MIDDLE POMERANIAN SCIENTIFIC SOCIETY OF THE ENVIRONMENT PROTECTION
ŚRODKOWO-POMORSKIE TOWARZYSTWO NAUKOWE OCHRONY ŚRODOWISKA

Annual Set The Environment Protection
Rocznik Ochrona Środowiska

Volume/Tom 20. Year/Rok 2018 ISSN 1506-218X 309-325

Occurrence and Survivability of *Escherichia coli* and Enterococci in Waters Used as Bathing Areas

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1. Introduction

Waters used as bathing areas are an important element of summer recreation. During the holiday season, they attract tourists for recreation purposes. However, these waters may become contaminated, and therefore pose a serious health risk to people who use them. They are also one of the most dynamically changing natural environments, due to the physical and biological factors affecting them (Kubera & Małecka-Adamowicz 2017). The source of microorganisms in recreational waters are improperly processed sewage, agricultural run-off, rainwater, human and animal excrements (Byappanahalli et al. 2003, Imamura et al. 2011) as well as boats, plant residues, contaminated groundwater, soil and sand from the beach (Sercu et al. 2009, Whitman & Nevers 2003). According to many authors, rainfall, storms and wind on land may cause a drastic increase in the concentration of microorganisms in the water (Olivieri et al. 2007, Reeves et al. 2004, Byappanahalli & Fujioka 2004, Mote et al. 2012, Crowther et al. 2001). The bacterial population in the aquatic environment is significantly influenced by conditions prevailing in particular months of the year (Frączek et al. 2015). Direct assessment of all pathogenic microorganisms occurring in water is very complicated and time-consuming, which caused the appointment of indicator bacteria. In Poland and in most countries, *Escherichia coli* and enterococci were used for the assessment of the microbiological contamination of waters used as batching areas (Olańczuk-Neyman 2003). The occurrence of these

bioindicators in the water, indicates the recent contamination of the reservoir with fecal microorganisms and hence, the possibility of other pathogenic bacteria, also of intestinal origin. (Walters et al. 2011), as well as numerous viruses (Soller et al. 2010), parasites and other pathogens. The study results of Viau et al. (2011) confirm the correlation between the occurrence of enterococci and pathogenic bacteria of the *Campylobacter* and *Salmonella* genus. Additionally, there was an explicit relation between the presence of streptococci in water and the and the number of cases of people using bathing areas, which in the summer season is definitely increasing. It may create a real epidemiological threat, therefore it is necessary to conduct systematic monitoring of microbiological status of waters used for bathing purposes (Attoui et al. 2016, Berninger et al. 2012, Zimoch & Paciej 2013).

The aim of the study was to assess the purity of water used for bathing purposes and to determine the survival time of *Escherichia coli* and *Enterococcus faecalis* bacteria in the water.

2. Material and methods

2.1. Subject, place and time of study

Samples of water for the study were taken from the Koronowo Reservoir located in the lower part of the Brda river, which is located within Kujawsko-Pomorskie Province. The reservoir has an extended shape with an area of approximately 1600 ha, a capacity of approx. 81 million m^3 and the maximum depth exceeds 20 m. Reservoir has a varied coastline, which has a total length of 100 m. The waters of the reservoir are used with high frequency, they also constitute an interesting recreational offer. Over the years, taking into account the state of cleanliness of the waters, the Koronowo Reservoir obtained the third class of purity. Due to heavy rainfall, which contributes to the increase of water levels in rivers, it is often possible to observe a significant increase in transport of humic compounds along with waters of Brda river which are responsible for giving the waters of the Koronowo Reservoir a brown color and contribute to the eutrophication process. Water samples of 2000 ml were taken from three bathing areas: Samociążek (bathing area I), Pieczyska (bathing area II), Kręgiel (bathing area III) three times in the months of July, August and September. The choice of sampling time was dependent

on the use of recreational beach resorts. Samples were collected 30 cm under the surface of water into sterile, glass bottles and then transported to the laboratory where microbiological analyzes were performed in accordance with the Polish Standard PN-EN ISO 9308-1 and PN-EN ISO 7899-2. The number of indicator bacteria *Escherichia coli* and enterococci was determined in the water samples. Additionally, each time the samples were taken, the water temperature and the number of bathers were measured.

