MODERN PROBLEMS AND SOLUTIONS IN ENVIRONMENTAL PROTECTION

Post-conference monograph of Current Environmental Issues 2019

edited by

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Contents

Dear Readers

The book you have received was created as a result of the 14th international interdisciplinary conference "Current Environmental Issues-2019" organized in cooperation with the Faculty of Biology and Ecology of the Yanki Kupala State University of Grodno in the period 24-26th September 2019 at the Faculty of Biology and Chemistry of the University of Bialystok.

It seems that at present social awareness of the importance of water, air, and soil conditions for the well-being of the population is very high. However, despite the educational efforts undertaken by relevant institutions, we are faced every day with various occurrences pointing out loopholes in the law, or the lack of awareness of the individual citizen regarding such prosaic matters as e.g. segregation of garbage or behavior in protected natural areas. Ecology is a field dealing with problems related to the coexistence of various species in a particular habitat. As a field of science, it is not easily classifiable because it covers a very diverse and extensive range of activities. It is as miscellaneous as the natural environment is heterogeneous. The scope of research of ecologists includes population research at the micro and macro levels, research on the effects of human economic activity, as well as ways of solving or avoiding ecological threats. So each of us, not only biologists dealing with population issues, but also microbiologists, physicists, chemists, as well as sociologists who study interactions on the human-natural environment border, falls within the scope of ecological research. Therefore, the content discussed in the monograph is very diverse.

The main goal of the prepared monograph was to present the current issues raised during the Conference related to the broadly understood protection of natural resources, the relationship between human economic activity and observed phenomena, as well as their impact on human health

The work begins with a chapter on issues related to an analysis of changes observed over a period of 30 years in the populations of alluvial and wetland birds in the area of south-eastern Poland.

The next chapter deals with problems related to the microbiological safety of surface waters. An analysis of bacterial species inhabiting the studied lakes and rivers is presented and an attempt has been made to point to the correlation between physicochemical parameters and the state of water purity as well as the number and type of bacteria species.

The third chapter presents a proposal for a quick new method for assaying cadmium in water matrices rich in humic substances. The described analytical procedure has been validated and can be used in routine testing of surface water.

The fourth chapter deals with problems related to the loss of viability of apple seeds. The authors present results of research on the variability of biotin levels in the embryonic axes of apple seeds during their storage, and carry out correlation tests on the biotin content and germination of seeds.

The next chapter deals with issues dealing with sociology and ecology. The research conducted by the authors indicates which initiatives taken by various

organizations contribute to raising public awareness of climate change and its prevention.

The next chapter is a typical experimental work devoted to the study of the kinetics and mechanism of the acidic dyes sorption process. The object of research were modified Lewatit and Amberlyst sorbents. The obtained results show that these sorbents can be successfully used for the decolorization of textile wastewater.

Chapter seven deals with the assessment of the viability and metabolic processes of algae of the species *Chlorella vulgaris* in an environment containing an excess of iron and manganese ions. The obtained results indicate the relationship between the level of magnesium in waters and the intensity of the eutrophication process taking place in an examined reservoir.

The next chapter is devoted to human health issues, in particular problems related to the treatment of malignant tumors. The theoretical and practical aspects of using highly purified free amino acids and mini-aminozoles for metabolic therapy of proliferative diseases is discussed in detail.

Chapter nine presents issues related to the pathogenicity of the fungus *Malassezia pachydermatis*, which displays a dual nature depending on its properties. These fungi may act as commensal, however in immunosuppressed patients and animals they often cause surface or systemic infections.

The tenth chapter is devoted to the use of a new ion-selective electrode for the determination of lead in liquid environmental samples. By using a membrane containing multi-walled carbon tubes and an ionic liquid as an internal electrolyte, a significant reduction in the detection limit and improved electrode selectivity compared to a conventional electrode was achieved.

The next chapter is focused on the relationships between climate and ecotypes of bacteria pertaining to the *Bacillus cereus* group. The authors prove that different environmental pressures lead to the selection of bacteria with distinct properties.

Another chapter discusses complications with coherent bacterial taxonomy and the definition of prokaryotic species. Although knowledge about bacterial biology is actually quite broad, still the environmental impact on bacteria, as well as horizontal gene transfer and diversity, remain explored only in part.

The next chapter is focused on the diversity of major histocompatibility complex class II (MHC II) DRB genes in moose. With the aid of molecular analyses, authors prove that the Biebrza population of this species is distinct, which suggests its relict character.

The last chapter discusses the characteristics and the mechanisms of action of polyene antimycotics in relation to the fungus *Candidia* sp., which is known as one of the most important causative factors of fungal infections in humans. New insights into the knowledge about the mode of action of these medicines and the problem of arising drug-resistance are of crucial importance.

We hope that the monograph we have prepared, due to the variety of topics, will interest many specialists dealing with various aspects of both ecology and environmental protection.

Editorial team

Szanowni Państwo,

Książka, która państwo otrzymaliście powstała jako wynik XIV Międzynarodowej Interdyscyplinarnej Konferencji „Current Environmental Issues-2019" zorganizowanej we współpracy z Wydziałem Biologii i Ekologii Grodzieńskiego Uniwersytetu Państwowego im. Janki Kupały w dniach 24-26 września 2019 roku na Wydziale Biologiczno – Chemicznym Uniwersytetu w Białymstoku.

Wydaje się, że w chwili obecnej społeczna świadomość znaczenia dobrej kondycji wód, powietrza i gleb dla dobrostanu populacji jest bardzo wysoka i o problemach ochrony różnych elementów środowiska nie warto dyskutować. Jednak pomimo działań edukacyjnych podejmowanych przez odpowiednie instytucje, codziennie spotykamy się z różnego rodzaju wydarzeniami pokazującymi luki w prawie, bądź brak świadomości indywidualnego obywatela dotyczących tak prozaicznych spraw, jak np. segregacja śmieci, czy zachowanie się na terenach chronionych.

Ekologia jest to dziedzina zajmująca się problemami związanymi ze współistnieniem różnych gatunków w określonym środowisku. Jako dziedzina nauki nie poddaje się łatwo klasyfikacjom, gdyż obejmuje bardzo zróżnicowany i obszerny zakres działań. Jest tak różnorodna jak różnorodne jest środowisko przyrodnicze. Zakres badań ekologów obejmuje zarówno populacje na poziomie mikro i makro, analizy efektów związanych z aktywnością gospodarczą człowieka, jak również sposoby rozwiązywania bądź unikania zagrożeń ekologicznych. Tak więc każdy z nas, nie tylko biolodzy zajmujący się zagadnieniami populacyjnymi, ale również mikrobiolodzy, fizycy, chemicy jak również socjolodzy, którzy badają interakcje na granicy człowiek-środowisko przyrodnicze wpisują się w zakres badań ekologicznych. Dlatego też poruszane w monografii treści są bardzo różnorodne. Celem postawionym przez zespół redakcyjny było przedstawienie poruszanych w trakcie Konferencji aktualnych problemów związanych z szeroko pojętą ochroną zasobów przyrodniczych oraz bogactw naturalnych, ich eksploatacji w kontekście ochrony oraz zrównoważonego rozwoju.

Przygotowana książka obejmuje bardzo szerokie spektrum tematyczne dotyczące ekologii. Pracę rozpoczyna rozdział dotyczący zagadnień związanych z analizą zmian obserwowanych w okresie 30 lat w populacjach ptaków łęgowych oraz wodno-błotnych na obszarze południowo-wschodniej Polski.

Następny rozdział porusza zagadnienia związane z bezpieczeństwem mikrobiologicznym wód powierzchniowych. Przedstawiona jest analiza gatunków bakterii zasiedlających badane jeziora i rzeki oraz podjęta została próba wskazania korelacji pomiędzy parametrami fizykochemicznymi i stanem czystości wód a liczebnością i rodzajem bakterii wskaźnikowych.

W rozdziale trzecim przedstawiono propozycję nowej szybkiej metody oznaczania kadmu w matrycach wodnych bogatych w substancje humusowe. Zaproponowana procedura analityczna została zwalidowana i może znaleźć zastosowanie w rutynowych badaniach wód powierzchniowych.

Czwarty rozdział dotyczy zagadnień związanych z utratą żywotności nasion jabłek. Autorzy przedstawiają badania zmienności poziomu biotyny

w zarodkach obecnych w nasionach jabłek w trakcie ich przechowywania oraz korelację z ich siłą kiełkowania.

Kolejny rozdział dotyczy zagadnień z pogranicza socjologii i ekologii. Przeprowadzone przez autorów badania wskazują jakie inicjatywy podejmowane przez różne organizacje wpływają na podniesienie świadomości społecznej dotyczącej zmian klimatu i ich zapobieganiu.

Następny rozdział jest typową pracą eksperymentalną poświęconą badaniu kinetyki i mechanizmu procesu sorpcji barwników kwasowych. Obiektem badań były modyfikowane sorbenty Lewatit i Amberlyst. Uzyskane wyniki pokazują, że sorbenty te mogą być z powodzeniem wykorzystane do dekoloryzacji ścieków włókienniczych.

Rozdział siódmy dotyczy oceny żywotności i przebiegu procesów metabolicznych glonów z gatunku *Chlorella vulgaris* w środowisku zawierającym nadmiar jonów żelaza i manganu. Uzyskane wyniki wskazują na związek pomiędzy poziomem magnezu w wodach a zachodzącym w danym zbiorniku procesem eutrofizacji.

Kolejny rozdział jest poświęcony zagadnieniom zdrowia człowieka, a w szczególności problemom związanym z leczeniem nowotworów złośliwych. Szczegółowo omówione zostały teoretyczne i praktyczne aspekty wykorzystania kompozycji wysoko oczyszczonych wolnych aminokwasów i mini-aminozoli do metabolicznej terapii chorób rozrostowych.

W rozdziale dziewiątym przedstawiono zagadnienia dotyczące chorobotwórczości drożdżaków *Malassezia pachydermatis*, które wykazują dualistyczną naturę, w zależności od swoich właściwości. Mogą pełnić rolę komensali jak również czynników chorobotwórczych, szczególnie u zwierząt oraz ludzi poddanych immunosupresji.

Rozdział dziesiąty jest poświęcony wykorzystaniu nowej elektrody jonoselektywnej do oznaczania ołowiu w ciekłych próbkach środowiskowych. Dzięki zastosowaniu membrany zawierającej wielościenne rurki węglowe oraz cieczy jonowej jako elektrolitu wewnętrznego uzyskano znaczne obniżenie granicy wykrywalności i polepszenie selektywności elektrody w porównaniu do elektrody klasycznej.

Następny rozdział jest poświęcony powiązaniom pomiędzy klimatem oraz ekotypami w naturalnych populacjach bakteryjnych na przykładzie bakterii należących do grupy *Bacillus cereus* sensu lato. Autorzy wykazali między innymi, że presja środowiskowa prowadzi do selekcji mikroorganizmów o ściśle określonych właściwościach.

Kolejny rozdział dyskutuje złożony problem spójnej taksonomii gatunków prokariotycznych. Mimo iż aktualna wiedza na temat biologii bakterii jest szeroka, wciąż takie czynniki jak wpływ środowiska na bakterie czy horyzontalny transfer genów pozostają częściowo nierozpoznane.

Kolejny rozdział dotyczy zróżnicowania sekwencji genu DRB (MHC II) u łosia. Przy pomocy analiz molekularnych autorzy wykazali, że biebrzańska populacja tego gatunku charakteryzuje się odmiennością, co dowodzi jej reliktowego charakteru.

Ostatni rozdział omawia charakterystykę i mechanizm działania polienów w stosunku do drożdżaków z rodzaju *Candida* sp., które są jednymi z najbardziej powszechnych czynników etiologicznych infekcji grzybiczych u ludzi. Nowe informacje o mechanizmach ich aktywności oraz narastającym problemie antybiotykooporności są obecnie niezwykle istotne.

Mamy nadzieję, że przedstawiona monografia, dzięki różnorodności zawartych w niej zagadnień, zainteresuje wielu specjalistów zajmujących się różnorodnymi aspektami ekologii i ochrony środowiska.

Zespół edytorski

POPULATION TRENDS OF BREEDING WATERBIRDS ON FISHPONDS IN SOUTH-EASTERN POLAND DURING 30 YEARS

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Abstract

The abundance of breeding waterbirds species on fishponds was analysed between 1985 and 2014 in south-eastern Poland. Significant decreasing trends were observed for *P. grisegena*, *F. atra*, *A. ferina*, *A. fuligula* and *P. nigricollis*. However, *C. olor*, *A. anser*, *M. strepera*, *A. platyrhynchos*, *T. ruficollis* and *P. cristatus* showed increasing trends. Although the studies were carried out on a regional microscale, the results for some species were convergent with European and national trends.

Key words: waterbirds, breeding period, fishponds, population trends

Introduction

The abundance and distribution of European bird species are constantly monitored in order to determine their global, European, and national trends, and conservation status. In this paper an attempt has been made to determine trends for some breeding water and wetland birds species on a regional scale. This group of birds was chosen because they are excellent bioindicators of the environment's status (Wetlands International 2012). Strong anthropopressure has had an unfavorable effect on natural wetlands and waterbodies. The disappearance of wetland habitats and negative changes in natural waterbodies connected with human activities has caused that fishponds are often a basic substitute for the natural environment for waterbirds. This phenomenon affects mainly the central and southern regions of Poland, which are the poorest in terms of the occurrence of natural reservoirs and a lack of lakelands but where there are the largest areas of carp ponds (Bocheński 1996, Guziur et al. 2003). The Lublin region is a farmland area where surface waters constitute only about 0.8%; however, it belongs among the leaders in Poland in terms of area occupied by fishponds, which cover 6707 ha (Uziak, Turski 2008, GUS 2017). Therefore,

reservoirs such as semi-natural fishponds, have become an important breeding habitat for birds in this region and they are an important component of some bird refuge of international importance in Poland (Wilk et al. 2010).

Many ponds were created in the Middle Ages, becoming a permanent element of the landscape together with birds, for which they became important habitats in Central and Eastern Europe (Dobrowolski 1995, Bocheński 1996, IUCN 1997, Kozulin et al. 1998, Kalivodova, Feriancova-Masarova 1999, Vogrin 1999, Kren 2000, Svazas, Stanevicius 2000). Fishponds are usually shallow and strongly eutrophic, with richly developed submerged and emergent vegetation (Michael 1987). Thanks to good management, they do not overgrow completely. As a result, they usually provide good conditions for bird reproduction and provide high species diversity of breeding water and wetland birds (Bukacińska et al. 1996). These types of habitats are willingly colonized by ducks, geese, swans, grebes and rails in the breeding period (Hagemeijer, Blair 1997, Konter 2001, Sikora et al. 2007).

Materials and methods

The analysis of population trends of breeding waterbirds was made on the basis of data from 16 fish pond complexes (total surface ca. 3150 ha), positioned in various parts of south-eastern Poland, mainly in the Lublin region and partly in the Podkarpackie voivodship (*50°30'-51°37' N, 22°03'-23°44' E*, Fig. 1.).

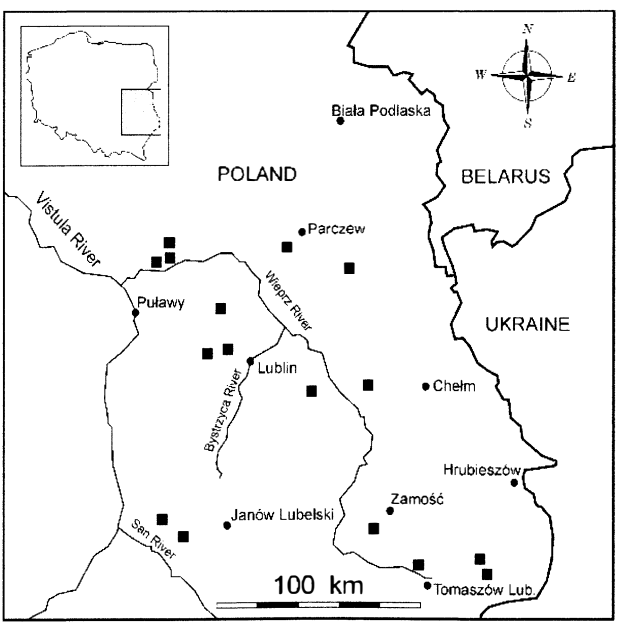

Figure 1. Location of the studied fishpond complexes represented by squares on the map.

The largest groups of fish pond complexes are situated in the north and north-east of the region in the Valley of the Lower Wieprz, Tyśmienica valleys, and Polesie Lubelskie; in the south in Janowskie and the Lipskie Forests; and in the west in the Lublin Upland close to the Vistula River. Individual complexes are scattered in the agricultural landscape of the Lublin Upland near Lublin and in the south between Zamość, Hrubieszów, and Tomaszów Lubelski. The surveys included complexes located in different landscapes, because the surroundings of ponds affect their biodiversity (Bukacińska et al. 1996). Most of the ponds are located in agricultural lands, which is dominant in this region with a various share of fields, meadows, settlements and forests in the vicinity of the complexes. The four fishpond complexes are situated completely in woodland.

The research was made to determine trends in the abundance and distribution of 15 species of birds belonging to waterfowl (Anatidae - 10 species), grebes (4 species), and for the coot *Fulica atra* under farm pond conditions. The data came from journals, MA theses, and reports, as well as from monitoring and scientific activity carried out by cooperating ornithologists from Lublin Ornithological Society and Lublin research institutions. The data were collected from two fifteen-year periods during 1985-1999 and 2000-2014. Fish complexes were selected, for which bird censuses and valuation numbers of breeding pairs were made in both periods and for all species taken into account in this paper, following standard ornithological procedures (Ranoszek 1983, Koskimies, Väisänen 1991, Chylarecki et al. 2015). To include the data from various breeding seasons in the studied period, the median was calculated from the number of breeding pairs of given certain species coming from many annual observations for each fishpond complex separately. The Wilcoxon paired-range test was used as a statistical method to compare differences in the abundance of breeding pairs between two studied periods of observations.

