Pol. J. Environ. Stud. Vol. 23, No. 2 (2014), 467-473

Original Research

Occurrence of F-Specific RNA Coliphages and Microbial Indicators in Municipal Lake Water

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Received: 24 October 2013
Accepted: 29 January 2014

Abstract

Recent studies have shown the increasing interest of F-specific RNA bacteriophages (FRNAPH) genotyping to identify major sources of faecal contamination in waters. The aim of the study was to evaluate the presence and relevance of FRNAPH genotyping in lake water. A comparison between the levels of all FRNAPH and each genogroup and that of the standard indicators of faecal pollution (total coliforms – TC and faecal (thermotolerant) coliforms – FC) was performed. Two faecal indicators were detected in lake water: total coliforms (TC) and faecal coliforms (FC). The standardized method was used to quantify these indicators and bacterial concentrations were expressed in the most probable number per 100 ml of water (MPN/10 ml). FRNAPH were determined by the single agar layer (SAL) method using the host strain *Escherichia coli* Famp (ATCC 700891) in accordance with U.S. EPA Methods 1602. To distinguished between RNA and DNA phages, each FRNAPH isolate was subjected to RNAse sensitivity. All FRNAPH isolates were subjected to the four SYBR Green-based real time PCRs to determine genogroups.

Keywords: aquatic environment, bacteriophages, bacteria, microbial source tracking (MST)

Introduction

Microbiological contamination of lakes, rivers, estuaries, and circumlittoral waters is the main threat to human health, from organisms inhabiting such waters, e.g. fish or crustaceans (being food for humans), through human contact with the environment, e.g. for recreational purposes [1-3]. Each time, upon the appraisal of an aquatic environment – water class – bacterial indicators are used, including generally appraised total coliforms (TC) and faecal coliforms (FC) [2, 3]. However, coliforms as tested do not provide any information as to source [3]. The microbiological assay does not include viral contamination, which results in the lack of very important information on microbial source tracking (MST) [1-7]. It is assumed that determination of microbial source of water contamination plays an important role not only for determining factual threat, but also creates the opportunity of effective control and remediation strategy [1-4, 8-10]. Furthermore, identifying dominant sources of faecal pollution is critical for accurate assessment of public health risks and implementation of best management practices [2].

Recently, many methods have been proposed, which by use of various microorganisms, such as *Escherichia coli*, *Bifidobacterium* sp., *Clostridium perfringens*, faecal streptococci, or *Rhodococcus coprophilus*, would serve to identify and differentiate among the sources of faecal contamination of surface waters [11]. It was also evidenced that F-specific RNA bacteriophages could serve that purpose [1, 2, 9, 11-17]. Such bacteriophages belong to the *Leviviridae* family, which has two types: *Levivirus* and *Allolevivirus*. These include four genogroups I-IV related to different sources of faecal contamination [1, 2, 9, 11-17]. It was evidenced that genogroups II and III were isolated principally

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from human faeces, while genogroups I and IV from animal faeces, hence they can serve to identify the microbial source [1, 2, 9, 11-17].

Due to limited research regarding waters in small municipal lakes up to 10 ha, the purpose of our study was the analysis of water samples originating from Rusałka Lake in the aspect of the number of F-specific RNA bacteriophages, total coliforms and faecal coliforms, as well as the level of contamination caused by such microorganisms, and the assessment of the impact of F-specific RNA coliphages on the number of bacteria indicative of sanitary condition, namely TC and FC. In connection with the above, the main objective of this paper was to estimate the sanitary condition of Rusałka Lake and to test F-specific bacteriophages RNA as indicators of faecal-polluted waters.

Material

The material for the study involved water samples originating from municipal Rusałka Lake (Fig. 1), which is located at the centre of the city of Szczecin, in the valley between the hills of Kasprowicz Park and Dendrological Garden. The surface of the lake amounts to 3.7 ha, with maximum width of 40 m, and depth of 2 m. The lake is of elongated shape, while its horizontal axis is west-east. This is a flow-through lake supplied by the Osówka River, affluent of which is located in the western part of the lake. The river brings in waters from the Osówka River catchment area, including the areas of Lasek Arkoński and the districts of Warszewo, Osowo, and Pogodno. In the eastern part of the lake waters flow out via an underground pipeline across Niecka Niebuszwska and the area of Warski Shipyard to the western Oder. Rusałka Lake used to act as an equalizing reservoir for nowadays non-existing water mill. In the reservoir, on the basis of the land relief and ecological interview, two sampling points were selected, A and B. Point A is located at the tributary to Rusałka Lake, while point B is on the opposite side of the lake, at its outlet. The samples were collected into autoclaved bottles from the surface layer at a depth of about 30 cm below the water surface, from January to December (monthly).