2.2. Procedures of microbiological tests

2.2.1. Methods for determination of the number of indicator bacteria

The determination of the number of *Escherichia coli* and enterococci bacteria in the tested water samples was performed by membrane filtration with usage of filtration apparatus and membrane filters of cellulose esters with a size of 47 mm and a pore size of 0.45 μm. Due to the significant microbiological contamination of the tested samples, a series of decimal dilutions in the range from 10^{-1} to 10^{-3} were made. After filtering 100 ml of water, the filter was transferred to a Petri dish with Lactose TTC Agar with Tergitol™-7 substrate and incubated for 24 hours at 37°C. After the incubation, all characteristic colonies of lactose-positive bacteria with yellow-orange approbate were counted, which caused yellow color on the substrate with the filter. All characteristic colonies were transplanted into Trypton Soy Agar (TSA) and tryptophan broth. TSA agar with inoculated bacteria was incubated for 24 hours at temperature of 37°C. After incubation, a cytochrome oxidase occurrence and indole production tests were performed. All colonies which exhibited a negative reaction to cytochrome oxidase and were producing indole, were counted as *E. coli* bacteria. In order to determine the number of enterococci, after filtering 100 ml of the water from sample tested, filter with microorganisms detained on it was transferred to a Petri dish with Slanetz and Bartley medium and incubated for 48 hours at temperature of 37°C. After incubation, convex colonies, either whole red or only in the middle, pink or maroon color, were considered to be typical *Enterococcus* bacteria. In the next stage, in order to confirm the results, the membrane filter was transferred together with the grown colonies on agar with kanamycin, esculin and azide and then incubated for 2 hours at temperature of 44°C. As a result of bacterial growth, occurs decomposition of esculin into glu-

cose and escullet, which reacts with the iron ions present in the medium, creating a dark brown or black complex, which is visible in the form of a characteristic darkening of the substrate (black sediment around grown colonies). Colonies of light brown to black color, clearly diffusing into the interior of the substrate were considered typical.

2.2.2. Survivability studies of *Escherichia coli* and *Enterococcus faecalis*

Water for testing of the survivability of indicator organisms was taken from the bathing areas of the Koronowo Reservoir. *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 19433 were used as indicator bacteria. By usage of System Vitex 1550 densitometer, a suspension was prepared with the above mentioned strains with a turbidity of 10^8 cells/ml. The result of the suspension was introduced into the test water in an amount of 5 ml per 5000 ml of water and left at room temperature for 1 hour and then the number of indicator bacteria in the inoculated water sample was determined. Samples of inoculated water were placed at temperature of 4°C and 20°C, and then the number of *Escherichia coli* and *Enterococcus faecalis* was determined at the specified intervals by usage of MPN method (most probable number of bacteria). In order to determine the number of *E. coli*, a lactose broth with bromocresol purple (incubation at temperature of 44°C/24-48h) was used in the preliminary tests. Positive and doubtful results were confirmed using Tryptone Bile Glucuronic Agar (TBX). The final confirmation consisted in usage of the API 20E microtest. For the determination of *Enterococcus faecalis*, broth with glucose and azide (incubation at temperature of 37°C/24h) was used in the first stage. The results were confirmed by usage of agar with kanamycin, esculin and azide, while the final identification was performed with API 20 Strep.

2.3. Statistical analysis of results

The Microsoft Excel 2010 and Statistica 2013.1 programs were used to calculations and to execute the charts. The results of the survivability studies of *Escherichia coli* and *Enterococcus faecalis* in water used for bathing purposes were digested and then subjected to statistical analysis based on changes in the number of bacteria tested at the time in accordance to formula 1:

$$y = ax + b \tag{1}$$

while:
y – logarithm of the number of indicator bacteria, elimination of bacteria in one day, a – the directional coefficient corresponding to the average change in the number of bacteria in the form of log per one, b – day the number of bacteria in the zero phase, x – time counted in days.