Results

The dominating species of birds were: mallard *Anas platyrhynchos*, coot, pochard *Aythya ferina* and tufted duck *Aythya fuligula* (mean share amounted to 11-32%), which were breeding on all complexes in both study periods (Tab. 1.).

Table 1. Occurrence of breeding species on fishpond complexes.

Species	Mean proportional abundance (%) of breeding pairs (1985-99)	Mean proportional abundance (%) of breeding pairs (2000-14)	Frequency (proportions) (1985-99)	Frequency (proportions) (2000-14)
Cygnus olor	1.2	2.2	0.8	0.8
Anser anser	0.6	2.2	0.3	0.4
Bucephala clangula	0.2	0.1	0.1	0.1
Aythya ferina	18.4	12.5	1.0	1.0

Species	Mean proportional abundance (%) of breeding pairs (1985-99)	Mean proportional abundance (%) of breeding pairs (2000-14)	Frequency (proportions) (1985-99)	Frequency (proportions) (2000-14)
Aythya fuligula	17.3	11.1	1.0	1.0
Spatula querquedula	1.1	0.9	0.6	0.6
Spatula clypeata	0.5	0.7	0.3	0.3
Mareca strepera	1.4	2.4	0.4	0.5
Anas platyrhynchos	22.1	31.6	1.0	1.0
Anas crecca	0.4	0.3	0.3	0.3
Tachybaptus ruficollis	2.2	4.8	0.9	0.8
Podiceps grisegena	4.1	1.1	0.9	0.4
Podiceps cristatus	4.9	6.3	0.9	0.9
Podiceps nigricollis	1.5	0.2	0.5	0.1
Fulica atra	24.0	23.5	1.0	1.0

The little grebe *Tachybaptus ruficollis*, great crested grebe *Podiceps cristatus* and mute swan *Cygnus olor* in both periods, and the red-necked grebe *Podiceps grisegena* in the first period, inhabited 81-94% of the complexes. The average percentage share of the mentioned species was low, diversified, and amounted to 1.1-6.3% depending on the studied period. The average percentage share of the other seven bird species did not exceed 3% in either period. They were the least frequently observed species (frequency ≤ 50%): goldeneye *Bucephala clangula* (observed only on two complexes) teal *Anas crecca*, greylag goose *Anser anser*, northern shoveler *Spatula clypeata*, black-necked grebe *Podiceps nigricollis* and gadwall *Mareca strepera*. Garganey *Spatula querquedula* also was not numerous but it appeared on over half of the complexes (56-63%).

Declining trends were observed in nine species but there were significant differences in abundant breeding pairs between the two periods only in the case of the red-necked grebe (Wilcoxon test, $z = 3.41$, $p < 0.001$), coot ($z = 3.01$, $p = 0.003$), black-necked grebe ($z = 2.52$, $p = 0.01$), pochard and tufted duck ($z = 2.53$, $p = 0.01$) (Fig. 2., Tab. 2.).

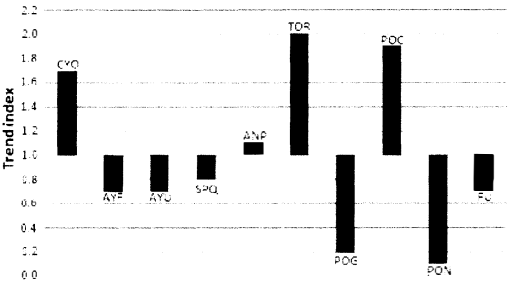

Figure 2. Waterbird species trends which occurred on the most fishpond complexes (frequency >50% in the first studied period). Quantitative changes in the breeding population were expressed in relation to the numbers from the first monitoring period as a means of trend indexes from particular complexes. Species abbreviations: *Cygnus olor* (CYO), *Aythya ferina* (AYF), *A. fuligula* (AYU), *Spatula querquedula* (SPQ), *Anas platyrhynchos* (ANP), *Tachybaptus ruficollis* (TOR), *Podiceps grisegena* (POG), *P. cristatus* (POC), *P. nigricollis* (PON), *Fulica atra* (FU).

The highest declining trend concerned the black-necked grebe (reduction of the number of breeding pairs by average 94% and widespread from 50% to 13%) and red-necked grebe (82% and from 94% to 44% respectively). Six species showed increasing trends; however, the comparison of results was not statistically significant. They were the common species of great crested grebe, little grebe, mute swan with a high increase, mallard with a slight increase, and a rare species of greylag goose and gadwall.

Discussion

The observed abundance trends of some described species of birds in the Lublin region were of a supra-regional nature (BirdLife International 2017, Chodkiewicz et al. 2018, BirdLife International 2019, see tab. 2.). Among the analysed species, only the pochard is threatened globally and its population is decreasing in Europe and in the world. In turn, the European and global population of the other common diving duck species on fishponds – that is, the tufted duck – has achieved a stable status. In Poland, a significant decrease in both pochard and tufted duck was in 2007-2009, and is currently rather regressing with slight fluctuations in numbers. In neighbouring countries, also in Belarus, a declining trend of both species of diving ducks was found, while in Ukraine their fluctuations were noted. However, the tufted duck has a growing trend in Lithuania. Coot was declining in 2007-2010 in Poland but now is an increasing trend like in Lithuania. The coot population is stable in Belarus but fluctuations are in Ukraine, while the European population is declining. The red-necked grebe shows decline across the country, as opposed to the European population. The situation of the black-necked grebe is uncertain in Europe. The

black-necked grebe recorded a significant decline in 2013-2017 in Poland. Species with growing trends in the region such as greylag goose, mallard, gadwall and great crested grebe are continually increasing in Poland. In the case of mute swan, greylag goose and gadwall, their European and global population shows an upward trend.

Table 2. Comparison of regional, national, European and global population trends of studied waterbirds breeding species based on this paper, Chodkiewicz et al. (2015, 2018) and BirdLife International (2019). Explanation of symbols: (+) increase, (-) decrease, (0) stable, (?) unknown or uncertain.

Species	Regional population trends	National population trends	European population trends	Global population trends
Cygnus olor	+	0	+	+
Anser anser	+	+	+	+
Bucephala clangula	–	+	0	0
Aythya ferina	–	–	–	–
Aythya fuligula	–	–	0	0
Spatula querquedula	–	–	–	–
Spatula clypeata	–	–	0	–
Mareca strepera	+	+	+	+
Anas platyrhynchos	+	+	0	+
Anas crecca	–	?	?	?
Tachybaptus ruficollis	+	?	0/?	–
Podiceps grisegena	–	–	+	–
Podiceps cristatus	+	+	–	?
Podiceps nigricollis	–	–	?	?
Fulica atra	–	+	–	+

Global drops in bird populations are related to threats such as hunting, intensification of agriculture and aquaculture, climate change, pollution, invasive species, diseases, natural system modifications and other human activities (BirdLife International 2015). There are a few significant problems, which have determined the trends of birds in the Lublin region. A significant reason for the drop in the number of birds was the change in the management of the ponds as a result of ownership transformations in the 1990s, in Poland and other post-communist countries. The intensification of fishing production could have led to negative pond habitat modifications (Houdková, Musil 2003, Fox et al. 2016). As a result, the production area of the ponds was increased which caused a decreasing diversity of habitats, reduction of reed beds surface, increasing the depth of ponds and size of fish stocks and removal of islands. Furthermore the

large surface share of ponds with one- and two-year old fish and late term filling of fry ponds impacted food and nesting conditions and consequently the distribution and breeding success of waterbirds (Kloskowski, Nieoczym 2015, Nieoczym, Kloskowski, 2018). It is also worth mentioning that from the 1970s, fishponds surface area was increasing, but now there is also a disturbing gradual decline in their surface area in the Lublin region (Uziak, Turski 2008, GUS 2014, 2017). This is a result of abandoning fish farming, which usually leads to the ecological succession of these ponds or their transformation into farmlands and fruit plantations.

Afterwards, nestling predation of birds (mainly corvids, gulls and different diurnal birds of prey) and mammals (mainly mustelids, canids and domestic cats) is an important factor determining the breeding success of ducks, grebes and coots (Chylarecki et al. 2018). An invasion of American mink could have been harmful to birds in wetlands in the north and north-east of the region (Zalewski, Brzeziński 2014). The American minks have been regularly observed and captured on the ponds in Polesie Lubelskie and in the Valley of the Lower Wieprz (personal observations, unpublished data). The disappearance of gull colonies as a protective shield in some ponds was the main cause of the decline in the number of black-necked grebes and diving ducks (Wójciak et al. 2005, Fox et al. 2016, Chylarecki et al. 2018).

In the case of sparsely observed duck species the obtained trends are not certain because fishponds may not be optimal nesting habitats for them. Garganey and the northern shoveler prefer meadows in river valleys and teal inhabit often eutrophic, small, forest lakes, so they are uncommon in Lublin region (Wójciak et al. 2005, Sikora et al. 2007). In turn, goldeneye is not common in south-eastern Poland, occurring only on forest ponds of Polesie Lubelskie (Wójciak et al. 2005, Sikora et al. 2007). Little grebe prefers small, shallow, eutrophiced ponds with developed submerged and emerged vegetation (Sikora et al., 2007, Kloskowski et al. 2010). It is a hidden species, so the increasing number could be affected by the growing effectiveness of their detection (Chylarecki et al. 2018). In turn, great crested grebe prefers different types of larger water reservoirs and also inhabits ponds but, compared to other smaller grebes, with larger fish (Sikora et al. 2007, Kloskowski et al. 2010). Effective protection and increased European population could have a positive effect on greylag goose and mute swan in Poland (Chylarecki et al. 2018). However, the increase in the number and spread of these species can be negatively perceived by agricultural or fish farmers due to feeding on crops in the vicinity of ponds and grains destined for fish. Greylag goose has been observed mainly on large complexes of fish ponds with rich rush vegetation and grasslands near the breeding sites, in typical nesting and feeding habitat for this species (del Hoyo et al. 1992). Mute swan until the mid-twentieth century nested mainly on large lakes, then it began to prefer artificial reservoirs in Poland (Sikora et al. 2007). In the Lublin region, 23% of breeding sites of mute swan were recorded on lakes, and 59% on fish ponds (Wójciak et al. 2005).

Conclusions

The shown abundance trends of some described bird species breeding in south-eastern Poland coincide with the observed avifauna changes in Poland and Europe. Despite the decline in numbers, pochard, tufted duck and coot are still the dominant breeding species on fishponds. The drastic decrease in red-necked grebe and black-necked grebe is alarming. Unfavorable global quantitative changes should encourage international action to protect birds in their breeding habitats, resting and feeding areas during migration, and wintering. Consequently, it is recommended to continue the monitoring of waterbirds and to extend it to further waterbodies in the Lublin region and to undertake transboundary research and protection initiatives in cooperation with ornithological societies and research institutions from Belarus and Ukraine.

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BACTERIAL CONTAMINATION IN THE WATERS OF TWO LAKE AND RIVER SYSTEMS IN NE POLAND IN RELATION TO THE PHYSICOCHEMICAL PROPERTIES OF WATER

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Abstract

Determination of the sanitary-epidemiological status of waters used for tourism purposes is very important, hence the aim of this research was to determine the sanitary status of various types of surface water used for recreation. The research area included 18 stands on the Ostróda-Elbląg Canal and 25 on the Augustów Canal. Water samples were taken during the summer period in 2017-2018. Microbiological analyses were performed in accordance with PN-EN ISO standards and included determining the total abundance of such bacteria as *E. coli*, coliforms, fecal enterococci and *P. aeruginosa*. In addition, basic physicochemical analyses of water were performed according to standard methods. The presence of indicator bacteria in the water indicates their fecal contamination and possible epidemiological threat. The general average abundance of bacteria for the waters of the Augustów Canal was 1,628,000±784,000 CFU/mL, while for the Ostróda-Elbląg Canal 2,242,800±964,300 CFU/mL. The abundance of *E. coli* bacteria in the waters ranged from 0 CFU/100mL to 6400 CFU/100mL, coliforms from 0 CFU/100mL to 7,200 CFU/ 100mL, fecal enterococci from 0 to 5,800 CFU/100mL. The maximum values of these indicators were recorded in lakes. The *P. aeruginosa* bacteria were isolated only from waters from an artificial section of the river-lake systems, and their abundance fluctuated in the 120-860 CFU/100mL range. Statistical analyses have shown that the abundance of indicator bacteria depends on some physicochemical parameters of water, such as: EC, pH, temperature, total carbon concentration, total nitrogen concentration. Summing up, it can be concluded that the highest values of the abundance of indicator bacteria occurred in lakes. The river sections were characterized by higher values of an abundance of coliforms and *E. coli* bacteria and the total abundance of bacteria compared to canal waters, which indicates a fresh inflow of pollutants to the tested systems. In turn, the canal sections were the only habitat of *P. aeruginosa*.

Key words: Augustów Canal, Ostróda-Elbląg Canal, total abundance of bacteria, indicator bacteria, water quality

Introduction

Microorganisms may occur as natural (autochtonous) microflora of water or allochthonous microflora, coming from other sources. Allochthonous bacteria are not usually able to reproduce in an aquatic environment and occur in a resting state in water (Cabral 2010). Bacteria play an important role in biogeochemical processes in water, e.g. in carbon and nitrogen cycles, and in maintaining water quality thanks to their involvement in the biodegradation of pollutants produced by households, industry and agriculture. However, although autochthonous bacteria are indispensable for aquatic ecosystems and help maintain water quality (Wetzel 2001, Briée et al. 2007, Newton et al. 2011), allochthonous bacteria – entering rivers and lakes with industrial waste-water and surface runoff (Kundzewicz et al. 2010), human and animal excrement (Ihejirika et al. 2011), and heavy rainfall (Vignesh et al. 2013) – may pose serious sanitary and epidemiological problems. Transported over long distances in water, they may pose a serious threat to public health (Marcheggiani et al. 2015); hence, monitoring bacterial contamination is of immense importance, especially in those water bodies which are used by people for recreational purposes. In addition, the results of monitoring may be used to further our understanding of local water ecosystems, in particular the role of microorganisms.

The aim of this study was to determine the sanitary status of the Augustów and Ostróda-Elbląg Canals, as examples of alternately located lake and river ecosystems characterized by continuous transport, exchange and accumulation of matter, as well as transport and exchange of microorganisms (Hillbicht-Ilkowska and Wiśniewski 1996). Additionally, the aim of the study was also to compare the river, lake, and artificial sections of the canals in terms of the total abundance of bacteria (including indicator bacteria), as well as selected physicochemical parameters.

Materials and methods

Field studies

The research area included 18 sites located on the Ostróda-Elbląg Canal and 25 sites on the Augustów Canal, in north-eastern Poland. The canals were selected because they include artificial sections (including locks) and natural lake and river ecosystems. Water samples were collected during the summer seasons in relatively similar weather conditions (e.g not after extreme events such as storms or torrential rains that could temporarily and significantly affect the levels of the studied parameters) in 2017-2018. Samples for microbiological and chemical analyses were taken using a Limnos extractor at the depth of 0.5 m. The samples were transported to the laboratory in glass bottles (1L) in a refrigerator with a temperature of 4°C (PN-EN ISO 19458, PN-ISO 5667-5:2003). The following water parameters were measured on site with the HQD 9200 from Hach Lange: electrolytic conductivity, water temperature, and pH.

Laboratory tests

Total organic carbon (TOC) and total nitrogen (TN) were determined in non-filtered water subject to high-temperature catalytic oxidation in Shimadzu's TOC-5050A analyzer.

The total abundance of bacteria expressed in colony forming units per milliliter of water (CFU/mL) was determined by filter incubation on a nutrient agar (PN-EN ISO 6222). The determination of indicator bacteria was carried out by filter incubation on a suitable selective medium with the use of biochemical confirmation tests according to the following standards: PN-EN ISO 9308-1:2004+Ap1:2005+AC:2009 for *Escherichia coli* and coliforms, PN-EN ISO 7899-2: 2004 for fecal enterococci and PN-EN ISO 16266: 2004 for *Pseudomonas aeruginosa*.

Statistical analyses

The results were subjected to statistical analysis using Statistica v. 13.3 software. The Kruskal-Wallis test was used to estimate the difference between means. The significance of correlations was estimated by Pearson's coefficient. Differences were considered as statistically significant at $p<0.05$.

Results

The electrolytic conductivity (EC) of the Augustów Canal waters ranged from 266 (±13.3) µS/cm in Studzieniczne Lake to 498 (±23.9) µS/cm in the Piecówka River. In comparison, the waters of the Ostróda-Elbląg Canal were characterized by EC ranging from 210 (±8.4) µS/cm (Pauzeńskie Lake) to 699(±34.9) µS/cm (section between the towns of Elbląg and Nowakowo) (Table 1). The water pH in the Augustów Canal was in the range of 7.12 (±0.35) – 8.31 (±0.27), while in the Ostróda-Elbląg Canal it ranged from 7.23 (±3.82) to 8.07 (±0.40) (Table 1). Water temperature in the Augustów Canal ranged from 14.8 (±0.75) ºC (section in the town of Augustów) to 22.8 (±0.91) ºC (Lake Mikaszewo). The waters of the Ostróda-Elbląg Canal were characterized by higher temperatures: from 21.4 (±0.53) ºC in the Iława Canal to 26.7 (±0.53) ºC in the Liksajny Canal (Table 1). Total organic carbon (TOC) in the waters of the Augustów Canal ranged from 6.81 (±0.34) mgC/L in Białe Augustowskie Lake to 18.14 (±0.90) mgC/L in Białe Augustowskie Lake, whereas total nitrogen (TN) ranged from 654 (±32.04) µgN/L in the Piecówka River to 1311 (±65.55) µgN/L in the Kamienny Bród River. TOC concentration in the waters of the Ostróda-Elbląg Canal was higher and oscillated between 15.83 (±0.79) mgC/L (Sambród Lake) and 54.32 (±2.66) mgC/L (Dręstwo Lock). The waters of the Ostróda-Elbląg Canal were also characterized by a high TN range of 894 (±26.82) – 3976 (±178.92) µgN/L (Table 1).