Methods

Assay of Total Coliforms

The assay was performed as previously described [18] using the test tube fermentation method, which relies on using the total coliform's capacity to ferment lactose with generation of acid and gas in the medium. It involves preliminary, confirmation, and supplementary analysis. The final result was read from Mc Cardy's probability charts, and presented in the form of the most probable number (MPN) of total coliforms in 100 ml of analyzed samples.

Assay of Faecal Coliforms

Detection of faecal coliforms was performed as previously described [19] using the test tube fermentation method, which relies on using the bacteria's capacity to ferment lactose with the generation of acid and gas in the

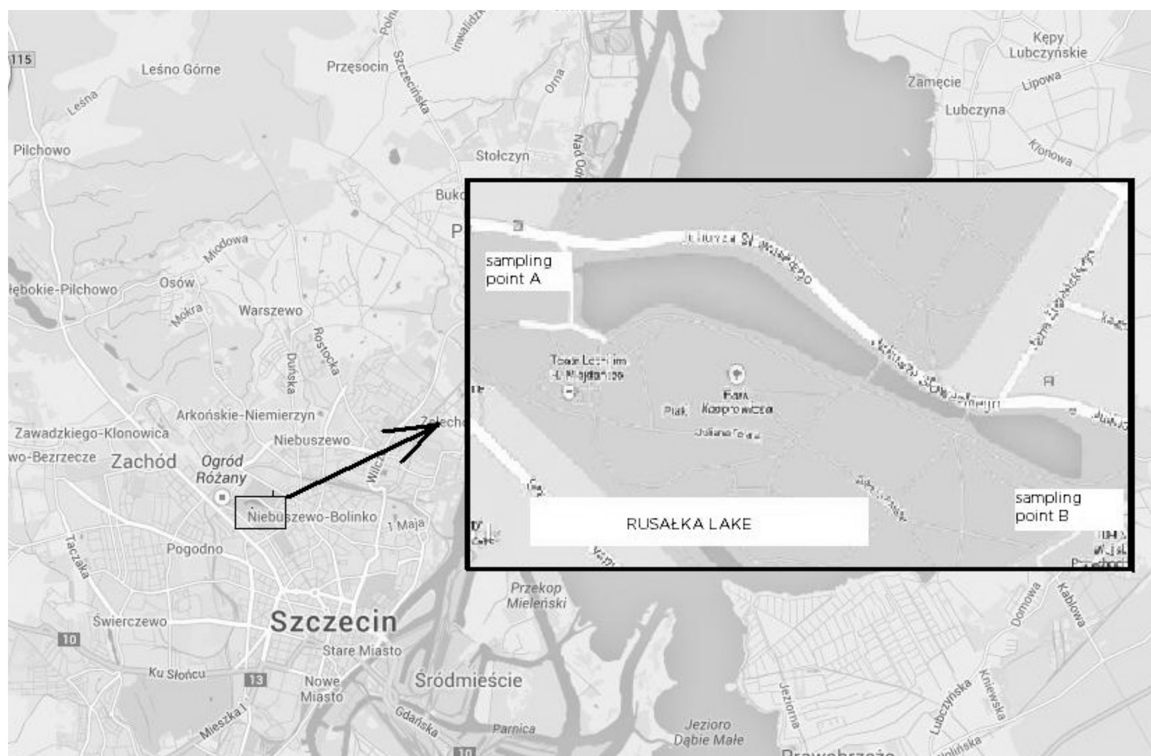

Fig. 1. Sampling sites of Rusałka lake.

Table 1. Sequence of primers used to detect FRNA phages.