3. Results and discussion

The results of the studies on the presence of indicator bacteria *Escherichia coli* and enterococci in water samples taken from three bathing areas are presented in Figures 1-2. The analyzes indicated that the tested water was characterized by significant microbiological contamination, because in the July the occurrence of *E.coli* bacteria was recorded at an average of 3697 cfu/100 ml (bathing area I) to 4539 cfu/100 ml (bathing area II). Additionally, in the samples of water taken from the bathing area III, the number of these bacteria was also high and amounted to 3836 cfu/100 ml (Fig. 1).

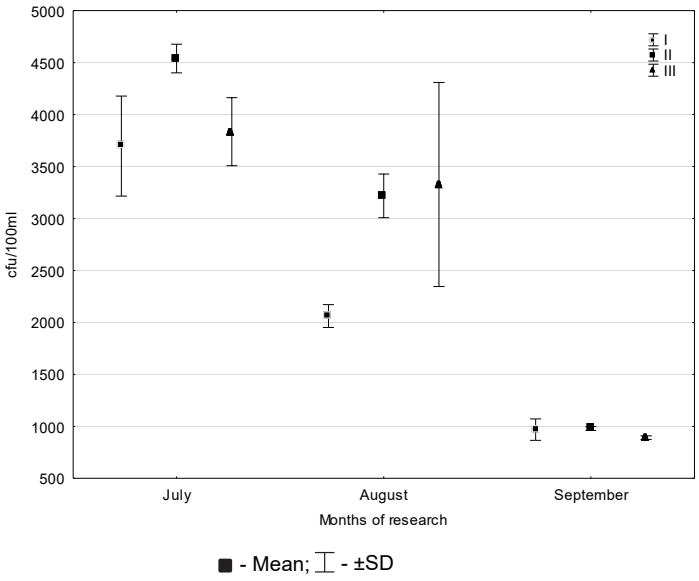

Fig. 1. Average number of *E. coli* in water from individual bathing areas
Rys. 1. Średnia liczba *E. coli* w wodzie z poszczególnych kąpielisk

However, in August, the number of these bacteria decreased and ranged from 2062 cfu/100 ml (bathing area I) to 3327 cfu/100 ml (bathing area III). Similar relations were noted by An et al. (2002), stating that the highest number of *E. coli* bacteria and coliform bacteria occurred in water samples from the lake during the summer. Own study indicated that the least number of *E. coli* was isolated from water samples taken in September, where the number of these bacteria was at a similar level in all bathing areas (892-980 cfu/100 ml). Analyzing the occurrence of enterococci, it was found that the majority of these microorganisms were isolated from water samples taken from bathing area I in July when their number was 412 cfu/100 ml and from bathing area III in August - 404 cfu/100 ml (Fig.2). In the rest of the samples of the analyzed water, the number of enterococci didn't exceed the normative value of 400 cfu/100 ml.

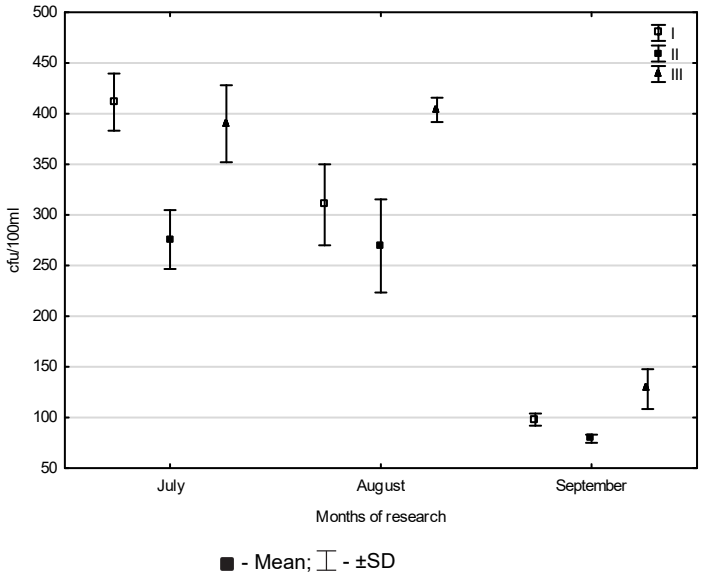

Fig. 2. Average number of enterococci in water from individual beach resorts
Rys. 2. Średnia liczba enterokoków w wodzie z poszczególnych kąpielisk