Table. 1. Physicochemical characteristics of waters of the river-lake system of the Augustów Canal and the Ostróda-Elbląg Canal (mean value ± SD, n = 4)

parameter / position	EC [µS/cm]	pH	Temperature of water [°C]	The concentration of total organic carbon (TOC) [mgC/L]	The concentration of total nitrogen (TN) [µgN/L]
Augustów Canal					
Dębowo Lock	410 (±18.4)	7.88 (±0.39)	19.2 (±0.67)	8.88 (±0.26)	666 (±26.64)
Sosnowo Lock	422 (±11.1)	7.69 (±0.38)	17.4 (±0.87)	12.67 (±0.63)	678 (±18.98)
Białobrzegi Canal	381 (±19.5)	7.94 (±0.23)	19.2 (±0.96)	8.56 (±0.35)	1,087 (±54.35)
Augustów Canal	398 (±17.9)	7.89 (±0.15)	14.8 (±0.75)	12.98 (±0.64)	1,190 (±59.50)
Przewięź Lock	275 (±13.7)	8.13 (±0.16)	19.7 (±0.98)	7.65 (±0.38)	605 (±18.15)
Gorczyca Lock	281 (±14.6)	8.05 (±0.28)	20.1 (±0.96)	9.11 (±0.35)	711 (±22.75)
Paniewo Lock	285 (±13.2)	8.01 (±0.40)	22.4 (±1.12)	12.22 (±0.61)	811 (±32.44)
Mikaszówka Lock	282 (±10.1)	7.77 (±0.31)	19.8 (±0.99)	7.32 (±0.21)	775 (±38.75)
Sosnówek Lock	296 (±12.8)	7.12 (±0.35)	18.4 (±0.92)	7.87 (±0.39)	961 (±48.05)
Tartak Lock	384 (±18.2)	7.42 (±0.17)	17.9 (±0.89)	9.12 (±0.44)	899 (±44.95)
Kudrynki Lock	388 (±19.4)	7.71 (±0.38)	18.2 (±0.70)	8.44 (±0.42)	768 (±28.41)
Netta River	402 (±13.1)	7.66 (±0.36)	18.4 (±0.92)	9.82 (±0.39)	980 (±36.26)
Kamienny Bród River	400 (±20.1)	8.12 (±0.29)	14.8 (±0.74)	10.14 (±0.50)	1,311 (±65.55)
Rospuda River	466 (±23.3)	7.88 (±0.27)	16.9 (±0.47)	7.88 (±0.27)	1,115 (±55.75)
Serwianka River	270 (±9.5)	7.99 (±0.39)	21.3 (±1.06)	7.01 (±0.35)	1,085 (±54.25)
Czarna Hańcza River	400 (±16.6)	7.70 (±0.38)	17.9 (±0.80)	9.23 (±0.25)	887 (±39.91)
Piecówka River	498 (±23.9)	7.69 (±0.20)	15.4 (±0.77)	18.14 (±0.90)	654 (±32.04)
Necko Lake	414 (±18.7)	8.13 (±0.40)	20.8 (±0.81)	9.99 (±0.49)	1,245 (±62.25)

parameter / position	EC [µS/cm]	pH	Temperature of water [°C]	The concentration of total organic carbon (TOC) [mgC/L]	The concentration of total nitrogen (TN) [µgN/L]
Sajno Lake	389 (±15.4)	8.09 (±0.36)	22.3 (±1.11)	12.7 (±0.44)	987 (±49.35)
Rospuda Lake	430 (±11.5)	8.20 (±0.41)	22.2 (±0.62)	13.4 (±0.67)	1,344 (±67.20)
Białe Augustowskie Lake	269 (±12.0)	7.91 (±0.39)	16.5 (±0.49)	6.81 (±0.34)	954 (±47.70)
Studzieniczne Lake	266 (±13.3)	8.11 (±0.34)	20.2 (±1.01)	9.09 (±0.18)	1,111 (±33.33)
Orle Lake	332 (±14.6)	8.31 (±0.27)	19.6 (±0.39)	11.99 (±0.35)	1,145 (±28.62)
Paniewo Lake	280 (±14.1)	8.20 (±0.17)	20.1 (±0.80)	11.04 (±0.55)	1,288 (±57.96)
Mikaszewo Lake	241 (±8.5)	8.31 (±0.41)	22.8 (±0.91)	10.03 (±0.50)	1,376 (±45.40)
Ostróda-Elbląg Canal					
Elbląg-Nowakowo Canal	699 (±34.9)	7.52 (±0.15)	24.6 (±1.23)	19.42 (±0.97)	1,233 (±34.52)
Elbląg Canal	662 (±33.1)	7.40 (±0.37)	25.3 (±1.26)	19.03 (±0.95)	894 (±26.82)
Canal before Buczyniec	568 (±28.4)	7.32 (±0.23)	23.2 (±0.69)	24.45 (±1.22)	1665 (±59.94)
Awajki Canal	433 (±17.32)	7.31 (±3.99)	22.8 (±1.14)	22.98 (±0.68)	1,436 (±71.80)
Małdyty Canal	411 (±20.55)	7.23 (±3.82)	23.9 (±1.19)	22.87 (±1.14)	1459 (±72.95)
Liksajny Canal	391 (±19.55)	8.00 (±0.40)	26.7 (±0.53)	18.98 (±0.94)	1,221 (±54.94)
Miłomłyn Canal, ul. Kościelna	439 (±21.9)	7.60 (±0.25)	23.3 (±1.16)	19.23 (±0.61)	923 (±26.76)
Miłomłyn Canal, ul. Pasłęcka	377 (±9.4)	7.61 (±0.26)	21.8 (±0.80)	19.88 (±0.99)	889 (±43.56)
Iława Canal	398 (±11.9)	7.44 (±0.37)	21.4 (±0.53)	21.19 (±1.05)	967 (±48.35)
Zielona Lock	420 (±21.0)	7.32 (±0.36)	24.2 (±1.21)	23.45 (±0.65)	1,178 (±58.90)
Dręstwo Lock	244 (±7.8)	7.50 (±0.29)	23.1 (±1.03)	54.32 (±2.66)	1345 (±48.42)
Ruś Mała Lock	339 (±16.9)	7.88 (±0.33)	22.9 (±0.91)	18.91 (±0.66)	986 (±34.51)
Sambród Lake	402 (±19.2)	7.42 (±0.37)	27.8 (±1.11)	15.83 (±0.79)	3336 (±166.80)

parameter / position	EC [µS/cm]	pH	Temperature of water [°C]	The concentration of total organic carbon (TOC) [mgC/L]	The concentration of total nitrogen (TN) [µgN/L]
Ruda Woda Lake	404 (±20.2)	7.30 (±0.36)	26.6 (±0.93)	24.56 (±1.22)	1,681 (±84.05)
Ilińsk Lake	409 (±8.1)	7.59 (±0.25)	25.3 (±1.21)	22.9 (±0.45)	1,811 (±48.89)
Dręstwo Lake	392 (±16.0)	7.80 (±0.15)	25.9 (±1.29)	22.4 (±1.12)	1456 (±40.768
Pauzeńskie Lake	210 (±8.4)	7.55 (±0.30)	24.8 (±1.24)	28.6 (±0.85)	3,976 (±178.92)
Szeląg Wielki Lake	306 (±11.6)	8.07 (±0.40)	22.7 (±1.13)	21.8 (±1.09)	1,288 (±51.52)

The total bacterioplankton abundance in the waters of the Augustów Canal was the lowest in Białe Augustowskie Lake – 143,666 (±37,554) CFU/mL, and the highest in the Canal's section in the town of Augustów– 2,976,667 (±643,920) CFU/mL (Fig. 1).

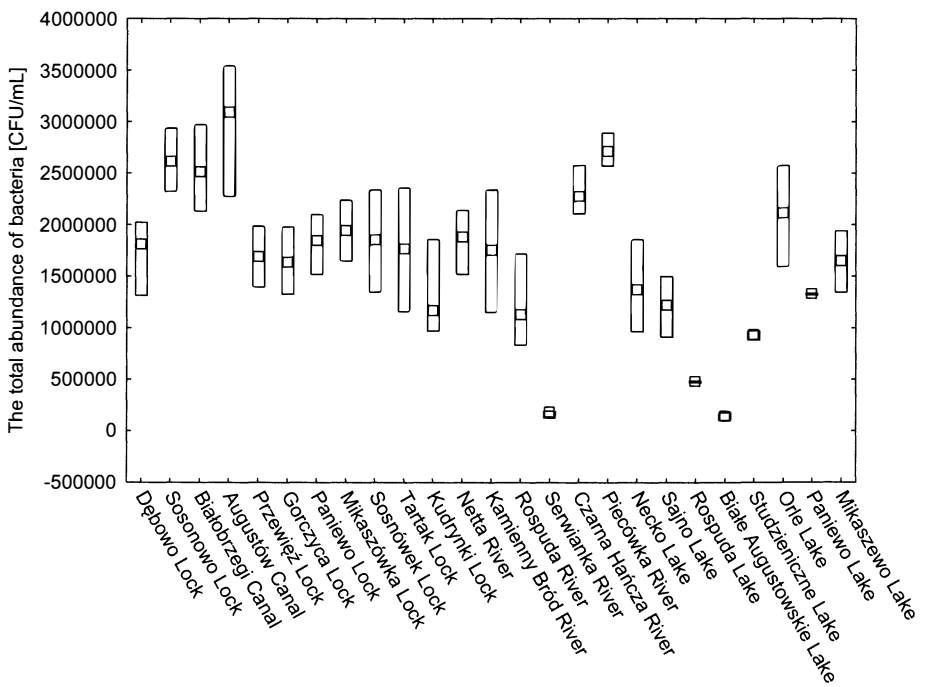

Fig. 1. The total number of bacteria in the waters of the Augustów Canal (n=4).
□ Median ▯ 25%-75%

The total abundance of bacteria in the waters of the Ostróda-Elbląg Canal ranged from 1,163,663 (±615 006) CFU/mL (the section in Miłomłyn at Pasłęcka Street) to 3,376,667(±290,229) CFU/mL (Dręstwo Lock) (Fig. 2). The abundance of coliforms and *E. coli* was the lowest in artificial sections of the canal – on average 1,012 (±666) CFU/100mL for coliforms and 523 (±276) CFU/100mL for *E. coli*. The highest abundance of coliforms and *E. coli* were recorded in lake ecosystems: 4,519 (±2,123) CFU/100mL and 3,631 (±2,121) CFU/100mL, respectively (Figure 3). The mean abundance of fecal enterococci was the lowest in artificial canal sections (465 (±2,12 CFU/100mL) and the highest in lake waters (4,350 (±1,317) CFU/100mL) (Figure 4). The presence of *P. aeruginosa* was recorded only in the artificial canal sections – on average 414 (±207) CFU/100mL (Fig. 4).

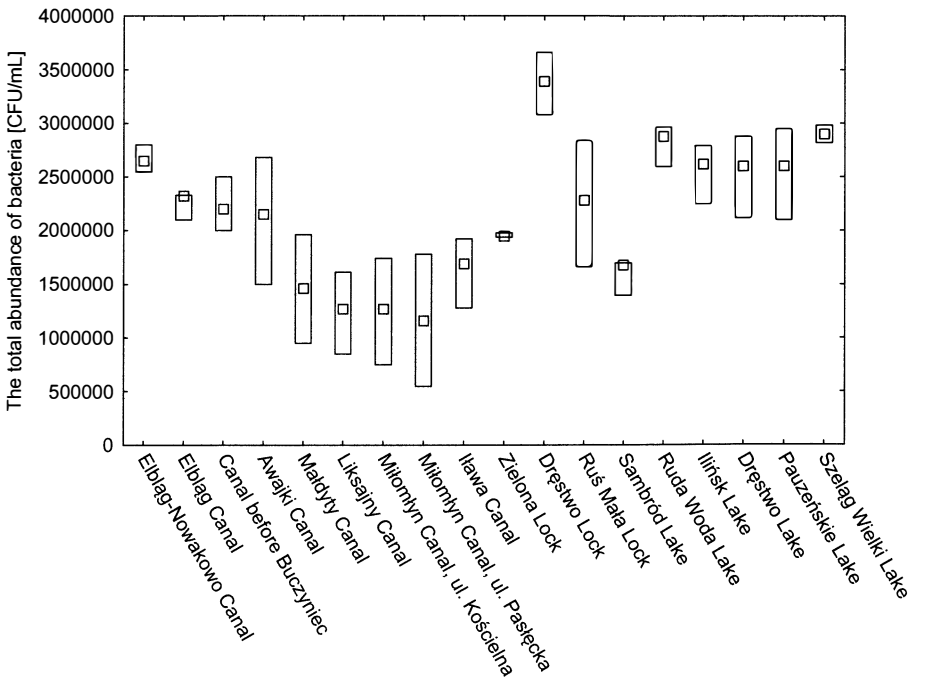

Fig. 2. Total number of bacteria in the waters of the Ostróda-Elbląg Canal (n=4). □ Median ▯ 25%-75%

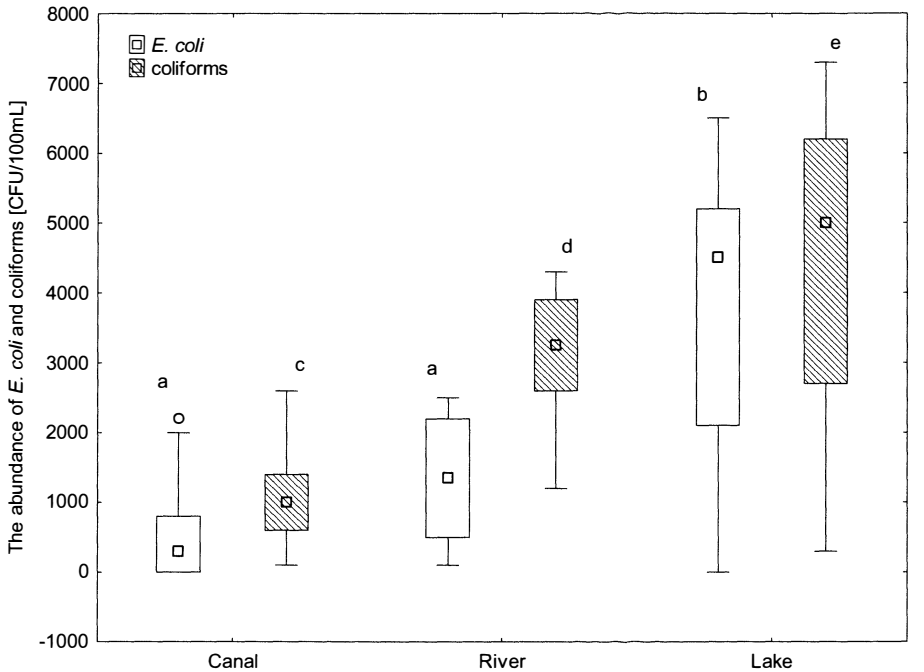

Fig. 3. The abundance of *E. coli* and coliforms in different types of waters of the river-lake system of the Augustów Canal and the Ostróda-Elbląg Canal.

□ Median ▯ 25%-75% ⌶ the range of non-rising values º- outliers

The same letters over a series of data mean that there are no statistically significant differences between them, while different letters mean statistically significant differences between the data (p<0,05).

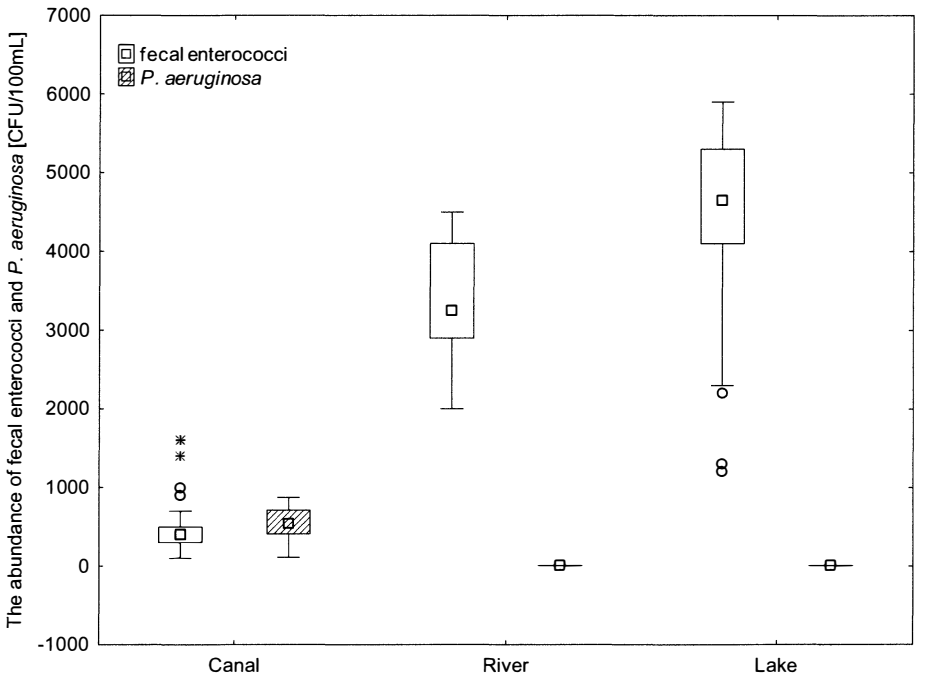

Fig. 4. The abundance of fecal enterococci and *P. aeruginosa* in different types of waters of the river-lake system of the Augustów Canal and the Ostróda-Elbląg Canal.