Phages	Primers names	Sequence	Length of amplicon (bp)	Tm [˚C]	References
Leviviridae	GGI (forward)	5'-CGTGGTTCCATACTGGAGGT-3'	177kb	62.6	[16]
	GGI (reverse)	5'- CTTTCGAGCACACCCACC-3'		61.1	
	GGII (forward)	5'-GGTTCAAGTTGCGGGATG-3'	184kb	60.2	
	GGII (reverse)	5'-GAAAACAAACCGTTGCCG-3'		59.0	
	GGIII (forward)	5'-CCGCGTGGGGTAAATCC-3'	113kb	62.2	
	GGIII (reverse)	5'-TTACGATTGCGAGAAGGCTG-3'		61.3	
	GGIV(forward)	5'-CGTGGAAGCATGCCTGT-3'	182kb	61.9	
	GGIV (reverse)	5'-TTCCAGCCRGGCTCGAT-3'		64.1	

medium at of 44ºC. The method involves preliminary and confirmation tests. The final result was read from McCardy's probability charts and presented in the form of the most probable number (MPN) of faecal coliforms in 100 ml of analyzed sample.

Assay of F-Specific RNA Bacteriophages

Detection of F-specific RNA bacteriophages (FRNA) was performed as previously described [20] using single agar layer (SAL) method, based on bacteriophage capacity of forming lysis zones (plaques) formed as a result of phage multiplication on the bacterial layer, initiated by infection of a single bacterial cell by the phage. The host for bacteriophages searched for is *Escherichia coli* F_{amp} (ampicillin-resistant) (American Type Culture Collection, ATCC 700891), having sex pili conditioned with the presence of F factor, via which FRNA bacteriophages permeate to the bacterial cell. The result testifying to the fact that the plaque isolated was a result of the presence of RNA bacteriophages was the presence of bacteriophage plaque in the medium without RNAse, and lack of plaque in the medium with the addition of RNAse.

RNAse Sensitivity Test

In order to differentiate between FRNA and FDNA bacteriophages, isolated plagues were subjected to RNAse sensitivity assay. The method involved preparation of bacteriophage lysates and dropping them on slides with TSA medium with the addition of RNAse and without it. The presence of RNA bacteriophages was testified to by the presence of bacteriophage plague in the medium without RNAse, and lack of plague in the medium with the addition of RNAse.

Classification of Isolated F-Specific RNA Bacteriophages into Genotype

Genetic analysis of the FRNA bacteriophages involved isolation of the RNA material from the previously prepared phage suspension, performance of reverse transcription reaction (RT-PCR), and then performance of real-time PCR reaction using Light Cycler 1.5 (Roche Diagnostics, Germany). In the first phase, genetic material of FRNA bacteriophages was isolated. This activity was performed using a ready High Pure Viral RNA Kit by Roche (Roche Diagnostics, Germany), according to manufacturer's recommendations. Next, reverse transcription reaction (RT-PCR) was performed using ready kits for this reaction, RevertAid™ H Minus (Fermentas, Lithuania), according to the procedure recommended by the manufacturer. After performing RT-PCR on the matrix of bacteriophage RNA, complementary strand of nucleic acid (cDNA) was obtained, which was then used as a matrix for amplification of specific fragments of genetic material of F-RNA bacteriophages using the real-time PCR technique. Amplification was performed using the reagent kit LC FastStart DNA Master SYBR Green I (Roche Diagnostics, Germany). Each reaction was performed in the end volume of 20µl containing 1x FastStart DNA Master SYBR Green I, 1 µM of each primer, 5 mM of $MgCl_2$ ions, and matrix with unknown cDNA concentration, which was added in the volume of 2 µl to the reaction mix. The primers (Table 1) were designed in a way to allow for identification of particular genogroups of bacteriophages on the basis of sequences coding in the case of genogroups I, II, and IV RNA replicase gene, while in the case of genogroup III – glycocalyx protein gene (TIB MOLBIOL, Germany). Positive control in the real-time PCR reaction for the *Leviviridae* family used bacteriophage genomes MS2, (ATCC 15597-B1), fr (ATCC15767-B1), and R17 (ATCC25868-B1) for genogroup I; GA (own material confirmed with sequencing) for genogroup II; Qβ (ATCC2363-B1) for genogroup III; and FI and SP (owing to the cordiality of Stephanie D. Friedman, research microbiologist Gulf Ecology Division U.S. EPA) for genogroup IV. The reaction was performed according to the conditions specified after optimization of the reaction in the aspect of concentrations of particular reagents and the time-temperature profile (Table 2).