It was also indicated that in every water sample from bathing areas, regardless of the month of research, *E. coli* bacteria were significantly more numerous than enterococci. Similar conclusions were obtained by Alm et al. (2003) who noticed over four times higher number of *E.coli*

bacteria in water used for bathing purposes than enterococci. Considering sanitary indicators in own study, it should be noted that only in September the analyzed water samples met the microbiological requirements for the assessment of the current quality of bathing water and the place used for bathing specified in the Regulation of the Minister of Health of 8 April 2011 on the supervision of the quality of bathing water and the place used for bathing (Dz.U. 2011 no 86 item 478; Dz.U. 2015 item 1510). Higher results were obtained by Kostecki et al. (2000), who studied the number of streptococci in the Dzierżno Duże reservoir. The highest values were noted at the river inflow, where they amounted to 1000 cfu/100 ml. According to the authors, the number of bacteria in water is always higher in the coastal zone of the lake than in the middle part. Niewolak & Gotkowska-Płachta (1999) after analyzing 20 samples of water taken from Hańcza Lake, found that the average number of streptococci in the summer was the highest in the July and was 43 cfu/100 ml for coastal waters, while the average number of streptococci from 80 samples taken from the deepest point of the lake reached its highest value in August: 14 cfu/100 ml. Own study indicated that there is a noticeable relationship between water contamination and the number of people taking baths, because in July the number of people using the water reservoir was the largest and ranged from 35 to 68 people (Table 1).

Table 1. Water temperature and the number of bathers
Tabela 1. Temperatura wody i liczba kąpiących się osób

Place of study	Temperature of water (°C)			Number of bathers		
	Months of study			Months of study		
	VII	VIII	IX	VII	VIII	IX
Bathing area I	25	21	18	35	30	3
Bathing area II	26	21	19	68	45	12
Bathing area III	24	20	18	36	26	9

Additionally, the highest number of *Escherichia coli* and enterococci in the tested water was also recorded this month. In August, the water temperature was lower than in month July by an average of 4-5°C,

while the number of bathers at the time of sampling was also lower than in the previous month. In August, the number of people taking baths ranged from 26 to 45 people. At the same time, the number of indicator bacteria in water samples decreased during this period. The number of people using bathing areas for recreational purposes had the greatest impact on the microbiological contamination of the reservoir. The argument confirming this fact was the last month of research (September), because microbiological indicators in samples of water were much below the acceptable standards. The relation between the number of bathers and the contamination of water and sand on the beach with *E. coli* bacteria was also found by Whitman & Nevers (2003).

Table 2. The survivability of *E. coli* and *Enterococcus faecalis* bacteria at temperature of 4 and 20°C
Tabela 2. Przeżywalność bakterii *E. coli* i *Enterococcus faecalis* w temperaturze 4 i 20ºC

Day of study	Temperature of 4°C		Temperature of 20°C	
	Number of bacteria [cfu/ml]			
	Escherichia coli	*Enterococcus faecalis*	*Escherichia coli*	*Enterococcus faecalis*
1	$4.5 \cdot 10^7$	$2.5 \cdot 10^7$	$4.5 \cdot 10^7$	$2.5 \cdot 10^7$
3	$2.5 \cdot 10^6$	$2.5 \cdot 10^6$	$3.0 \cdot 10^6$	$2.5 \cdot 10^6$
7	$1.5 \cdot 10^6$	$1.5 \cdot 10^6$	$4.5 \cdot 10^2$	$4.5 \cdot 10^5$
10	$1.5 \cdot 10^5$	$1.5 \cdot 10^5$	$4.5 \cdot 10^1$	$1.5 \cdot 10^3$
15	$1.5 \cdot 10^4$	$1.5 \cdot 10^5$	$4.5 \cdot 10^1$	$4.5 \cdot 10^2$
20	$4.5 \cdot 10^3$	$1.5 \cdot 10^4$	0	$1.5 \cdot 10^2$
25	$2.5 \cdot 10^3$	$1.5 \cdot 10^4$		0
30	$4.5 \cdot 10^1$	$1.5 \cdot 10^3$		
35	$4.5 \cdot 10^1$	$4.5 \cdot 10^2$		
40	$1.0 \cdot 10^1$	$2.5 \cdot 10^1$		
45	0	0		

Due to the long survivability of pathogenic microorganisms in the aquatic environment, studies have been undertaken to determine the survival time of *E. coli* and *Enterococcus faecalis* in water used for bathing purposes. The obtained results are presented in Table 2 and Figures 3-6.

