB	**0.388***	**0.124***	**0.186***	1.000	– 0.088	– 0.045	**0.209***	0.089	**0.264***	**0.182***	**0.156***	**0.208***	**0.103***	**0.329***
Reducing NO_3-N to NO_2-N	**– 0.129***	**– 0.128***	**– 0.208***	– 0.088	1.000	**0.297***	**0.378***	**– 0.116***	**0.295***	– 0.078	**– 0.141***	– 0.003	– 0.062	0.075
Denitryfying	– 0.064	– 0.014	**0.138***	– 0.044	**0.297***	1.000	**– 0.163***	**0.123***	**0.366***	**0.193***	**– 0.340***	**0.012***	**0.145***	**0.295***
Azotobacter sp.	**0.153***	**0.321***	**0.126***	**0.209***	**– 0.378***	**– 0.163***	1.000	0.033	**0.266***	– 0.107	**0.351***	– 0.028	– 0.052	0.089
Clostridium pasteurianum	– 0.018	– 0.093	0.042	0.089	**– 0.116***	**0.123***	0.033	1.000	**– 0.217***	**– 0.221***	**– 0.277***	– 0.018	0.297	0.095

* statistically significant correlations ($p<0.05$)

– the quantitative occurrence of denitrifying microorganisms with AOB, bacteria-reducing NO_3-N to NO_2-N, *Azotobacter* sp. and *Clostridium pasteurianum*;
– the counts of all the assayed groups of microorganisms versus values of temperature and oxygen saturation of the water samples collected from the Drwęca;
– the quantitative occurrence of proteolytic, AOB, NOB, denitrifying and *Clostridium pasteurianum* bacteria versus water pH values;
– the quantities of proteolytic, ammonifying, AOB, NOB, denitrifying bacteria versus concentrations of ammonia and nitrate nitrogen;
– the quantitative occurrence AOB, NOB, versus concentrations of nitrite nitrogen.

Discussion

The counts (means) of the nitrogen cycle bacteria assayed in the waters of the River Drwęca varied within a few orders of magnitude depending on the physiological group of microorganisms and the sampling site. This was confirmed by a statistical analysis which showed, in the majority of cases, the presence of statistically significant differences between bacteria counts and the sampling sites (Kruskal-Wallis test) and mutual positive and negative dependencies between the particular physiological groups. In most samples, the counts of bacteria changed in correlation with the physico-chemical properties of water, which was evidenced by Spearman's correlation ranges. In the years 2004–05 the counts of the assayed physiological groups of microorganisms in the particular sampling sites differed from 2 to 100 times from the counts determined in previous analogous studies of the waters of the River Drwęca [26].

The smallest were the counts of AOB and NOB as well as atmospheric nitrogen fixing *Azotobacter* sp. (aerobic) and *Clostridium pasteurianum* (anaerobic) bacteria. However, the microorganism groups showed similar tendencies (rising or falling) in the same sampling sites. Relatively small numbers of autotrophic nitrifying bacteria (AOB, NOB) found at particular sampling sites could have been conditioned by several biotic and abiotic factors. This was confirmed by a statistical analysis which showed significant correlations not only between the counts of these microorganisms but also their dependency on the majority of the assayed physico-chemical parameters. In the case of the shallow and unshielded sites I, II and III, very small counts of such bacteria in these places may have been caused by the bactericidal effect of sunshine, which is particularly toxic to *Nitrobacter winogradsky* [27]. Besides, low temperatures of the river water recorded at all the sampling sites (0.0–20.8°C) may have considerably reduced counts of *Nitrosomonas* and *Nitrobacter* bacteria, which are considered to be mesophilous organisms. In addition to this, low levels of such chemical parameters, like water-dissolved oxygen saturation (5.90–12.80 mg $O_2 \cdot dm^{-3}$) and concentrations of NO_2-N (0.00–0.062 $NO_2 \cdot dm^{-3}$) did

not encourage the growth of those bacteria [28]. On the other hand, the negative correlation between the numbers of NOB and concentrations of NH_4-N in the waters of the River Drwęca suggests that in excess of 0.1 mg NH_4-N·dm^{-3} may have been toxic to some bacteria, especially to those of the genus *Nitrobacter* [29]. In addition, lower numbers of AOB than NOB could be connected with their ability to produce extracellular polymers, which may help survival in starvation conditions or facilitate nitrification at low pH [30]. Elevated numbers of both groups of the nitrifying bacteria determined in water samples collected at the sites above the fish farms confirm the fact that counts of those bacteria may rise alongside an increased influx of organic matter to a water reservoir [31, 32] or due to some motion of bottom sediments caused by bentos organisms [33, 34]. The counts of AOB and NOB in the waters of the River Drwęca were similar to the ones in the waters of Lake Szeląg Wielki (respectively: 1–400 and 0–10 MPN·100 cm^{-3}) [4] and the oligotrophic Lake Wigry (3–450 and 5–79 MPN ·100 cm^{-3}, respectively) [7] and assayed waters of the Czarna Hańcza River [35].