□ Median [ ] 25%-75% I the range of non-rising values °outliers
* extreme values

The same letters over a series of data mean that there are no statistically significant differences between them, while different letters mean statistically significant differences between the data ($p<0,05$).

Discussion

In our study, the waters of the Ostróda-Elbląg Canal had a much higher total abundance of bacteria than the Augustów Canal. This difference may be attributed to the presence of poorly accessible moorlands and rushes near the Ostróda-Elbląg Canal, which serve as shelters and breeding grounds for multiple species. In addition, the Canal includes a waterway popular among tourists (Furgała-Selezniow et al. 2006); waters flowing through areas of intensive human use are particularly vulnerable to bacteriological contamination (Adewoye 2010). Finally, the Ostróda-Elbląg Canal flows mainly through urban and agricultural areas, which increases the risk of bacteriological contamination due to

increased exposure to waste and run-off from the fields. All this contributes to increased microbiological contamination, additionally confirmed by elevated EC, total organic carbon, and total nitrogen.

The average abundance of coliforms and *Escherichia coli* was higher in the waters of the Ostróda-Elbląg Canal than in the waters of the Augustów Canal. The highest levels were recorded in lakes, probably due to the intensive use of these waters by people during the holiday season and discharge of sewage, as well as the run-off of fertilizers from fields (Hoyer et al. 2006, McLellan et al. 2007, Bradshaw et al. 2016). In general, the increased abundance of bacteria in lakes may be related by their constant supply by the rivers flowing into them. Finally, a significant factor is the presence of numerous bird species (Fogarty et al. 2003) – both the Augustów Canal and the Ostróda-Elbląg Canal are the habitats of many birds. The lowest abundance of coliforms and *E. coli* were recorded in artificial canal sections, especially those located at the beginning of the canal. Similar results were obtained by Barakat et al. (2013), who analyzed channels in the city of Beni-Mellal (Morocco). This indicates that the gradual deterioration of canal water status is most strongly influenced by human activity in the canal's catchment area, such as discharges from local sewage treatment plants and surface run-off from adjacent areas, especially urban agglomerations (Augustów, Elbląg, Ostróda).

A very important indicator of microbiological water pollution are fecal enterococci, which are a sign – in contrast to *E. coli* – of contamination distant in time (Anderson et al. 2005, Byappanahalli et al. 2012). The average abundance of enterococci was higher in the waters of the Ostróda-Elbląg Canal than in the waters of the Augustów Canal. Similarly to the coliforms and *E. coli*, the highest abundance of these bacteria were recorded in lakes, followed by rivers and artificial canal sections. Many species of fecal enterococci can be found in the sand on the beach and on aquatic plants (Boehm and Sassoubre 2014). Moreover, it has been shown that biogenic compounds are carried to lakes along with tributaries, which causes deterioration of water quality in flow lakes (Biedka and Wawrentowicz 2011). Along with rivers and watercourses flowing into lakes, bacteria can also get, hence the strong domination of fecal enterococci, into lake ecosystems. Enterococci are also abundant in animal feces (Layton et al. 2010), and since the rivers feeding the Augustów Canal flow through agricultural areas where animals graze, a higher abundance of these bacteria was recorded in river sections, compared to the artificial sections of the investigated canals. Another species of bacteria treated as an indicator of fecal water pollution is *P. aeruginosa*, present in soil, water, sewage, feces of warm-blooded animals, and on plants, from which they enter surface waters during rainfall (Niewolak and Opieka 2000). These bacteria can be found in increased amounts in rivers near urban areas (Mena and Gerba 2009). In our study, *P. aeruginosa* was found only in artificial canal sections, which confirms that the main source of these bacteria is water coming from urban areas.

Our study showed that the total bacterioplankton abundance and the occurrence of indicator bacteria depended on the physicochemical properties of waters. Water temperature is a particularly important parameter, as it modifies

metabolic processes in the cells of microorganisms, thus regulating their abundance. The most sensitive to changes in water temperature are mesophilic bacteria because they are allochthonous organisms and water is not their natural habitat (Chomutowska 2009). In our study, an increase in water temperature correlated with a decrease in the abundance of *Escherichia coli* ($r^2 = -0.69$, $p<0.05$) and coliforms ($r^2 = -0.71$, $p<0.05$). In contrast, a positive correlation with water temperature was observed for *P. aeruginosa* ($r^2 = 0.64$, $p< 0.05$). These results are in line with observations that *Escherichia coli* survive in cooler water environment for longer than in the waters with higher temperatures that occur in late summer (Sampson et al. 2006).

The electrolytic conductivity value (EC) is an indicator of the amount of ions reaching water reservoirs (Suchowolec and Górniak 2006). High EC values indicate high chemical pollution of water. In our study, sites with high bacterial abundance were also characterized by high EC ($r^2 = 0.72$, $p<0.05$). EC also positively and statistically significantly correlated with the occurrence of *P. aeruginosa* ($r^2 = 0.67$, $p<0.05$). Another important factor determining the occurrence of indicator bacteria is water pH, with a slightly alkaline environment being optimal for aquatic bacteria (Chomutowska 2009, Augustynowicz et al. 2015). This was confirmed by a positive correlation between the abundance of coliforms ($r^2 = 0.62$, $p<0.05$) and the abundance of *E. coli* ($r^2 = 0.78$, $p<0.05$) and the pH of water in our study. However, no statistically significant correlation was observed between pH and total bacterioplankton abundance, probably due to the fact that many species of bacteria prefer water with lower pH (Starliper et al. 2015).

Total nitrogen (TN) and total organic carbon (TOC) are also important parameters determining the structure of bacterioplankton, which is an important component of the microbiological loop and actively participates in the decomposition of organic matter (Siuda and Chróst 2002). A large abundance of bacteria in water indicates an increased content of organic compounds in the water (Chomutowska 2009). Not surprisingly, our study showed that in waters with increased TOC concentrations there was a higher total abundance of bacteria ($r^2 = 0.56$, $p<0.05$) and P. *aeruginosa* ($r^2 = 0.61$, $p<0.05$). The Dręstwo Lock on the Ostróda-Elbląg Canal and the Piecówka River flowing into the Augustów Canal were characterized by the highest TOC concentrations. In the case of the Piecówka River, this may have been due to the run-off of water from agricultural lands along the river. It was also shown that TN concentrations in water positively correlated with the abundance of *E. coli* ($r^2 = 0.64$, $p<0.05$), coliforms, and fecal enterococci ($r^2 = 0.69$, $p<0.05$). The lakes of the Ostróda-Elbląg Canal were characterized by the highest concentrations of total nitrogen and total organic carbon in water. These compounds enter lake waters mainly as a result of sewage discharges and fertiliser run-off from farmlands (Hijosa-Valsero et al. 2016). As heterotrophic bacterioplankton can use them for its own metabolic needs (Zehr et al. 2002), it comes as no surprise that the abundance of bacteria was also highest in the lakes of the Ostróda-Elbląg Canal.

Conclusions

Our results showed that the Ostróda-Elbląg Canal was characterized by a much higher microbiological contamination in comparison to the river and lake system of the Augustów Canal, which is related to the higher fertility of its waters, as evidenced by the increased values of EC, total nitrogen, and total organic carbon. The highest values of total bacterial count and indicator bacterial count were recorded in lakes. River sections were characterized by a higher abundance of total bacterioplankton, coliforms, *E. coli* and fecal enterococci in comparison to the artificial sections of the canals, with the lowest microbiological contamination recorded in the initial sections of both canals. Moreover, *P. aeruginosa* was found only in the artificial sections, which indicates that its main sources were discharge from local sewage treatment plants and surface run-off from adjacent areas, especially from urban agglomerations.

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SIMPLE AND FAST METHOD FOR DETERMINATION OF TRACE CADMIUM IONS IN ENVIRONMENTAL WATER SAMPLES CONTAINING HUMIC SUBSTANCES

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Abstract

A simple and fast anodic stripping voltammetric procedure for trace determination of cadmium in environmental water samples is described. The interferences caused by humic substances were removed by mixing an analysed sample with resin in a voltammetric cell. The following optimum conditions were found: supporting electrolyte 0.1 mol L^{-1} HCl, the 10 mL of sample was mixing with 0.5 g of resin for 5 min. The proposed procedure was successfully applied for the detection of cadmium in environmental water samples.

Key words: cadmium, environmental waters, humic substances, voltammetric determination

Introduction

The increasing concentration of heavy metals in natural waters is one of the most important elements of environmental contamination and, if they are present in concentrations above norm, may have a negative effect on living organisms and plants. One such metal is cadmium, which as a result of human activity has become one of the main chemical pollutants of the environment.

Cadmium is relatively poorly distributed in the Earth's crust; the average content is about 0.00004% and is present in air, water, soil, and plant and animal tissues. In nature, the metal is not present in a free state, but is present mainly in sulphide ores of zinc, copper, or lead. Rich in cadmium in particular are zinc ores (3%). Their mining and processing releases significant quantities into the atmosphere, hydrosphere, and soil, which contributes to the contamination of the human environment. Cadmium is also present in fossil fuels, for example coal. The share of the combustion process of carbons in cadmium pollution in urban areas is almost 10% [1-3]. Cadmium is also used in many technological processes in various branches of industry and agriculture. In the industry, cadmium is used for the production of dyes and stabilizers for plastics, galvanic protective coatings, solder and alloys, cadmium rods. It is also used for the production

of alkaline nickel-cadmium batteries, fireworks, fluorescent inks. A significant source of cadmium in the environment are fertilizers, which are contaminated with this metal in an amount of 10 to 100 mg/kg. Their long-term and widespread use leads to contamination of the soil with cadmium. Cadmium, once introduced into the environment, is not subject to degradation and remains in continuous circulation. Environmental exposure can lead to the absorption of large amounts of cadmium and the toxic effect of this element on the body [4-7].

Cadmium is an extremely harmful element for living organisms. The greatest damage is caused in organs where it is easily accumulated, i.e. liver, kidneys, bones, testes. Acute poisoning caused by a single high dose of metal in humans is rare. The poisoning caused by the long-term effect of cadmium on the body is much more common. In humans, long-term exposure of the organism to cadmium and the absorption of small amounts of this metal leads to chronic poisoning, which very often for a long time (about a year) may be asymptomatic [4,8-10].

Because, as mentioned above, long-term exposure is very harmful to humans, even at very low concentrations of cadmium, there is a constant need for simple, cheap and fast methods to determine even the lowest concentrations of cadmium in environmental waters. One of the techniques that meets all these requirements is stripping voltammetry. Stripping voltammetry is an electrochemical technique that measures the intensity of a current flowing through an electrode as a function of the potential applied to it after the earlier accumulation of the determined element on this electrode. This method has many advantages, such as fast measurement, cheap equipment, and low detection limits. However, this method has a disadvantage related to interference caused by the presence of humic substances in environmental samples [11-13].

Humic substances (HS) are commonly occurring matrix components in environmental samples. Among humic substances we can distinguish fulvic acids (FA) which are water soluble under all pH, humic acids (HA) which are soluble in water at higher pH, and humins which are water insoluble [14]. Humic substances are a heterogeneous mixture of natural organic substances that are widely distributed in soil, sediments, and different environmental waters, such as river water or lake water. These substances are formed by humification, i.e. the microbiological decomposition of plant and animal residues. The structure of humic substances is complex and diverse and depends on the conditions in which the humification occurred. Humic substances found in environmental water in normal concentrations are not harmful to human and animal health but even their small concentrations, as repeatedly proven in literature, can cause interference in voltammetric measurement [15].

Recently, a few voltammetric procedures have been reported for the determination of cadmium in different samples. However, in these procedures it is necessary to remove or destroy the organic matter present in the environmental samples. This is usually accomplished using ultraviolet irradiation, which takes a long time [16-18]. Therefore, the aim of this work will be to present a fast and simple method for determining the trace of cadmium ions in waters containing HS. In order to reduce the interference associated with the presence of HS, it

was proposed to introduce Amberlite XAD-7 resin into the measuring cell. In this case, HS present in the analysed sample do not block the working electrode on which cadmium determination takes place because they are adsorbing on the resin. Optimization of the proposed procedure was aimed at choosing a number of parameters for obtaining the most efficient removal of HS by means of a resin and at the same time quantitatively leaving cadmium ions in solution. The following parameters were selected: supporting electrolyte composition, the ratio of the resin mass to the volume of the sample, the time of mixing the resin with the sample.

Materials and methods

Apparatus

All voltammetric measurements were carried out with an Autolab PGSTAT 10 analyzer (Utrecht, Netherlands) and a hanging mercury drop electrode (HMDE) (MTM Cracow, Poland). A three-electrode cell, volume 10 mL, consisting of HMDE as a working electrode, a Pt as auxiliary electrode and an Ag/AgCl as reference electrode was used.

Reagents

All chemicals used were of analytical reagent grade or Suprapur. A working standard solution of 1×10^{-4} mol L^{-1} Cd(II) was prepared from a dilution of 1 g L^{-1} Cd(II) standard solution (Merck, Darmstadt, Germany). Humic acid sodium salt (HA) was obtained from Aldrich. The fulvic acid (FA) and natural organic matter (NOM) were obtained from the Suwannee River and purchased from the International Humic Substances Society. The Amberlite XAD-7 resin was obtained from Sigma (St. Louis, MO, USA), and the resin was washed four times with triply distilled water and dried at the temperature 50 °C. All solutions were prepared using triply distilled water.

Measurement procedure

The following components were introduced into the volumetric cell:

- analyzed sample or synthetic sample containing Cd(II) and various amounts of humic substances
- 1 mL of 1 mol L^{-1} HCl
- an appropriate amount of H_2O to make up to 10 mL volume
- 0.5 g resin.

The solution prepared in this way was mixed using a magnetic stirrer for 5 min. with simultaneous deoxidization using nitrogen gas. At that time, humic

substances adsorbed onto the resin while Cd(II) ions remained in solution. After this time, a voltamperometric measurement was carried out in two steps:

- accumulation, performed using a potential of -0.9 V for 30 s with stirring; during this time Cd(II) was reduced and deposited as metallic cadmium on the mercury electrode
- after 5 s equilibration time, the differential pulse voltammogram was recorded changing potential towards positive values from -0.9 V to -0.4 V; during this time accumulated cadmium was oxidized with the intensity of the obtained peak on the voltammogram directly proportional to the concentration of Cd(II) in the solution. The peak of cadmium appeared at ~ -0.62 V.

Results

The following parameters were examined in the selection of the most ideal conditions of the proposed procedure: supporting electrolyte composition, the ratio of the resin mass to the volume of the sample, the time of mixing the resin with the sample.

Supporting electrolyte composition

Literature data show that the best effect in the removal of humic substances by Amberlite XAD-7 resin is obtained in an acidic environment [19-21]. Therefore, several acids have been selected for initial testing, such as CH_3COOH, HCl, HNO_3, H_3PO_4. The conducted research was aimed at investigating in the presence of which acid humic substances are most effectively removed from the analyzed sample. This was assessed by carrying out a series of measurements in which the cadmium signal was tested for each acid in the presence of increasing HA concentrations. It was found that the highest cadmium signal in the presence of increasing concentrations of HA was obtained in the presence of HCl. It can be deduced from this that in such a case the humic substances are removed to the greatest extent, thanks to which the cadmium signal is disturbed to the least degree.

The ratio of the resin mass to the volume of the sample

At first, for research aimed at selection, the ratio of the resin mass to the volume of the sample humic acids as the interferent was chosen. The measurements were performed for 10 mL of solution and different mass of resin: 0.2 g, 0.3 g, 0.4 g, 0.5 g and 0.6 g. The time of mixing the resin with the sample was 5 min. The solutions contain a fixed concentration of Cd(II) 5×10^{-7} mol L^{-1}, 0.1 mol L^{-1} HCl and an increasing concentration of HA in the range from 0.25 mg L^{-1} to 4 mg L^{-1}. The obtained results are presented in Fig. 1. As can be seen, as the

resin mass increases, the cadmium signal is less diminished with the increasing concentration of HA. Since the differences in the obtained cadmium signals for 0.5 g and 0.6 g of resin are small at the most optimal conditions, it was proposed to add 0.5 g of resin to 10 mL of the solution.

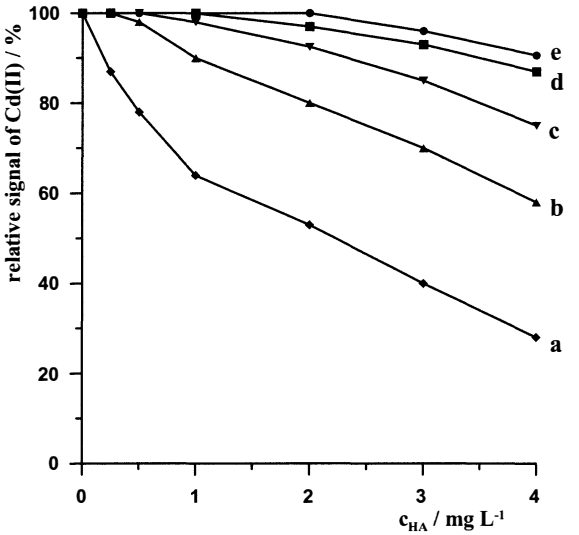

Figure 1. The influence of concentration of humic acid (HA) on Cd(II) relative signal for mixing sample with different masses of resin: 0.2 g (a); 0.3 g (b); 0.4 g (c); 0.5 g (d); 0.6 g (e). The time of mixing the resin with the sample was 5 min. Concentration of Cd(II) 5×10^{-7} mol L^{-1}, accumulation potential and time -0.9 V and 30 s, respectively.