At the beginning of the study the number of *E. coli* bacteria in the initial sample was $4.5 \cdot 10^7$ cfu/ml for water stored at temperature of 4°C and also 20°C. A significant decrease in the number of these bacteria at temperature of 4°C by more than 3 logarithmic units was recorded after 15 days of the study. In the next days of the study, successive decreases in the *E. coli* number were registered, while in the 45th day of the study, the presence of these microorganisms wasn't found in the water samples. A definitely faster elimination of these bacteria was found in water at temperature of 20°C and after 15 days of study the number of *E. coli* decreased to the level of $4.5 \cdot 10^1$ cfu/ml (Table 2).

The equation of regression indicated that the daily rate of elimination of these bacteria in water was 0.16 log cfu at temperature of 4°C (Fig. 3) and 0.45 log cfu at temperature of 20°C (Fig. 4). The prognostic calculations indicated that these indicator bacteria survived 45 days at temperature of 4°C, while at temperature of 20°C they survived for 29 days shorter. Wcisło & Chróst (2000) determined that *E. coli* bacteria can survive in an aqueous environment for 30 days. The reason for this phenomenon is probably the ability to adapt these bacteria to adverse conditions. At low temperature, the volume of microorganisms decreases a few times, and vital functions are slowed down, which makes their survival time additionally longer even up to several months (Stojek 2010).

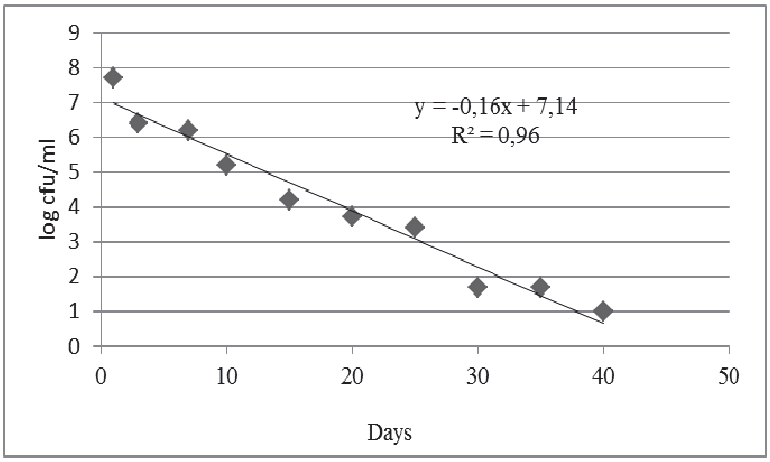

Fig. 3. The survivability of *E. coli* at temperature of 4°C
Rys. 3. Przeżywalność *E. coli* w temperaturze 4°C

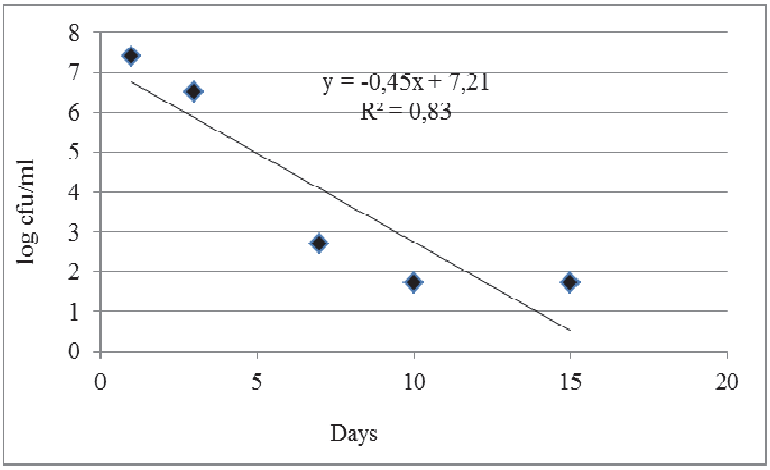

Fig. 4. The survivability of *E. coli* at temperature of 20°C
Rys. 4. Przeżywalność *E. coli* w temperaturze 20°C