Table 2. The thermal conditions of real-time PCR during detection of FRNA phages.

Thermal conditions		
Denaturation		95ºC for 10 min
Amplification Program (45 cycles)	Denaturation	95ºC for 10 sec
	Annealing	60ºC for 10 sec
	Elongation	72ºC for 7 sec
Melting	Denaturation	95ºC for 0 sec
	Annealing	65ºC for 15 sec
	Elongation	95ºC for 0 sec
Cooling		40ºC for 30 sec

Statistical Analysis of Bacteriological and Virusological Studies

The results of the studies were subjected to statistical analysis using STATISTICA 9.0 software. Within the statistical analysis, W Shapiro-Wilk test was applied, on which basis it was determined that the parameters analyzed do not adopt normal distribution. Therefore, for correlation calculations, non-parametric statistics were applied, and R Spearman's rank correlation coefficient was calculated. All the calculations were performed with the significance level $p < 0.05$.

Results

The values of studied parameters in samples of water from Rusałka Lake for sampling points A and B in every month are presented in Table 3 and, in the form of average values of annual minimum and maximum values, in Table 4. Results of R. Spearman's rank correlation coefficients for the number of total coliforms, faecal coliforms, and the number of F-specific RNA bacteriophages analyzed in water samples from Rusałka Lake are presented in Table 5.

In the period analyzed, as regards the number of TC, it was recorded that at point A the values of the parameter ranged from MPN 700 (April) cells in 100 ml of water to MPN 2,400,000 (December) cells in 100 ml of water. In turn, at point B, values for TC amounted to from MPN 240 (October) to MPN 24,000 (November, March, April). Average annual values for this parameter at point A amounted to MPN 507,020, while at point B: MPN 10,684 (Tables 3, 4). As regards FC, at point A, the values of MPN <5 (May) to MPN 2,400,000 (December) were recorded, while at point B – from MPN <3 (October) to MPN 24,000 (March). Average annual values for this parameter at point A amounted to MPN 519,280, while at point B: MPN 6,807.5 (Tables 3, 4). When analyzing the values obtained, it must be noticed that at point A, higher values of the parameters analyzed were recorded than at point B, although the level of contamination by such organisms is rather high at both points. As regards determination of the number of F-specific RNA bacteriophages (FRNA), it was recorded that at point A the values of this parameter ranged from 11 PFU/100 ml (June) to 2,840 PFU/100 ml (April), while at

Table 3. Values of studied parameters in samples of water from Rusałka Lake for sampling points A and B.

Month of analysis	Sampling point – A					Samplin point – B				
	TC (MPN/100ml)	FC (MPN/100ml)	FRNA (PFU/100ml)	Air temp. (ºC)	Water temp. (ºC)	TC (MPN/100ml)	FC (MPN/100ml)	FRNA (PFU/100ml)	Air temp. (ºC)	Water temp. (ºC)
I	62,000	62,000	1,022	-5	2	2,400	2,400	980	-5	2
II	70,000	70,000	319	0	1	2,400	130	237	0	2
III	2,400	≥2,400	309	3	2	24,000	24,000	102	3	1.5
IV	700	62,000	2,840	8.5	9	24,000	700	449	8.5	10
V	62,000	<5	25	16	18	6,200	<5	0	11	15
VI	62,000	60,000	11	15	19	2,400	60	0	16	19
VII	62,000	6,200	19	17	18	2,400	620	4	17	17
VIII	62,000	23	42	23	21.5	2,400	23	7	18.1	20
IX	6,200	13,000	130	16	17	6,200	2,400	2	14	16
X	62,000	9	307	10	9	240	<3	72	10	9
XI	620,000	13,000	46	8.5	8.8	24,000	6,200	31	8	8
XII	2,400,000	2,400,000	296	2.5	2.5	620	620	38	4	4

TC – total coliform, FC – faecal coliform, FRNA – F-specific bacteriophages RNA, MPN – most probably number, PFU – plague forming units

Table 4. The values of annual average, minimum, and maximum results of bacteriological and virological parameters, air and water temperatures in samples of water from Rusałka Lake for sampling points A and B.