For comparison, identical measurements for selecting the ratio of the resin mass to the volume of the sample using fulvic acids (FA) and natural organic matter (NOM) as interferents were performed. The experiments were carried out using different masses of resin: 0.2 g, 0.3 g, 0.4 g, 0.5 g, 0.6 g and different concentrations of FA or NOM in the range from 0.25 mg L^{-1} to 4 mg L^{-1}. The results obtained were similar to those for HA. So with the increase in resin mass, the voltammetric cadmium signal corresponding to its content in the solution is less suppressed by FA or NOM. Because also in this case the results obtained for 0.5 g and 0.6 g of resin were very similar, it was found that the most optimal resin and resin introduced into 10 mL of the solution is 0.5 g of resin.

The time of mixing the resin with the sample

At first in order to choose the optimal time of mixing resin with the sample, humic acid as an interferent was selected. The measurements were performed for 10 mL of solution and 0.5 g of resin while the time of mixing the resin with the sample was changed from 3 min. to 7 min. The solutions contain a fixed

concentration of Cd(II) 5×10^{-7} mol L^{-1}, 0.1 mol L^{-1} HCl and increasing concentrations of HA in the range from 0.25 mg L^{-1} to 4 mg L^{-1}. The obtained results are presented in Fig. 2. As can be seen, as the mixing time increases from 3 to 5 min. the signal of cadmium increases too; for a mixing time longer than 5 min. the cadmium signal did not change. That is why 5 min. was chosen for further research as the time for mixing the solution with the resin, because further prolonging the mixing time did not increase efficiency in the removal of humic substances.

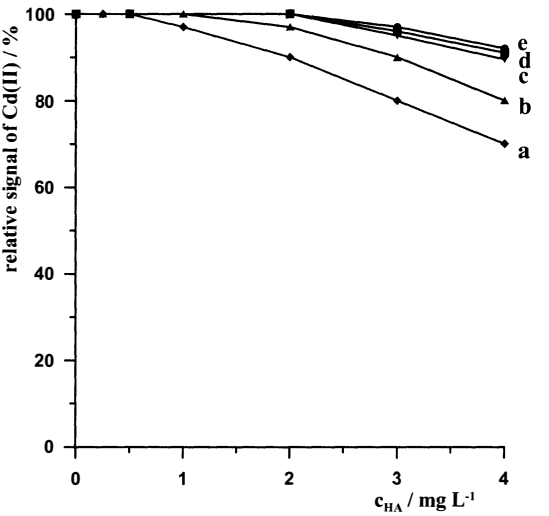

Figure 2. The influence of concentration of humic acid (HA) on Cd(II) relative signal for different times of mixing the resin with the sample: 3 min. (a); 4 min. (b); 5 min. (c); 6 min. (d); 7 min. (e). The mass of resin was 0.5 g. Concentration of Cd(II) 5×10^{-7} mol L^{-1}, accumulation potential and time -0.9 V and 30 s, respectively.

For comparison, identical measurements for selecting the time of mixing the resin with the sample using fulvic acids (FA) and natural organic matter (NOM) as interferents were performed. The experiments were carried out using different mixing times: 3 min., 4 min., 5 min., 6 min. and 7 min. and different concentrations of FA or NOM in the range from 0.25 mg L^{-1} to 4 mg L^{-1}. The results obtained were similar to those for HA. So with the increase of mixing time, the voltammetric cadmium signal corresponding to its content in the solution is less suppressed by FA or NOM. Since, as in the case of HA, differences in the cadmium signal in the presence of both FA and NOM for mixing times of 5 min. 6 min. and 7 min. are insignificant, 5 min. was chosen as the most optimal mixing time. In this case, an important rule was that the total time of the measurement procedure was as short as possible.

Validation

As is known, the best way to validate a procedure is to analyze a certified reference material. It is best that the matrix of such material corresponds to the matrix of samples for analysis, which a given procedure is dedicated to. Therefore, choosing SPS-WW1 Waste Water as a certified reference material seems to be a good choice to validate the proposed procedure. In addition to the organic matrix characteristic for waste water, this material contains, besides cadmium, twelve other elements with concentrations traceable to ultra pure metals or stechiomerically well-defined substances. Concentrations of all elements were as follows: 2000 ± 10 ng/mL Al, 100.0 ± 0.5 ng/mL As, 20.0 ± 0.1 ng/mL Cd, 60.0 ± 0.3 ng/mL Co, 200 ± 1 ng/mL Cr, 400 ± 2 ng/mL Cu, 1000 ± 5 ng/mL Fe, 400 ± 2 ng/mL Mn, 1000 ± 5 ng/mL Ni, 1000 ± 5 ng/mL P, 100.0 ± 0.5 ng/mL Pb, 100.0 ± 0.5 ng/mL V and 600 ± 6 ng/mL Zn. Results of cadmium determination in certified reference material SPS-WW1 Waste Water are presented in Table 1. In order to test the possibility of using the proposed method in samples with more complicated matrices, the SPS-WW1 Waste Water was additionally spiked with different concentrations of humic acid, fulvic acid, and natural organic matter. As can be seen both for the certified reference material and the material enriched in various concentrations of humic substances and natural organic matter, satisfactory results were obtained and the concentrations of determined cadmium ranged from 18.8 ± 0.7 to 20.5 ± 0.4 ng/mL.

Table 1. Results of Cd(II) determination in certified reference material SPS-WW1 Waste Water with certified value of Cd(II) 20.0 ± 0.1 ng/mL. Experiments were performed at the recommended conditions.

Sample	Concentration of added substance [mg L^{-1}]			Cd(II) determined [ng/mL]
	HA	FA	NOM	
SPS-WW1 Waste Water	-	-	-	19.6 ± 0.5
	1	-	-	19.2 ± 0.6
	-	1	-	20.5 ± 0.4
	-	-	1	19.4 ± 0.5
	0.5	0.5	0.5	18.8 ± 0.7

Analytical application

In order to examine the performance of the proposed procedure, it was applied to the determination of trace amounts of cadmium in Bystrzyca river water samples (collected from eastern areas of Poland). To prove that the proposed method of humic substances removal by means of Amberlite XAD-7 resin was proper, the samples of Bystrzyca river water were spiked with 5×10^{-7} mol L^{-1}

Cd(II) and different amounts 1 or 2 mg L^{-1} of HA, FA or NOM. The samples were analysed using the standard addition method. The obtained recoveries of cadmium were in the range from 94.3 to 97.3% with relative standard deviation between 5.2 and 6.4%, confirming that the proposed procedure is a suitable tool for analysis of environmental water samples for cadmium monitoring.

Conclusions

Combination of the anodic voltammetric method for determining trace amounts of cadmium with the simultaneous introduction of resin into the voltammetric cell to the analyzed solution made it possible to elaborate a simple and fast procedure in which interference from humic substances was minimalized. Its main advantage is the possibility of low-cost direct determination of Cd(II) in environmental water samples containing humic substances without the necessity of preliminary UV-irradiation of the samples. This drastically reduces analysis time. The proposed procedure looks promising and can be recommended for monitoring the environment.

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WARM STRATIFICATION OF APPLE SEEDS LEADS TO ALTERATIONS IN BIOTIN-CONTAINING PROTEIN LEVELS

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Abstract

Warm stratification stimulates apple (*Malus domestica* Borkh.) seeds' ageing. In our work, warm stratification was used as a treatment leading to the controlled seeds' deterioration. We examined the levels of biotin-containing proteins (indicators of seeds' viability) and ubiquitin-marked proteins in the embryonic axes of aged seeds. Alterations in the level of biotin-containing proteins depended on the duration of warm stratification. No change in the content of ubiquitinated proteins was observed.

Key words: biotinylated proteins, seeds ageing, ubiquitin-marked proteins, warm stratification

Introduction

Seeds are the main mobile form of Spermatophyte, enabling dispersal in the environment. Seeds' longevity, defined as the total time span during which seeds remain viable, is a significant trait for ecology, agronomy, and economy (Walters et al., 2010, Morscher et al., 2015). Seed quality impacts germination and seedlings' development. Seed viability depends on plant species; e.g. long-term (more than 2,000 years) seed longevity has been described for the date palm (*Phoenix dactylifera* L.) (Sallon et al., 2008). However, prolonged seed storage leads to ageing; even favourable conditions (low humidity and low temperature) do not prevent viability loss (Walters et al., 2010, Morscher et al., 2015). Inappropriate storage conditions accelerate seed deterioration. During seeds' loss of viability, adverse metabolic changes, such as increased membrane permeability and genetic/proteomic damage, occur.

Seeds which tolerate dehydration (orthodox seeds) are more resistant than recalcitrant seeds (sensitive to desiccation) to adverse environmental factors, so they age slowly. Ageing of orthodox seeds can be artificially induced at high temperatures and high relative humidity. Accelerated ageing has been recognized as a good predictor of the loss of seeds' storability (Priestley, 1986). Alterations in the level of specific proteins could be used as markers of seed longevity (Rajjou et al., 2008, Sano et al., 2016). It has been proposed that a group of late embryogenesis abundant (LEA) proteins participate in modulating seed longevity (Sano et al.,

2016). As was demonstrated for pea (*Pisum sativum* L.) seeds, seed-specific biotinylated protein (SBP65, a member of the 3 LEA proteins) is accumulated at the end of the maturation state (Duval et al., 1994a). The decrease of SBP65 was observed during pea seeds' loss of dormancy, pointing to the involvement of these proteins in metabolic processes at early phases of germination (Duval et al., 1994b). Reduction of SBP65 levels was also demonstrated for the embryos of viviparous pea mutant (*vip-1*), which germinate precociously in the pods (Dehaye et al., 1997). It is suggested that this protein is engaged in maintaining seed longevity by scavenging free biotin, which is a cofactor of several carboxylases and decarboxylases (Sano et al., 2016). Nevertheless, there is no information about changes in the levels of biotin-containing proteins in seeds after artificially induced ageing.

In the literature, the mechanism of the degradation of biotin-containing proteins in plant cells is not well described. Proteolysis could be related to ubiquitination – labelling of proteins for degradation in proteasome (Stuttmann et al., 2009). There is no data concerning alteration in ubiquitin-marked protein content in seeds subjected to warm stratification.

Apple (*Malus domestica* Borkh.) seeds belongs to the orthodox type and are characterised by deep dormancy (Lewak, 2011) which can be broken by cold stratification (at 4°C in wetted sand) (Lewak, 2011, Dębska et al., 2013). On the contrary, embryos isolated from seeds subjected to long-term warm stratification (at 25°C in wetted sand) do not germinate well. Moreover, seedlings developed from those embryos have morphological anomalies (Dębska et al., 2013).

The aim of the work was to investigate the levels of biotin-containing proteins and ubiquitin-marked proteins isolated from embryonic axes of warm stratified apple seeds to evaluate loss of vigour during artificially induced ageing. The obtained data provide more insights in the relationship between seed longevity and storage conditions.

Material and methods

Apple (*Malus domestica* Borkh.) seeds were stratified at 32ºC for 7, 14, 21 and 40 days. Embryos isolated from stratified seeds were placed on glass Petri dishes containing filter paper wetted with distilled water. Embryos were cultured in a growth chamber at 20°C with 12/12 h (light/dark) photoperiod and light intensity 50 μmol PAR m^{-2} s^{-1}. After 24 h of the culture, embryonic axes were isolated and used for analysis.

Detection of biotin-containing proteins and ubiquitin-marked proteins

Isolation of biotin-containing proteins was done as described by Krasuska et al. (2016). Protein samples (3 μg) were placed on a nitrocellulose membrane. The blocking step was done using 5% (w/v) nonfat dry milk in Tris buffered saline with 0.05% (v/v) Tween-20 (TBST). After incubation the nitrocellulose membrane was washed with TBST and marked with alkaline phosphatase conjugated with streptavidin (S2890 Sigma-Aldrich) solution in 100 mM Tris-HCl pH 9.5 containing

100 mM NaCl and 5 mM $MgCl_2$. Visualization of the spots was performed using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

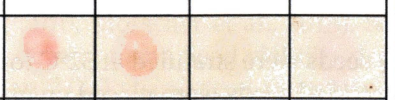
A similar procedure was done for ubiquitin-marked proteins. After insetting the protein probes on a nitrocellulose membrane and a blocking step, the nitrocellulose was washed with TBST. Then, the nitrocellulose membrane was incubated with a polyclonal anti-ubiquitin antibodies (AS08 307 Agrisera) solution in TBST (1:10,000) for 1 h. After another washing step with TBST, the nitrocellulose membrane was incubated with secondary antibodies (anti-Rabbit-Alkaline Phosphatase conjugated, A3687 Agrisera) 1:30,000. Visualisation of the protein spots was done as described above.

Both detection procedures were done in three independent experiments and the representative results are shown. After a dot blot test, the intensity of the spots was estimated using a densitometry analysis (ImageJ programme).

Results

Densitometry analysis indicated that the content of biotin-containing proteins in axes of apple embryos isolated from seeds stratified for 7 days was 148. A similar level of biotin-containing proteins in homogenates from embryonic axes isolated after 14 days of warm stratification was observed (Fig. 1). Prolonged warm stratification (21 and 40 days) decreased the content of these proteins. Moreover, no significant differences in the intensity of visualised protein spots for these probes were revealed (Fig. 1).

The level of ubiquitin-marked proteins isolated from embryonic axes of seeds stratified for 7, 14, 21 and 40 days was similar (Fig. 1).

	Biotin containing proteins				Ubiquitin marked proteins			
Days of warm stratyfication	7	14	21	40	7	14	21	40
Dot blot								
Densitometry analysis	148±5	145±12	118±5	120±7	235±10	228±15	240±15	248±13

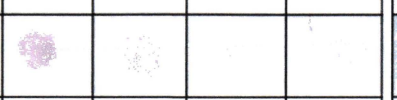
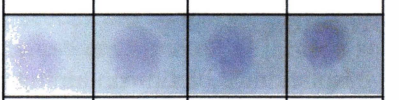
	Biotin containing proteins				Ubiquitin marked proteins			
Days of warm stratification	7	14	21	40	7	14	21	40
Dot blot								
Densitometry analysis	148±5	145±12	118±5	120±7	235±10	228±15	240±15	248±13

Figure 1. The levels of biotin-containing proteins and ubiquitin-marked proteins in axes of apple embryos isolated from seeds stratified at 32°C for 7, 14, 21 or 40 days. Densitometry analysis ± SD.

Discussion

The exposure of seeds to high temperature and high humidity is known as the accelerated ageing procedure and can be performed to indicate seeds' vigor and storability. A rapid deterioration process of seeds points to limited ability to germinate (McDonald, 1999). As was demonstrated for sunflower (*Helianthus annuus* L.) seeds, storage at 45°C and 100% RH reduced germination and led to the growth of atypical seedlings. Finally, such conditions resulted in the seeds' death (Corbineau et al., 1988). In apple seeds, warm stratification, opposite to cold stratification, does not alleviate dormancy (Lewak et al., 2011, Dębska et al., 2013). Furthermore, prolonged warm stratification leads to seeds' ageing and the formation of abnormal seedlings or even their death (Dębska et al., 2013).

Proteins and mRNA are stored in mature seeds as they are sufficient for the first phases of germination (Rajjou et al., 2004). Proteomic analyses done for Arabidopsis (*Arabidopsis thaliana* (L.) Heynh.) seeds subjected to controlled deterioration have revealed the linkage between loss in seed vigour and protein alterations in the dry seeds. During germination, the proteome of seeds with low vigor displayed some abnormalities (Rajjou et al., 2008). Rajjou et al. (2008) demonstrated a decreased level of the dehydrin/RAB (Responsive to abscisic acid) group of LEA proteins during seeds' ageing. The authors also proposed that this group of LEA is the most involved in seed longevity; h wever, they did not exclude other LEA proteins' participation in maintaining seed vigour. Our results indicated that biotin-containing protein levels in apple embryonic axes decrease as the warm stratification period is prolonged. This could point to a link between apple embryo vigour and the amount of such proteins. A lower abundance of biotin-containing proteins was observed during germination of apple embryos (Krasuska et al., 2016) and during pea seeds' loss of dormancy (Duval et al., 1994b), indicating the involvement of these proteins in metabolic pathways during early phases of seed germination. Moreover, the increased lev l of oxidation of pro ein homologous to SBP65 isolated from Arabidopsis deteriorated seeds has been demonstrated (Rajjou et al., 2008). It has been proposed that the survival of dry, aged Arabidopsis seeds can be altered due to the oxidation of biotin-containing proteins (Rajjou et al., 2008). The increase of protein oxidation is commonly linked to stress conditions and elevation of reactive oxygen species (ROS) levels, which are also high in aged tissues (Rajjou et al., 2008, Dębska et al., 2013). During warm stratification of apple seeds, the increase of ROS production and protein carbonylation (type of oxidation) was observed (D bska et al., 2013). Oxidation of proteins may stimulate their degradation. As we did not observe any changes in ubiquitin-marked proteins' levels, we suspect that the decrease of biotin-containing proteins' levels may be due to their oxidation and proteolysis.

In conclusion, the presented data indicate the relationship between the warm stratification-induced loss of viability of apple seeds and the decrease of seeds' biotin-containing proteins.

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INFORMING PEOPLE ABOUT CLIMATE CHANGE

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Abstract

The article estimates the need to implement a strategy of sustainable development. It says that it is possible to achieve the premises of sustainable consumption by stimulating demand for products manufactured with the use of energy-efficient technologies, non-toxic materials, etc. The article also outlines the urgency of the problem of waste accumulation in Ukraine. The necessity of solving the problem of solid household waste management is determined. The quality of household waste management determines the living conditions of people and is an indicator of the maturity of a society. We strongly believe that promotion of sustainable consumption ideas will help to reduce the amount of solid waste and will help prevent climate change. The purpose of the present study was to identify the main points that climate change related organizations and initiatives can develop to increase people's knowledge about the topic. Informational and educational programs on climate change should be conducted to increase the level of ecological culture and awareness of the global problem. Several areas of activity have been identified: savings and restrictions on the use of fossil fuels; informing about renewable energy sources; sorting household solid waste, etc.

Key words: climate change, ecological education, solid waste, sustainable consumption.