Enterococci are used as indicators of fecal contamination of recreational waters around the world (Boehm & Sassoubre 2014). In the own study on the survivability of *Enterococcus faecalis*, it was indicated that these bacteria were also inactivated in the test water. At the beginning of the study, the number of streptococci was at the rate of $2.5 \cdot 10^7$ log cfu/ml at temperature of 4°C and 20°C. In the first days of the study, a slight decrease in the number of these bacteria at temperature of 4°C was observed, while a significant elimination of *Enterococcus faecalis* to the rate of $1.0 \cdot 10^1$ cfu/ml was recorded on the 40th day of the study. In water at temperature of 20°C, a rapid decrease in the number of these bacteria was found on the 20th day of the study, since these bacteria were isolated from the water in the number of $1.5 \cdot 10^2$ cfu/ml (Table 2). With usage o equation of regression, it was calculated that the maximum survival time of *Enterococcus faecalis* in water at temperature of 4°C was 52 days, while at temperature of 20°C it was 26 days (Fig. 5-6). The obtained results proved that the daily rate of elimination of these bacteria in water at both temperatures was slower as compared to *E. coli* and amounted to 0.14 log (4°C) and 0.28 log (20°C).

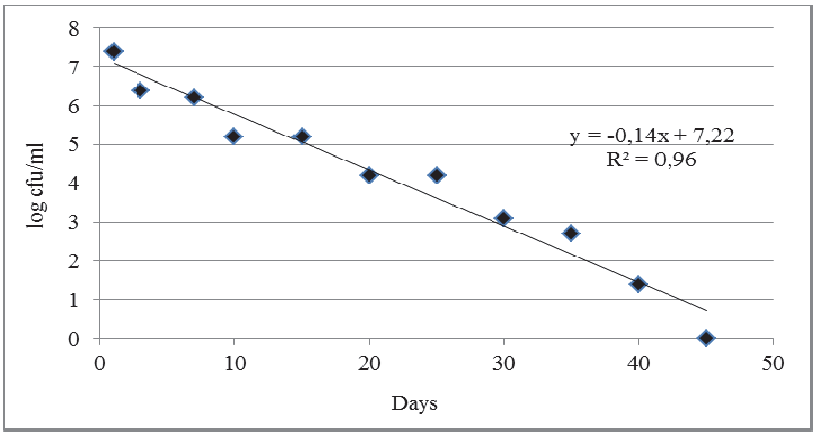

Fig. 5. The survivability of *Enterococcus faecalis* at temperature of 4°C
Rys. 5. Przeżywalność *Enterococcus faecalis* w temperaturze 4°C

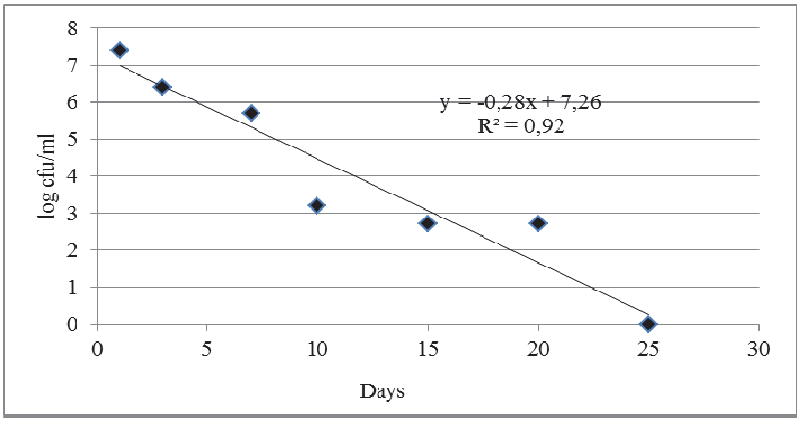

Fig. 6. The survivability of *Enterococcus faecalis* at temperature of 20°C
Rys. 6. Przeżywalność *Enterococcus faecalis* w temperaturze 20°C