Sampling point	TC (MPN/100ml)			FC (MPN/100ml)			FRNA (PFU/100ml)			Air temp. (ºC)			Water temp. (ºC)		
	Min.	Max	Mean	Min.	Max	Mean	Min.	Max	Mean	Min.	Max	Mean	Min.	Max	Mean
A	700	2,400,000	507,020	<5	2,400,000	519,280	11	2,840	447.1	-5	23	9.5	1	21.5	10.6
B	240	24,000	10,684	<3	24,000	6,807.5	0	980	160.1	-5	18.1	8.7	1.5	20.1	10.7

TC – total coliform, FC – faecal coliform, FRNA – F-specific bacteriophages RNA, MPN – most probably number, PFU – plague forming units, MIN – minimal value, MAX – maximal vale, mean – mean value

Table 5 Spearman's rank correlation coefficient for TC, FC, air and water temperature and FRNA in water samples from Rusałka Lake.

Parameters / Sampling points	Sampling point – A	Sampling point – B
	FRNA	
TC	-	-
FC	0.59	0.36
Air temp. (ºC)	-	-0.77
Water temp. (ºC)	-	-0.73

TC – total coliform, FC – faecal coliform, FRNA – F-specific bacteriophages RNA

point B the values ranged from 0 PFU/100 ml (May, April) to 980 (January). Average annual values at point A amounted to 447.16, while at point B: 160.16 (Tables 3, 4). When analyzing prevalence of FRNA bacteriophages in the samples from Rusałka Lake, it must be stated that they were actually present in the reservoir analyzed throughout the period of study in particular months. The genetic analysis of occurrence of four genogroups GI, GII, GIII, and GIV of FRNA phages obtained in water samples from Rusałka Lake has allowed to observe 75%, 58%, 54%, and 29%, respectively. One may conclude that there were the fewest FRNA bacteriophages belonging to genogroup IV (29%), while the most FRNA phages from genogroup I (75%). Considering the high percentage of the four genogroups of FRNA phages, it can be concluded that contaminants permeating Rusałka Lake are of human and animal origin.

Discussion of Results

Concerns over water quality have increased in recent years, but it involves only water containers that are the source of drinking water or lakes of over 50 ha in area. Small municipal lakes represent sites that enrich landscapes and provide towns with character and form elements of green fields and parks. Due to their locations, small municipal lakes may also pose a biological and epidemiological threat. Furthermore, those lakes are often receivers of municipal wastewater. In the area of Szczecin city several small water containers are present which are not covered by the environmental monitoring program and there are no reports on their sanitary state. Rusałka Lake is an example of a small reservoir which is situated in a park in the centre of the city of Szczecin, and by its location is a place frequented by the local population. Taking into consideration the above arguments, we decided to define sanitary state of Rusałka Lake by determining number of coliforms (TC and FC) and FRNA coliphages. Analyzing values of bacteriological parameters, one may conclude that Rusałka is highly polluted. Values of TC are similar from January to October and increase on November and December at sampling point A. However, they are different at sampling point B. First of all, values are lower than at sampling point A and increased only on March, April, and November. We didn't notice seasonal changes, which means that pollution is constantly high. As far as FC is concerned, one may conclude that values are differential in every month and each sampling point. When analyzing average annual values for bacteria indicating the sanitary condition, it must be pointed out that in most cases they are higher at sampling point A, which is located in the direct vicinity of Osówka River inlet, as compared to point B, located at the outlet from the lake. Such observation allows for stating that the volume of contaminants reaching Rusałka with waters of the Osówka is significant. The indicator organisms presently used for the monitoring of water environment in developed countries are total coliforms, faecal coliforms, and/or *E. coli*. The use of coliforms as bacterial indicators of microbial water quality is based on the hypothesis that coliforms are present in elevated numbers in faeces of humans and other warm-blooded animals [3]. With a few exceptions, the coliform group of bacteria is not considered to be a health risk, but their presence indicates that faecal pollution may have occurred and pathogens might be present in water environment as a result [3]. However, total coliforms may be considered unreliable indicators of faecal pollution because many of them are capable of growth in both the environment and in drinking-water distribution systems [3].