The counts of atmospheric nitrogen fixing bacteria (under aerobic and anaerobic conditions) determined in the waters of the River Drwęca were in general similar to the quantitative occurrences of AOB and/or NOB at the analogous sampling sites. During the whole period of time covered by our investigations the counts of *Azotobacter* sp. cells did not exceed 500 cfu·1 cm^{-3}, whereas those of *Clostridium pasteurianum* were up to 40 NPL·100 cm^{-3}. They were similar to those found in the samples of the waters of Lake Szeląg Wielki [4] and 10- to 100-fold lower than their counts determined in the mesotrophic Lake Długie Wigierskie [8] or the eutrophic Lake Bartąg [36]. The counts of *Azotobacter* sp. cells being 5- to 10-fold higher than those of *Clostridium pasteurianum* detected in the water samples taken from the River Drwęca might be attributed to the thermal and oxygenation conditions, which were more favourable to aerobic nitrogen fixing bacteria. This was confirmed by statistical analysis, which revealed positive relationships between *Azotobacter* sp. cell counts and the temperature or oxygen saturation of the river water. In contrast, those two physicochemical parameters were negatively correlated with the counts of *Clostridium pasteurianum*. Elevated counts of bacteria fixing atmospheric nitrogen under aerobic and anaerobic conditions sporadically detected during our investigations may have been caused by the penetration of those microorganisms from the surface layers of the river's bottom sediments to water, either stimulated by bentos organisms [34], [8] or else by intensive runoff of surface waters.

The presence of NO_3-N to NO_2-N reducing and denitrifying bacteria in the waters of the River Drwęca ranged within 0–140,000 MPN·100 cm^{-3} and differed 10- to 100-fold from counts of those bacteria in Hańcza Lake [34], Hańcza River [35] and Bartąg Lake [36]. In the authors' own research several-fold differences in the counts of bacteria-reducing NO_3-N to NO_2-N and denitrifying bacteria found at all the sampling sites may have resulted from their different demands regarding physiochemical conditions of the environment and other groups of nitrogen cycle bacteria, which was confirmed by statistical analysis. The minimum and maximum numbers of NO_3-N to NO_2-N reducing and denitrifying bacteria were significantly positively correlated with water temperature and negatively correlated with water-dissolved oxygen concentrations. The lowest amounts of denitrifying bacteria were negatively correlated with the values of water temperature, pH and concentration of dissolved oxygen. Differences in the quantitative presence of both groups of bacteria could also have stemmed from such factors as access to organic matter, torrential rainfall and occurrence of anaerobic microzones due to oxygen depletion caused by respiratory processes of heterotrophic microorganisms [37]. In addition, in the water samples of the River Drwęca the quantitative occurrence of other groups of bacteria (such as proteolytic, ammonifying, AOB and *Clostridium pasteurianum*) could have been depressing of some denitrifying bacteria [3]. It was observed in the numbers of NO_3-N to NO_2-N reducing bacteria, especially the ones which were significantly negatively correlated with above groups of microorganisms. Those factors can largely elevate the amounts of N-NO_3 to N-NO_2 reducing and denitrifying bacteria as well as their increased activity [38].

Microorganisms participating in the decomposition of organic nitrogen compounds were detected in the waters of the River Drwęca in amounts varying from 1 to 10^3 cfu·1 cm^{-3} (proteolytic bacteria) or from 10^3 to 10^6 MPN·100 cm^{-3} (ammonifying bacteria), depending on the sampling site and physicochemical conditions. Their highest counts would typically correspond to higher temperatures, oxygen saturation and ammonia nitrogen values. This tendency was confirmed by statistical analysis, which showed significant positive correlations between the above groups of bacteria and most of the analyzed physicochemical factors. During the whole research, in the same water samples the counts of proteolytic bacteria determined were 5- to 10-fold lower than ammonifying bacteria. The quantitative presence of both groups of microorganisms detected in our own research were similar to the data found in references discussing determinations of proteolytic and ammonifying bacteria in various water reservoirs [7, 8, 34, 35, 36, 39]. The differences in the counts of proteolytic and ammonifying bacteria found between particular sampling sites, and in particular their highest counts detected at sites VII and VIII (outflows from the two fish farms in Rychnowska Wola), may suggest that intensive fish farming has some local and/or seasonal effect on the quantitative and/or qualitative composition of microorganisms involved in mineralization of organic nitrogen compounds [40].

Conclusions

1. Mean numbers of the analyzed groups of bacteria participating in nitrogen metabolism determined in the

water samples collected from the River Drwęca varied within a few orders of magnitude. The variation depended on the physiological group of microorganisms studied and the sampling site. Among the nitrogen cycle bacteria assayed in the waters of the River Drwęca autotrophic nitrifying bacteria as well as aerobic (*Azotobacter* sp.) and anaerobic (*Clostridium pasteurianum*) atmospheric nitrogen-fixing bacteria were found in smaller numbers (0–10^2 cfu·1 cm^{-3} or MPN·100 cm^{-3}), whereas ammonifying bacteria were the most numerous (10^3–10^6 MPN·100 cm^{-3}).

2. The quantitative occurrences of all the determined groups of microorganisms varied depending on the physicochemical properties of water, which was evidenced by statistical analysis.
3. Elevated counts of particular groups of nitrogen cycle microorganisms found in the water samples taken from the River Drwęca at various sampling sites suggest some local and/or seasonal influence of environmental factors, man-made pressure and fish farming on the microbiological status of the river.

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