Introduction

According to reports by the Intergovernmental Panel on Climate Change (IPCC), human activity has caused an increase of average global temperature in the world by 1°C [1]. The upper limit for raising the global temperature, enshrined in international agreements, is 1.5-2°C above pre-industrial levels [2]. It is believed that warming is caused by anthropogenic activity. Beyond this limit will have great negative consequences. The main reason for the increased temperature is that the concentration of greenhouse gases has changed. Scientists consider that concentration changes because of the use of fossil fuels, the destruction of forests, the accumulation of solid household waste, intensive agricultural activities, etc. Climate change will affect all areas of human activity.

Irreversible changes are transforming all of nature: air, water, and soils. For this reason, the issue of prevention and adaptation to climate change should be relevant to people, regardless of place of residence, education, or sphere of activity.

Materials and methods

On the basis of our research aim, we derived a few criteria for data collection and analysis. Free access to the results of the research on climate change was the first criteria. Therefore, the basic information on the topic was collected from open sources and included the reports of international research groups, including the NASA Goddard Institute for Space Studies and the Intergovernmental Panel on Climate Change. The data of the studies whose aim was to assess public awareness of environmental problems and the willingness to take certain actions to prevent it were also included in the analysis. Also, we should admit that the theoretical, methodical, and applied statements as for solid waste management, allowing for ecological and economic criteria, has been investigated.

There are many problems and obstacles that Ukraine faces in its path towards integration into the community of European countries. On the one hand, such problems should not prevent attempts to integrate environmental and social elements into economic development; on the other hand, such obstacles should be identified in order to develop appropriate measures, including the environmental problems of waste management in urban and rural areas. Scientists are focusing on resource conservation and minimizing the negative environmental impacts of waste. However, despite the large number of scientific papers and the relevance of the issue, the environmental and economic problems of waste management have not yet been resolved. A sociological method was used to demonstrate the need for the development of an organizational mechanism for solid waste management and adaptation to climate change.

The purpose of the present study was to identify the main points that climate-change-related organizations and initiatives can develop to increase people's knowledge about the topic. Accordingly, we designed questionnaires and got 385 valid responses.

Results

The state of the environment is the only indicator of the ecological consciousness of a population living in a certain territory, and the main factor in ensuring the health and well-being of people. Over the past ten years, a number of social studies connected with environmental problems have been carried out in Ukraine [3, 4, *etc.*]. The above-mentioned studies gave a fairly complete picture of the attitude of citizens towards the environment in the context of their willingness to support the environmental aspects of reforms within the framework of the implementation of the Association Agreement between Ukraine and the EU; or in the context of the implementation of the Agenda for Development

until 2030 – Sustainable Development Goals. Social studies demonstrate support for environmental values, which means that most Ukrainians regard environmental protection as an important area. In addition, most people are aware of the impact of environmental factors on the quality of their lives and health. At the same time, the readiness of Ukrainians to change their habits and to take action to protect the environment is quite low.

It has been proved that the spread of sustainable consumption is a way to preserve the environment and improve the quality of life. Sustainable consumption is the use of goods and services that satisfy basic needs and improve quality of life with minimal use of natural resources and with the least damage to the environment. The main purpose here is to draw attention to the problems of health and the environment.

Research shows that the main motive behind the introduction of environmental practices is the possibility of saving when paying for utilities (saving consumption of the main types of resources: water, gas, heat and electricity). In particular, the most commonly used practices of responsible consumption are reduction of energy consumption, the sorting of most of the waste for disposal, the choice of an environmentally friendly type of transport (walking, cycling), partial waiving of the purchase of disposable plastic products, reducing household water consumption, etc. [3, 4].

In some social studies, climate change as a global issue is considered in the context of the respondent's acquaintance with this tendency, as well as with reasons and its consequences. Thus, the survey shows that more than half of Ukrainians believe that climate change is the result of human economic activity [3]. Such results could be interpreted as a sufficient degree of awareness. But there is a rather negative tendency. In the last five years (in 2014 – the first study, 2018 – the second study), the share of Ukrainians who consider climate change as a result of natural processes has increased, while the proportion of those who believe that climate change is a result of human activity has decreased. This tendency is even more negative in the context of increasing the dissemination of information on climate change in the media, social networks, and so on.

Many people in the world realize the problem of climate change and all the consequences. Now we have to make changes in our everyday life to survive.

Conducted sociological surveys in Zhytomyr (Ukraine) showed that 88% of respondents are concerned about climate change. We asked them what consequences are the most manifested (Fig. 1). This research shows that the biggest part of Zhytomyr's population (75%) is concerned about drought. Half of respondents (50%) worry about drinking water and strongly believe that drought happens because of climate change. Only 7% of people feel temperature anomalies, which we find very interesting because people usually talk about temperatures that have never happened before.

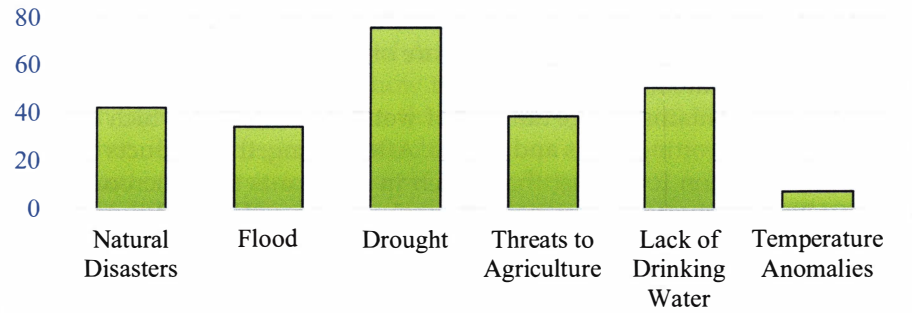

Figure 1. The consequences of climate change felt by the inhabitants of Zhytomyr

According to Eremenko I., Vynyarskaya M., Melnik Yu. the issues of adaptation to and prevention of climate change include measures in the sectors of energy, environmental protection, housing and communal services, agriculture, transport and other spheres of human activity. For Ukraine, energy efficiency and energy conservation are the most pressing. 67% of measures aimed at reducing greenhouse gas emissions are in this sector. It is precisely there that, according to the Energy Community, the scope of energy efficiency activities are the best for Ukraine [5].

The fact that in Ukraine for the last 4 years tariffs for domestic consumption of resources (in particular gas and heat) have been raised, plays a role in that achievement. Unfortunately, the reason for the introduction of energy and resource-saving measures by the Ukrainian population is not a transition in behavior according to the principles of responsible consumption. Therefore, the need to disseminate information on the consequences and dangers of climate change, as well as ways to adapt and prevent the deterioration of the situation, remains evident.

Solid waste is a common problem for all countries in the world. It is a great environmental threat, which causes climate change as well. We should admit that solid waste has an affect on water and air pollution. Also, garbage creates aesthetic problems. The amount of waste in Ukraine is rising every year. We strongly believe that sustainable consumption can solve the problem listed above. In this study, it is advisable to focus on the issue of solid waste management. Despite the fact that in the total amount of waste generated in the country, solid waste is less than 10%, the quality of its management, on the one hand, determines the living conditions of people and, on the other, is an indicator of the maturity of society.

Everybody knows about the Great Pacific Garbage Patch, which is the largest accumulation of plastic in the ocean. It contains about 80,000 tons of plastic or about 1.8 trillion plastic pieces [6]. In Ukraine, one person produces approximately 300 kg of solid waste in a year. However, there is a big difference in the amount of solid waste in rural and urban territories. The worst is that 98% of

it is not recycled and simply accumulates on piles of garbage. Currently, over 10,000 hectares of land are occupied by landfills (7,000) in the country. However, it should be noted that the data provided are official statistics that can be significantly underestimated.

Solid waste contains a large amount of wet organic matter, which while decomposing emits rotting odors and filtrate. After drying, the products of incomplete decomposition form dust that is rich in pollutants and microorganisms. As a result we have intense contamination of soil, air, surface and groundwater. Besides, in most rural areas of our country we do not have any garbage collection systems. In this case, household waste is stored in natural relief formations - beams, ravines, and river valleys. This poses an environmental risk because the pollutant-rich sewage enters the water bodies.

In Ukraine people throw away 20 million plastic bottles a year (that decompose over about 400 years). Therefore, we can assume that our country is part of the problem of The Great Pacific Garbage Patch. The main point is that we should not wait until the government will solve that problem; we should start right now and act.

Over the past 50 years, polymer manufacturing has accelerated by almost 200 times and is expected to double in the next 20 years [7]. Plastic is definitely dangerous for different groups of living organisms. However, we should emphasize that, under the sun, plastic emits methane and ethylene (greenhouse gasses). Dr. Sarah-Jeanne Royer says that for a long time we thought that the link between plastic and climate change was mainly focused on fossil fuels used to produce plastic. Now we are talking about greenhouse gasses emission from plastic degradation in the environment [8]. All of the polymer types tested by the scientists produce methane (CH_4) and ethylene (C_2H_4) when exposed to ambient solar radiation. The amount of greenhouse gasses produced depends on time under the sun [8]. Packaging and bottles are the main sources of polymer in everyday life. In the summer we have a great problem with disposable tableware that is usually burned or buried in the forest.

We assume that plastic causes a couple of problems:

- it increases the amount of solid waste;
- it emits greenhouse gasses in the process of degradation;
- it is dangerous for species;
- it creates aesthetic problems.

We conducted another sociological research in Zhytomyr (Ukraine) that showed that only 3.5% of inhabitants understand the reason why garbage should be recycled. The rest of the respondents do not realize the necessity and throw all waste in one dump. The main reason is that people see how all waste goes to the same container even when it is sorted. Moreover, all respondents said that they were not aware of the danger of luminescent bulbs and batteries. People throw that kind of garbage in the same dump.

The purpose of the present study was to identify the main points that climate-change-related organizations and initiatives can develop to increase people's knowledge about the topic. Our sample was composed of 385 respondents by the citizens of Zhytomyr. As told previously, most of our respondents were

concerned about climate change (88 per cent of our respondents were aware of climate change). Respondents were most concerned about the following effects of climate change: drought, lack of drinking water, and natural disasters. In addition, the results of the study show that it is better to inform the public about climate change adaptation actions through video reports in the media, posts on social networks, articles in print media.

The context of growing concern about climate change has increased the number of different climate and environmental organizations and initiatives in Ukraine. Their aim is to create a strong and high value for a clean environment in people's minds. Such organizations and initiatives bring much in climate activism as they make people willing to pay more attention to their contribution to climate problems.

We strongly believe that the main reason for the problem listed above is a lack of information. People do not realize the danger from the garbage that they produce every day. We can live without thousands of things that we buy (plastic packaging, bottles, disposable tableware etc.). It is possible to achieve sustainable development when the population's environmental awareness is raised. It is well-known that for profound change it is important to formulate restrictions, prohibitions, and changes in many behavioral habits that will be voluntarily accepted. Changes in the way of life of the population and the mechanisms of the economy are also necessary. It is important to start environmental education of the population at an early age.

Last year we started a "Climate Change Agents" campaign for 6-8th grade students, where we teach them the basics of sustainable consumption. They understand that their way of living can influence future generations' level of life. It was a great experience. High school students have been taught activities that they can carry out on a regular basis, namely: conservation of electricity; water conservation; sorting of plastic and paper waste; visits to shops and markets with reusable shopping bags; use of environmentally friendly types of transport; ecological tourism; garbage disposal after camping. During our classes, we teach students (climate agents) that we can use bottles, bags, and cups multiple times. That is the main step that society can take to reduce the amount of solid waste in the world. Moreover, we talked about the money economy while using non-disposable things (in Ukraine we pay for plastic bags in the supermarket).

The effects of climate change are manifested not only in violation of the normal state of natural ecosystems, but also in the changing living conditions of people. According to the State Statistics Service of Ukraine, more than 70% of the population live in the cities. Therefore, the problems and consequences of climate change are particularly relevant for urban areas. Cities of Ukraine, irrespective of their size and location, are more or less vulnerable to the effects of climate change. In addition, urban structures and energy sources can cause a worse effect and affect the research of the local warming [*9*].

Discussion and conclusions

Our results show that in recent years environmentally concerned organizations and initiatives have been developing rapidly in the largest cities of Ukraine (Kyiv, Poltava, Lviv, etc.). The case of Zhytomyr shows that climate and environmental organizations and initiatives are mainly represented by NGOs, individuals, and research institutions. At this point we realize that the population of our town is not ready to sort waste (one way to reduce the amount of solid waste). We specify a couple of common reasons such as governmental (conditions were not created) and educational (lack of information). We attribute some climate change with waste accumulation. Our research shows that people understand and feel climate changes. In addition, they can specify the consequences, which is why we have to show ways to solve the problem.

Then it could be said that the role of climate and environmental organizations and initiatives in informing people about climate change could have almost the same benefit as the official activities. NGOs and initiatives could generate the following effects:

- showing links between climate change and people's behavior;
- involving people in climate and environmental activism;
- involvement in providing strategies for embedding climate change adaptation in cities.

Obviously, it is necessary to develop and implement an integrated approach to addressing the problem of providing information and education actions on a regular basis and involving all stakeholders in their implementation. At this stage, we understand the necessity of ecological education and information availability. It is obvious that humanity must realize its responsibility for the impact of its activities on the environment. Promotion of sustainable consumption ideas among the population will achieve the goal.

We find that informational and educational programs on climate change are conducted to increase the level of ecological culture and awareness of the global problem. An international experience, such information support campaigns should include materials in the media, awareness-raising campaigns, involvement of educational institutions, and printed instructive materials. In Ukraine, in the field of climate change prevention, several areas of activity of civic organizations and international donors should be singled out:

- informing about the causes and consequences of climate change;
- saving and restrictions on the use of fossil fuels;
- informing about renewable energy sources;
- adopting and implementing programs and projects in the area of energy efficiency and transition to renewable energy sources. Our findings can be used as simple pieces of advice for organizations and initiatives related to climate.

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INFLUENCE OF FUNCTIONAL GROUPS OF POLYSTYRENE-DIVINYLBENZENE ADSORBENTS ON THE EFFECTIVENESS OF ACID DYE REMOVAL

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Abstract

For removal of C.I. Acid Red 18 (cochineal red A, ponceau 4R) from aqueous solutions, polystyrene Amberlyst A 21 and Lewatit VPOC 1064 were used. C.I. Acid Red 18 sorption was performed using static and dynamic methods. The parameters of the Freundlich and Langmuir isotherm models were calculated. The sorption rate constants were determined based on the pseudo-first-order, pseudo-second order and intraparticle diffusion models. The working and total exchange capacities, and bed and weight distribution coefficients were designated in the column system.

Key words: acid red, polystyrene adsorbents, dye removal

Introduction

Among the organic pollutants of the aquatic environment an important group are dyes and pigments. They are emitted into sewage from various industries including paint, textile, cosmetics, and paper. Dyes present in water, even in low concentrations, can stain significant areas of water giving it an unsightly appearance. They hinder the penetration of sunlight and thus inhibit photosynthesis and the development of biocenosis (1, 2). Textile wastes are a mixture of both organic and inorganic substances. They may contain not only significant amounts of dyes but also auxiliary agents (e.g. salts, acids, alkalis, surface active substances) as well as specific impurities (e.g. fats, waxes, casein, bleaching agents, starch) (3). These substances end up in sewage in the amount in which they are introduced into a dyeing bath and what is more, they do not wear out in the process of fiber dyeing as opposed to dyes. Table 1 shows the amounts of chemicals and auxiliaries (except dyes) contained in textile wastewater released into the environment in Europe.

Table 1. Content of auxiliaries in textile wastewaters in Europe (4).

Type of auxiliaries	Residue in sewage after the dyeing process
Carboxylic acids	15000-20000
Natural fibers contamination	50000-100000
Surfactants and detergents	20000-25000
Fats, esters, waxes	25000-30000
Dressings	80000-100000
Salts	200000-250000

The amount of unbound dye remaining in the dyeing bath depends inter alia on the type of dye, type of dyed fiber and dyeing method. The content of dyes in textile sewage after the dyeing process is from 2 to 50% depending on the type of dye used (5, 6). Table 2 shows the effects of the type of dye on the degree of its binding to fibers.

Table 2. The amount of dye entering textile sewage from dyeing baths (4).

Types of dye	Residual in sewage after the dyeing process (%)
Basic	2-3
Direct	5-20
Vat	5-20
Acid	7-20
Sulfur	20-40
Reactive	20-50

Acid dyes belong to one of the most numerous groups of dyes. They are most often used in the form of sodium salts of sulphonic acids, less frequently in the form of salts of carboxylic acids. The group of these dyes belongs to strong electrolytes, which in aqueous solutions are completely dissociated into coloured anions. Their permanent bond with fiber can be achieved by adding acids to the dye bath. In terms of chemical structure, they are azo dyes (mono- and diazo) (7). Azo dyes are the most important class of synthetic organic dyes used in the textile industry and therefore they can be considered as common industrial pollution. They are produced in large quantities and get into the environment during production processes. They are intended for dyeing wool and leather, and are also used in so-called household chemistry for colouring shampoos and lotions for baths, dishwashing liquids, toilet blocks as well as for coloring wood and paper (8). Cabrera and co-workers (9,10) obtained cochineal red A from dried and milled insects, and for its removal they used weakly basic anion exchangers of gel (Amberlite IRA 67) and macroporous (Amberlite IRA 96) structures. The determined sorption capacities were 0.318 kg and 0.271 kg cochineal

for 1 kg of Amberlite IRA-67 and 1 kg of Amberlite IRA-96, respectively. The modified clinoptilolite was used by Mirzaei et al. (11) for C.I. Acid Red 18 removal from aqueous solutions. The maximum adsorption capacity equaled 11 mg/g and was calculated from the Langmuir isotherm equation. In addition, kinetic studies showed that the adsorption was in agreement with the Elovich model. The proposed mechanism for removing the dyes are electrostatic interactions. Moreover, an attempt was made to remove C.I Acid Red 18 on a material based on titanium dioxide (As500). The monolayer capacity determined from the Langmuir equation was 24.92 mg/g. The time necessary to achieve the state of dynamic equilibrium was 180 minutes. In the case of dye solutions at a concentration of 100 mg/L and with increasing concentrations in the system, the period of equilibrium time increased. Experimental data were best described by the pseudo-second order model (12). Due to the complex structure of molecules, dyes are difficult to decompose by physical, chemical, and biological methods, as a result of which small amounts of toxic or carcinogenic products may be formed. One of the effective methods for removing dyes from water and sewage, both in the range of low and high concentrations, is their adsorption on porous synthetic sorbents (e. g. active carbons, ion exchange resins) (13). In connection with the above, the aim of the paper was to evaluate the effectiveness of Lewatit VPOC 1064 (VPOC1064) and Amberlyst 21 (A21) in the sorption process of C.I. Acid Red 18 (AR18, Fig. 1a) from aqueous solutions.