We aimed to establish the use of FRNA bacteriophages as indicators of faecal pollution and assessment of the original faecal source. The environmental samples from sampling point A that was most likely impacted by faecal pollution yielded higher levels of FRNA phages. Therefore, FRNA phages can be considered as indicators of faecal pollution in the watershed.

Referring to the results on the prevalence of particular genogroups among FRNA bacteriophages in the waters of the lake analyzed, it must be pointed out that the percentage

of particular genogroups in samples analyzed was similar as regards genogroups II and III, the highest number in the samples belonging to bacteriophages from genogroup I, while the lowest number – from genogroup IV. Genogroups II and III are highly associated with humans [14] and their presence confirms the human impact on lake pollution. Genogroups I and IV testify to the impact of animals on pollution [14] and the presence of these genogroups may be related to animals living there, especially with a large number of ducks. The highest number of genogroup I can be associated with their longer survival in lake water. It was shown that the various strains of FRNA phages demonstrated statistically significant differences in survival in source water microcosms and genogroup I survived statistically longer than all the other FRNA phages [21]. The relatively poor survival observed for genogroup IV is consistent with their infrequent isolation from the watershed, and with reported low isolation frequencies [4]. This suggests that genogroup IV may be useful for indicating the presence of recent animal contamination. Similarly to genogroup III, which demonstrated the least variability in survival, the study showed the applicability of genogroup III phages to indicate sporadic inputs of human faecal material [4]. Information on the human or animal origin of faecal pollution is important because it gives an indication of the possible risk of infection and the possible water treatment that may be needed to control the transmission of potential waterborne diseases [15]. FRNA bacteriophages have particularly attractive features as models of human enteric viruses because their physical structure, composition, and morphology closely resemble those many human enteric viruses [2, 15]. In addition, many experiments confirmed that the resistance of FRNA phages to unfavorable conditions and disinfection processes resemble or exceed that of most human enteric viruses [22-24]. In our study, enumeration of phages showed seasonal variation. Winter samples contained the highest concentration of FRNA phages while summer samples had the least or were not present. This may be related to the fact that FRNA bacteriophages are less resistant to sunlight and warmer temperatures [25]. The analysis of Spearman's rank correlation coefficients for the number of FRNA bacteriophages and the number of TC and FC revealed the interdependence between the number of bacteriophages analyzed and the number of FC. The recorded positive average correlation testifies to the impact of bacteriophages on the FC analyzed, and that together with the number of bacteriophages, the number of bacteria increases.

Conclusions

To conclude on the results of the Rusałka Lake waters analysis, the following must be stated:

1. The values of bacteriological parameters (TC, FC) obtained in the study of Rusałka Lake testify to prevailing contamination of the reservoirs.
2. When referring to results of virusological studies (F-specific RNA bacteriophages) in Rusałka Lake waters, it must be stated that their number is high, which confirms the results of bacteriological tests and testifies to the poor sanitary condition of the reservoir. The occurrence of four genotypes within FRNA phages in the analyzed water samples is an indicator differing the sources of water contamination and points to contamination of human origin (genogroup II and III), and contamination of animal origin (genogroup I and IV).
3. The correlation (recorded on the basis of statistical analysis) between the bacteriophages analyzed and the analyzed bacteria is evidence that the bacteriophages affect the abundance of TC and FC, and can regulate their number, which proves the need for marking them in the aquatic environment.
4. It is determined that monitoring water quality in small lakes, although also probably in other aquatic environments, should occur not only through bacteriological indices, e.g.: bacteria indicating sanitary condition, but also by determining bacteriophages specific to them, as they affect bacteria, namely the factor determining the assessment of ecological conditions of waters. Furthermore, assay of the number of such FRNA bacteriophages can also serve to indicate the prevalence of mammal viruses, principally enteroviruses, which react similarly to FRNA bacteriophages to environmental factors, which can be of additional importance to determining water cleanliness [2, 15, 17, 26, 27] .

Acknowledgements

The paper was supported by research grant NCS N304 018540.

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