As reported by many authors, enterococci are characterized by longer survivability in water than other bacteria, including *Escherichia coli* (Boehm & Sassoubre 2014, Craig et al. 2004, Byappanahalli at al. 2003). Lleò et al. (2005) explain the cause of this phenomenon by the ability of enterococci to transition into VBNC state (Viable But Not Culturable) and resuscitation capacity. The authors state that this strategy of survival of enterococci should be taken into account when assessing the microbiological quality of water, especially when these bacteria act as

sanitary indicators. Researchers found that the number of enterococci found in recreational waters was strongly correlated with the number of people suffering from gastrointestinal diseases. The presence of pathogenic bacteria in surface waters used for recreational purposes is particularly dangerous due to epidemiological reasons. Many people have access to infected water during the holiday season, which poses a very high risk for them due to the rapid spread of pathogenic microorganisms (Boehm & Soller 2011).

4. Conclusions

1. Performed study has indicated that there are numerous *Escherichia coli* bacteria in water from three bathing areas, especially in the months of July and August, where the number of these bacteria has exceeded the normative values several times.
2. It was found that the number of enterococci in the water from bathing areas was definitely lower compared to the *E. coli* bacteria. In the samples of water coming from two bathing areas in the months of July and August, the number of these bacteria was noted at the level slightly exceeding the sanitary requirements.
3. It was indicated that in September, the water from all the examined bathing areas met the microbiological requirements specified in the applicable legal acts.
4. The obtained results proved that the number of indicator bacteria was determined by the temperature of water and the number of bathers. Prognostic studies indicated that *Escherichia coli* and *Enterococcus faecalis* lasted longer in water at temperature of 4°C, respectively: 45 and 52 days, while at temperature of 20°C their survival time was shorter by 29 and 26 days.
5. The study indicated the necessity to perform permanent sanitary control of water used for bathing purposes in the aspect of epidemiological threats.

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Występowanie i przeżywalność *Escherichia coli* i enterokoków w wodach wykorzystywanych jako kąpieliska

Streszczenie

Wody kąpieliskowe w sezonie wakacyjnym wykorzystywane są z dużą częstotliwością w celach rekreacyjnych. Należy podkreślić, że ulegają one zanieczyszczeniu mikrobiologicznemu, w związku z czym stanowią poważne zagrożenie zdrowotne dla korzystających z nich osób. W związku z powyższym podjęto badania, których celem była ocena stanu czystości wody wykorzystywanej w celach kąpieliskowych oraz ustalenie w badanej wodzie czasu przeżywalności bakterii *Escherichia coli* i *Enterococcus faecalis*. Próbki wody pobierano z trzech kąpielisk Zalewu Koronowskiego: Samociążek (kąpielisko I), Pieczyska (kąpielisko II), Kręgiel (kąpielisko III) trzykrotnie w miesiącach lipcu, sierpniu oraz we wrześniu. W próbkach wody oznaczono liczbę bakterii wskaźnikowych *Escherichia coli* i enterokoków. Dodatkowo przy każdym poborze próbek dokonywano pomiaru temperatury wody oraz liczbę kąpiących się osób. Oznaczenie liczby bakterii *Escherichia coli* oraz enterokoków w badanych próbkach wody przeprowadzono metodą filtracji membranowej. W bada-