Materials and methods

Macroporous resins with a polystyrene matrix (Fig. 1b.) were used in the research: Lewatit VPOC 1064 (without the functional groups) and Amberlyst A21 (with the tertiary amino functional groups). Amberlyst A21 was prepared by washing with 1 M HCl and distilled water to remove impurities and convert the ionic form to chloride. In contrast, Lewatit VPOC 1064 was washed with 50% methanol with 4 M HCl at the ratio of 1:5 as well as with distilled water. Then both sorbents were dried at room temperature to a constant mass. The detailed information on the sorbents used is given in Figure 2.

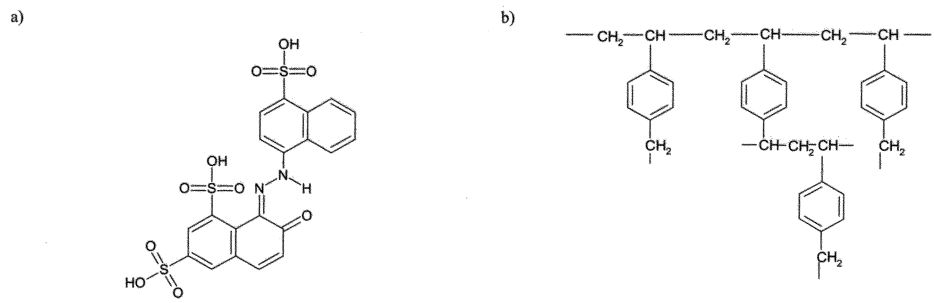

Figure 1. Structural formulae of dye (a) and polystyrene-divinylbenzene matrix (b) of the resins used.

Acetic acid, sodium chloride and sodium sulfate were purchased from POCh (Poland). The anionic surfactant sodium dodecyl sulfate (SDS) and the non-ionic surfactant 4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol (Triton X-100) were purchased from Sigma-Aldrich (Germany). C.I. Acid Red 18 (Sigma-Aldrich, Germany) is a synthetic azo dye. The molecular structure of C.I. Acid Red 18 ($C_{20}H_{11}N_2Na_3O_{10}S_3$, molecular weight 604.48 g/mol) is shown in Figure 1a. Solutions containing AR18 were prepared by dissolving a precisely weighed amount of dye in 1 liter of distilled water.

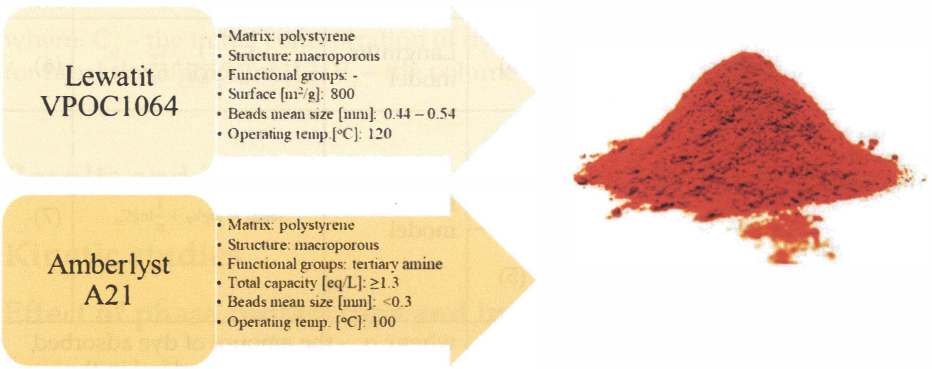

Figure 2. Physicochemical properties of the resins.

Static method

To perform laboratory tests using the static method, 100 ml conical flasks were used. The flasks were filled with 50 mL of a suitable dye solution and the resin of 0.5 g (± 0.0005). The flasks were then placed in a mechanical shaker (Elphin + 358S, Poland) with a vibration amplitude of 8 units at room temperature and stirred (180 cpm) from 1 to 240 min (kinetic test) or 24 h (equilibrium test). After shaking the sorbent was separated from the solution. The filtrate was analyzed using a spectrophotometer (Cary 60 UV-Vis, Agilent, USA) and then calculated:

– the amount of dye adsorbed at the equilibrium q_e [mg/g]

$$q_e = \frac{(C_0 - C_e)}{m} \cdot V \tag{1}$$

– the amount of dye adsorbed at the time t q_t [mg/g]

$$q_t = \frac{(C_0 - C_t)}{m} \cdot V \quad \text{for t = 1-240 min} \tag{2}$$

where: C_0 – the initial concentration of dye [mg/L], C_t – the concentration of dye after sorption time t [mg/L], V – the volume of solution [L], m – the mass of adsorbent [g].

In order to understand better the dynamics of the adsorption process and its mechanism, the following equations were used: pseudo-first order (PFO) (14), pseudo-second order (PSO) (15) and intraparticle diffusion (ID) (16). The kinetic parameters of C.I. Acid Red 18 sorption on the polystyrene adsorbents were calculated. Adsorption isotherms were determined using the Langmuir (17) and Freundlich models (18) (Table 1).

Table 1. Kinetics and equilibrium parameters.

Kinetic parameters		No.	Equilibrium parameters		No.
Pseudo-first order (PFO) model	$\log(q_e - q_t) = \log(q_e) - \frac{k_1}{2.303}t$	(3)	Langmuir model	$\frac{C_e}{q_e} = \frac{1}{Q_0 b} + \frac{C_e}{Q_0}$	(6)
Pseudo-second order (PSO) model	$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{1}{q_e}t$	(4)	Freundlich model	$\log q_e = \log k_F + \frac{1}{n}\log C_e$	(7)
Intraparticle diffusion (ID) model	$q_t = k_i t^{1/2} + C$	(5)			
where: k_1 – the adsorption rate constant [1/min], t – the time [min.], q_e, q_t – the amounts of dye adsorbed at equilibrium and time t, respectively [mg/g], k_2 – the adsorption rate constant [g/mg min], C – the constant illustrating the effect of the boundary layer on the sorption process			where: q_e – the amount of dye adsorbed per unit mass of the adsorbent in the equilibrium state [mg/g], C_e – the equilibrium dye concentration [mg/L], Q_0 – the Langmuir constant associated with the maximum sorption [mg/g], b – the Langmuir constant associated with the sorption energy [L/mg], K_F [mg/g], n – the Freundlich constants		

The effect of salt (Na_2SO_4), acetic acid (CH_3COOH) and surfactants (SDS, Triton X-100) on the AR18 sorption process on the Lewatit VPOC1064 and Amberlyst A21 was studied using the following conditions:

- Na_2SO_4 concentrations: C = 0-25 g/L Na_2SO_4, C_0 = 500 mg/L AR18, t = 15 min, V = 50 mL, m = 0.5 g, A = 8, room temperature,
- CH_3COOH concentrations: C = 0-2 g/L, C_0 = 500 mg/L AR18, t = 15 min, V = 50 mL, m = 0.5 g, A = 8, room temperature,
- SDS and Triton X-100 concentrations: C = 0-0.5 g/L, C_0 = 500 mg/L AR18, t = 15 min, V = 50 mL, m = 0.5 g, A = 8, room temperature.

The dye concentration after the sorption process was measured spectrophotometrically at the maximum wavelength.

Dynamic method

Laboratory tests were carried out using the dynamic method with a specially assembled set consisting of an ion exchange column with a diameter of

1 cm connected to a glass container by means of a rubber hose with a squeeze which allows the speed of the solution to be adjusted through the column. 10 mL of swollen resin was placed in the columns. Prepared solutions of AR18 at the concentrations of 100, 300 and 500 mg/L at a rate of 0.6 mL/min were passed through the column bed. The concentration of the dye in the leak from the column was determined by the spectrophotometric method. Based on the breakthrough curves, the working capacity (q_r) was calculated:

$$q_r = \frac{C_0 \cdot V_p}{V_s} \tag{8}$$

where: C_0 – the initial concentration of dye [mg/L], V_p – the volume of solution for breakthrough point [L], V_s – the volume of sorbent in the column [mL].

Results and Discussion

Kinetic studies

Effect of phase contact time and initial dye concentration

Figure 3 shows the effect of contact time (from 1 to 240 min) on C.I. Acid Red 18 sorption using Amberlyst A21 and Lewatit VPOC1064. The effect of the phase contact time on AR18 sorption on the above sorbents was examined taking into account the change in the initial dye concentration in the range of 100-500 mg/L as a function of time at room temperature with a constant mass of sorbents (0.5 g). For the weakly basic anion exchanger Amberlyst A21 the phase contact time needed to reach equilibrium was shorter compared to that for Lewatit VPOC1064 and was 20 min in an aqueous solution containing 100 mg/L of dye whereas for the initial dye concentrations 300 mg/L and 500 mg/L it was 40 and 80 min, respectively. The amount of AR18 adsorbed at time t by Amberlyst A21 is 10.03 mg/g, 30.33 mg/g and 51.17 mg/g for the initial concentrations 100, 300 and 500 mg/L, respectively. The sorption of AR18 on Lewatit VPOC1064 was significantly slower. For the initial concentrations of dye 100, 300 and 500 mg/L, the amounts of AR18 adsorbed were equal to 6.63 mg/g, 12.88 mg/g and 16.70 mg/g, respectively. The time needed to reach equilibrium was 140 min, 160 min and 200 min.

Analyzing the effect of phase contact time and the initial concentration of AR18 on the amount of dye absorbed by Lewatit VPOC1064 and Amberlyst A21, it can be concluded that the above-mentioned parameters had a significant effect on the q_t value. The time necessary to reach equilibrium was shorter using the resin with the tertiary amine functional groups - Amberlyst A21.

Pseudo-first order, pseudo-second order and intraparticle diffusion kinetic models

The mechanism of adsorption of AR18 on Lewatit VPOC1064 and Amberlyst A21 was estimated using the most commonly applied kinetic models such as PFO, PSO and ID. They are based on defining the amount of substance adsorbed by the unit of adsorbent mass after conditioning time (q_t). Based on the calculated kinetic parameters (Table 3), it can be concluded that the best fit of experimental data was obtained for the PSO model.

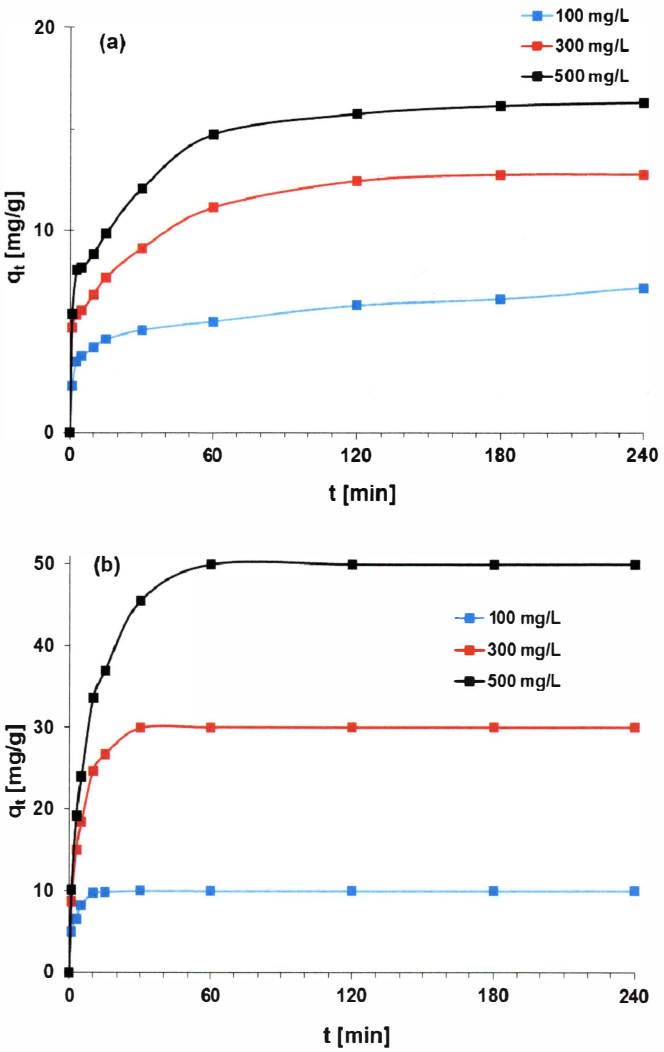

Figure 3. Influence of phase contact time and initial concentration on C.I. Acid Red 18 sorption on Lewatit VPOC1064 (a) and Amberlyst A21 (b).

Table 2. Kinetic parameters determined from the pseudo-first order kinetic, pseudo-second order, and intraparticle diffusion equations in the C.I. Acid Red 18 sorption process on Lewatit VPOC1064 and Amberlyst A21.

Resin	C_0 [mg/L]	$q_{e,exp}$ [mg/g]	PFO			PSO			ID	
			q_e [mg/g]	k_1 [1/ min]	R^2	q_e [mg/g]	k_2 [g/mg min]	R^2	k_{int} [mg/g $min^{0.5}$]	R^2
Lewatit VPOC1064	100	7.20	3.65	0.0123	0.917	6.63	0.0254	0.998	0.560	0.880
	300	12.80	7.82	0.0262	0.991	12.88	0.0097	0.999	0.877	0.992
	500	16.30	8.88	0.0229	0.988	16.70	0.0087	0.999	1.239	0.954
Amberlyst A21	100	10.00	0.89	0.0489	0.615	10.03	0.1463	0.999	1.108	0.769
	300	30.00	5.03	0.0399	0.686	30.33	0.0159	0.999	4.701	0.913
	500	49.99	18.66	0.0419	0.798	51.17	0.0044	0.999	7.815	0.962

The pseudo-second order model is based on the solid phase capacity. If the kinetics of the sorption process is better described by the pseudo-second order model, then the t/q_t vs t graph gives a linear relationship from which q_e and k_2 can be determined and knowledge of any parameters is not necessary (Fig. 4.).

In addition, the values of $q_{e.exp}$ are close to those calculated from the PSO equation and indicate that they fit this model perfectly. The values of determination coefficients R^2 during sorption of AR18 on Lewatit VPOC1064 were in the range of 0.998-0.999 while during sorption on Aberlyst A21 $R^2 = 0.999$. The PFO equation did not apply to the description of AR18 sorption kinetics on Lewatit VPOC1064 and Amberlyst A21 due to the lack of a linear dependence of $\log(q_e-q_t)$ vs t, confirmed by small R^2 values and differences between the determined sorption capacities ($q_{e,exp}$) and that calculated from the PFO equation (q_e). The studies also evaluated the usefulness of the intraparticle diffusion (ID) model due to the porosity of the resin. However, the kinetic parameters calculated from the ID equation based on the graphs q_t vs $t^{0.5}$ referring to the studied systems are characterized by very small values of the determination coefficients; therefore it was not applied in the description of kinetic data.

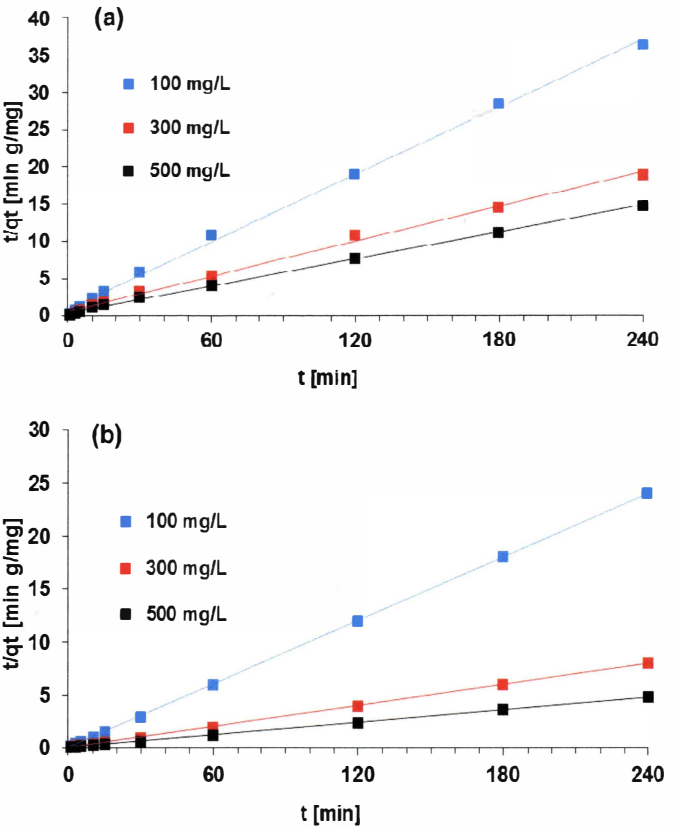

Figure 4. Dependence t/q_t vs t determined from the linear form of the PSO equation (AR18 - Lewatit VPOC1064 (a) and AR18 - Amberlyst A21 (b) systems).