niach dotyczących ustalenia czasu przeżywalności wykorzystano dwa szczepy *Escherichia coli* ATCC 25922 oraz *Enterococcus faecalis* ATCC 19433. Zmiany liczebności bakterii wskaźnikowych oznaczono metodą NPL (najbardziej prawdopodobna liczba bakterii). Przeprowadzone analizy wykazały, że badana woda charakteryzowała się znacznym zanieczyszczeniem mikrobiologicznym, ponieważ w miesiącu lipcu odnotowano występowanie bakterii *E. coli* średnio na poziomie od 3697 cfu/100 ml (kąpielisko I) do 4539 cfu/100 ml (kąpielisko II). Ponadto w próbkach wody pobranych z kąpieliska III liczba tych bakterii była wysoka i wynosiła 3836 cfu/100 ml. Z kolei w sierpniu liczba tych bakterii zmniejszyła się i kształtowała się w zakresie od 2062 cfu/100 ml (kąpielisko I) do 3327 cfu/100 ml (kąpielisko III). W przypadku enterokoków stwierdzono, że najwięcej tych drobnoustrojów wyizolowano z próbek wody z kąpieliska I w lipcu, liczba ich wynosiła 412 cfu/100 ml oraz z III kąpieliska w miesiącu sierpniu – 404 cfu/100 ml. W pozostałych próbkach analizowanej wody liczba enterokoków nie przekroczyła wartości normatywnej 400 cfu/100 ml. Wykazano, że w miesiącu wrześniu woda ze wszystkich badanych kąpielisk spełniała wymagania mikrobiologiczne określone w obowiązujących aktach prawnych. Uzyskane wyniki dowiodły, że liczba bakterii wskaźnikowych determinowana była temperaturą wody i liczbą kąpiących się osób. Na podstawie równań regresji ustalono że bakterie *Escherichia coli* i *Enterococcus faecalis* dłużej przeżywały w wodzie o temperaturze 4°C odpowiednio: 45 i 52 dni, natomiast w temperaturze 20°C czas ich przetrwania wynosił 52 i 26 dni. Przeprowadzone badania wskazują na konieczność ciągłego monitoringu wody wykorzystywanej w celach kąpieliskowych w aspekcie zagrożeń epidemiologicznych.

Abstract

Bathing waters during the holiday season are used with great frequency for recreational purposes. It should be noted that they are subject to microbiological contamination, and therefore pose a serious health risk to the people using them. In accordance with the above, study which was undertaken aimed at assessing the purity status of water used for bathing purposes and determining the survival time of *Escherichia coli* and *Enterococcus faecalis* bacteria in the test water. Water samples were taken from three bathing areas of the Koronowo Reservoir: Samociążek (batching area I), Pieczyska (bathing area II), Kręgiel (bathing area III) three times in the months of July, August and September. The number of indicator bacteria *Escherichia coli* and enterococci was determined in the water samples. Additionally, each time the samples were taken, the water temperature and the number of bathers were measured. The determination of the number of *Escherichia coli* and enterococci bacteria in the tested water samples was performed by membrane filtration. Two strains of *Escherichia coli* ATCC

25922 and *Enterococcus faecalis* ATCC 19433 were used in studies to determine the survival time. Changes in the number of indicator bacteria were determined by usage of MPN method (most probable number of bacteria). The analyzes indicated that the water tested was characterized by significant microbiological contamination, because in July the occurrence of *E.coli* bacteria was recorded at an average of 3697 cfu/100 ml (bathing area I) to 4539 cfu/100 ml (bathing area II). Additionally, in the samples of water taken from the bathing area III, the number of these bacteria was high and amounted to 3836 cfu/100 ml. However, in August the number of these bacteria decreased and ranged from 2062 cfu/100 ml (bathing area I) to 3327 cfu/100 ml (bathing area III). In the case of enterococci, it was found that the majority of these microorganisms were isolated from water from bathing area I in July and their number was 412 cfu/100 ml and from the bathing area III in August – 404 cfu/100 ml. In the remaining samples of the analyzed water, the number of enterococci didn't exceed the normative value of 400 cfu/100 ml. It was indicated that in September the water from all the examined bathing areas met the microbiological requirements specified in the applicable legal acts. The obtained results proved that the number of indicator bacteria was determined by the temperature of water and the number of bathers. In accordance to regression equations, it was found that *Escherichia coli* and *Enterococcus faecalis* lasted longer in water at temperature of 4°C respectively: 45 and 52 days, while at temperature of 20°C their survival time was 52 and 26 days. The study indicated the necessity to perform permanent sanitary control of water used for bathing purposes in the aspect of epidemiological threats.

Słowa kluczowe:
woda kąpieliskowa, bakterie wskaźnikowe, przeżywalność, *Escherichia coli*, *Enterococcus faecalis*

Keywords:
bathing water, indicator bacteria, survivability, *Escherichia coli*, *Enterococcus faecalis*