Isotherms

To determine the balance between adsorbate concentration in the solid state and its concentration in the liquid phase, the adsorption isotherms were determined. The maximum sorption capacities of the resins in relation to AR18 were determined from the most popular adsorption models such as Langmuir and Freundlich (Fig. 5).

Based on the C_e/q_e vs C_e and $\log q_e$ vs $\log C_e$ the plots of isotherm parameters were determined and are given in Table 3. The best fit of the experimental data was obtained using the Langmuir model in the AR18-Amberlyst A21 system as evidenced by the high value of the determination coefficient R^2 (0.999). In the case of dye adsorption on the sorbent deprived of functional groups (Lewatit VPOC1064) a better match fit of the experimental data to the Freundlich

model was obtained. The value of the determination coefficient of R^2 was 0.690. Wawrzkiewicz et al. (19) removing C.I. Direct Yellow 50 with the aid of various sorbents confirmed that those without functional groups are better described by the Freundlich model.

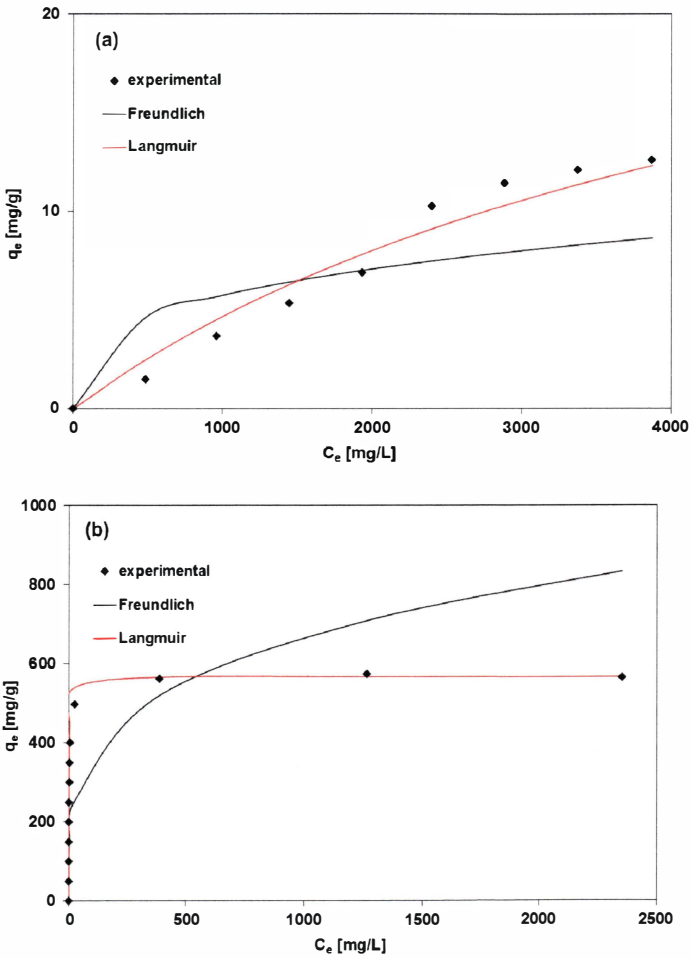

Figure 5. Adsorption isotherms of C.I. Acid Red 18 on the Lewatit VPOC1064 (a) and Amberlyst A21 (b) resins.

Table 4. Parameters determined from the Langmuir and Freundlich isotherms in the sorption process of C.I. Acid Red 18 by means of Lewatit VPOC1064 and Amberlyst A21.

Resin	Langmuir model			Freundlich model			
	R^2	Q [mg/g]	b [L/mg]	R^2	n	1/n	k_F [mg/g]
Lewatit VPOC1064	0.221	28.91	0.00019	0.690	3.29	0.303	0.705
Amberlyst A21	0.999	566.84	0.81365	0.233	3.81	0.262	109.018

Among the polystyrene resins used in the study the largest sorption capacity (Q_0) in relation to AR18 was found using Amberlyst A21, which amounted to 566.84 mg/g. Considering the presence or absence of functional groups it can be concluded that the Lewatit VPOC1064 adsorbent deprived of functional groups showed a much lower sorption capacity (Q_0 = 28.91 mg/g) compared to the adsorbent having functional groups – which leads to the conclusion that the presence of functionalities and their basicity increase the degree of adsorption in relation to the tested dye. Greluk and Hubicki (20) used anion exchangers with different basicity of functional groups to remove C.I. Acid Orange 7 from aqueous solutions. They observed that the strongly basic anion exchanger Amberelite IRA 458 exhibits much better sorption properties compared to the weakly basic Amberlite IRA 67 anion exchange resin as evidenced by the seven times higher Q_0 values 1211.3 mg/g and 168.2 mg/g, respectively.

Effect of sodium sulphate, acetic acid and surfactants presence on dye removal

Auxiliaries which include inorganic electrolytes (acids and salts) and surfactants are used in the processing of fibers. However, they remain in sewage at a concentration very close to the initial one in the dyeing bath. Depending on the type of dye, the dyeing process requires the use of other auxiliaries. Acid dyes belong to the most numerous group of dyes. The dyeing process using these dyes takes place in an environment of pH 4.5-5 acidified with acetic acid (21). Thus it is useful to study the effect of various concentrations of acetic acid, salts and surfactants on adsorption of AR18 (Fig. 6). The influence of auxiliaries on the effectiveness of sorption of AR18 on Lewatit VPOC1074 and Amberlyst A21 was studied using a static method in the system containing 0.5 g of resin and 50 mL of a dye solution (500 mg/L). The AR18 solutions additionally contained from 5 g/L to 25 g/L of Na_2SO_4, from 0.5 to 2 g/L of acetic acid and from 0.1 to 0.5 g/L of surfactants (SDS, Triton X-100). The amount of dye adsorbed by the resin was tested after 15 min of phased contact time.

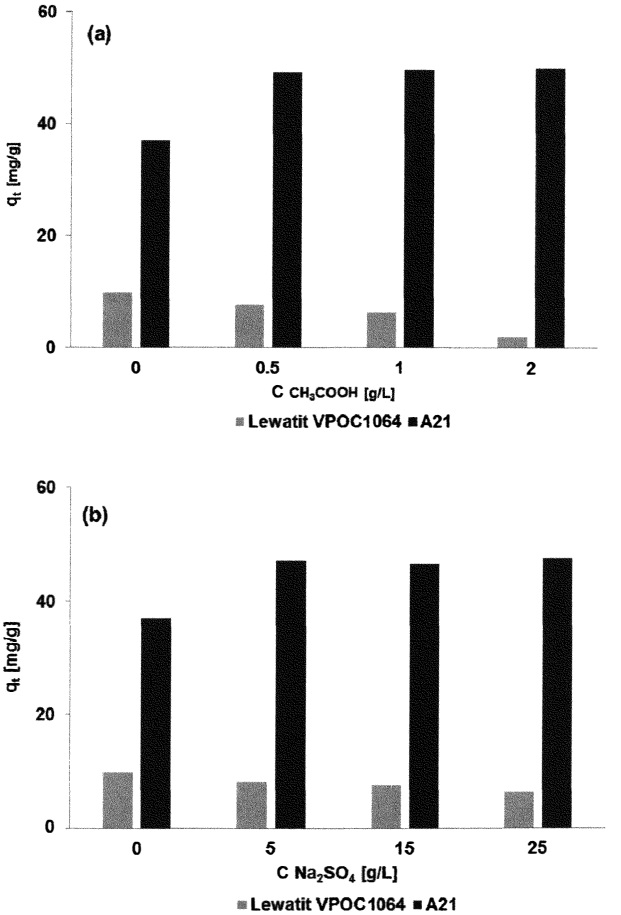

Fig. 6. The effect of salt and acid concentrations on the amount of C.I. Acid Red 18 adsorbed by the Lewatit VPOC1064 and Amberlyst A21.

The presence of acid in the system had an effect on the sorption process of AR18 on Lewatit VPOC1064 and Amberlyst A21. With increasing acetic acid concentration the values of q_t decrease during sorption on Lewatit VPOC1064, and q_t increases during sorption on Amberlyst A21 (Fig. 6a.). In the conducted studies, reduction of q_t values in the AR18-Lewatit VPOC1064 system and an increase of q_t in the AR18-Amberlyst A21 system with an increasing Na_2SO_4 concentration were observed (Fig. 6b.). The decrease in sorption capacity along with the increase in the concentration of salt or acid in the system may be caused by the competitive sorption of sulphate or acetate ions with the anionic form of the dye.

The effects of the SDS and Triton X-100 surfactants on AR18 sorption on Lewatit VPOC1064 and Amberlyst A21 were also studied (Fig. 7.). As the concentration of anionic surfactant (SDS) increases, q_t increases during sorption of AR18 on Lewatit VPOC1064 and Amberlyst A21. On the other hand,

the presence of non-ionic Triton X-100 caused a decrease in q_t values (9.81-1.59 mg/g) during sorption of AR18 on Lewatit VPOC-1064 and an increase in q_t (36.97-48.30 mg/g) during the sorption of AR18 on Amberlyst A21.

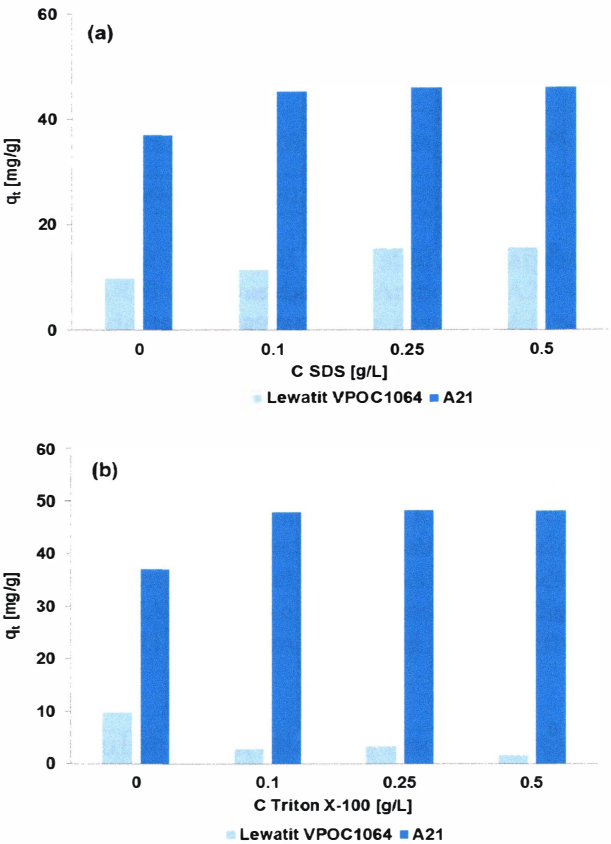

Figure 7. Effect of anionic SDS (a) and non-ionic Triton X-100 (b) surfactants on C.I. Acid Red 18 adsorbed by the Lewatit VPOC1064 and Amberlyst A21.

Column studies

Ion exchange reactions in industry are carried out in a column system under various conditions. Therefore it is quite important to determine the working ion-exchange capacity because the results obtained from such tests give the possibility of qualifying the adsorbent to be tested in a suitable technological process. On the basis of the breakthrough curves (Figs. 8 and 9), the working ion exchange capacities (C_w), weight (D_w) and bed (D_b) distribution coefficient were determined, the values of which are presented in Table 5:

$$D_w = \frac{(U - U_0 - V_v)}{m} \quad (9)$$

$$D_b = \frac{(U - U_0 - V_v)}{v} \quad (10)$$

$$C_w = \frac{V_{bp} \cdot C_o}{v} \quad (11)$$

where: U – the volume of the eluate for C/C_0= 0.5 [mL], U_0 – the dead volume of the column (liquid volume in the column between the bottom edge of the resin bed and the outlet of the column under the process conditions U_0=2 mL), V_v – the free volume (intergranular) in the resin bed (approx. 0.4 bed volume) [mL], m – the dry resin mass in the column [g], v – the swollen resin volume in the column [mL], V_{bp} – the column leakage volume [mL], C_0 – influent concentration [mg/L].

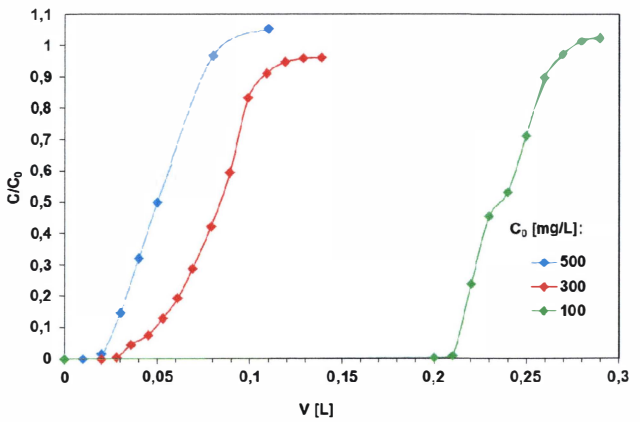

Figure 8. Breakthrough curves in the C.I. Acid Red 18 – Lewatit VPOC1064 system.

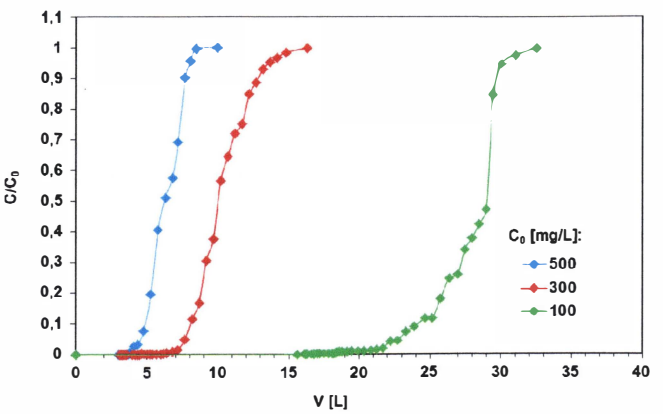

Figure 9. Breakthrough curves in the C.I. Acid Red 18 – Amberlyst A21 system.

Table 5. Comparison of the weight and bed distribution coefficients and working ion exchange capacities in the dye-resin systems.

Resin	C_0 [mg/L]	D_w	D_b	C_w [mg/mL]
Lewatit VPOC1064	100	76.1	22.9	2.1
	300	25.3	25.2	0.84
	500	14.3	4.3	0.5
Amberlyst A21	100	10102.4	2899.4	186
	300	3494.4	999.4	185.1
	500	2088.5	599.4	183.5

Conclusions

On the basis of the experiments it was found that polystyrene Amberlyst A21 of tertiary amine functional groups has a higher sorption capacity compared to the non-functionalized polymeric resin Lewatit VPOC1064. The Amberlyst A21 monolayer capacity determined from the Langmuir isotherm is 566.84 mg/g. The effectiveness of the dye sorption increases with the increasing contact time of the phases and the concentration of the initial dye until a plateau is reached. The kinetics of the AR18 sorption process on the resins was better described by the pseudo-second order model due to the high values of determination coefficients (the linearity of t/q_t vs t occured), and the sorption capacity determined experimentally ($q_{e,exp}$) is coincided with the capacities calculated (q_e) from the PSO equation. The presence of auxiliaries in the system, i.e. salts, acid and surfactants has an impact on the sorption of AR18 on Lewatit VPOC1064 and Amberlyst A21. The obtained results are of great application importance especially in dyes containing wastewater treatment technology.

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GROWTH AND METABOLISM OF *CHLORELLA VULGARIS* UNDER THE INFLUENCE OF MANGANESE AND IRON

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Abstract

The aim of this study was to investigate the effect of manganese(II) and iron(III) on the growth and basic metabolic processes of the unicellular alga *Chlorella vulgaris*. To this end, alga cultures were treated with ions of iron(III) and manganese(II) at concentrations 0.1, 0.5, 2, 5, 10, 20 and 50 mg L^{-1} and then analyzed in terms of changes in the number of cells, content of monosaccharides, proteins, chlorophyll and the activity of antioxidant enzymes: superoxide dismutase and glutathione reductase. The development of *C. vulgaris* was influenced more by manganese(II) than iron(III). The largest increase in the number of cells and concentrations of the studied biochemical parameters and antioxidant enzyme activity was induced by 20 mg L^{-1} manganese(II). The experiment also showed that an appropriate amount of iron(III) helped control the level of manganese(II) thanks to the co-precipitation of metals, and so could help in tackling the worldwide problem of eutrophication. In the case of introduction of 50 mg L^{-1} of both iron(III) and manganese(II), the number of *C. vulgaris* cells decreased, and thus the concentration of the biochemical parameters in water. The results of these experimental studies well collaborate with a study conducted earlier of surface water, which showed the existence of a strong correlation between the concentration of manganese in the water and algal biomass, whose determinant is chlorophyll *a*.

Keywords: *Chlorella vulgaris*, manganese, iron, eutrophication process

Introduction

In plants, iron is mainly associated with energy conversion required for photosynthesis and other processes. For example, iron protein compounds in chloroplasts are involved in the conversion of light into chemical energy. Iron also stimulates the formation of chlorophyll, although it is not included in its composition. It participates in a variety of redox reactions in plants that are associated with multiple metabolic processes: respiration (a component of many enzymes, e.g. cytochrome oxidase, reductase), photosynthesis, metabolism of nitrogen compounds, or takes part in the metabolism of nucleic acids. Ions of iron(II) play a very important role in biochemical processes carried out by

cyanobacteria as they activate hydrogenases and enzymes of the photoreduction and respiration systems.

Luka and Aegerter (1993) found that iron concentrations lower than 140 μg L^{-1} resulted in the slow proliferation of cyanobacteria while microcystin concentrations increased by an average of 30%; exactly the inverse relationship was observed by Utkilen and Gjølme (1995). Iron taken up by plants is mostly iron(II) in chelates. Iron is usually transported in plants in organic compounds, e.g., citric acid, or in anionic form. Environmental iron(III) is less digestible than iron(II) and therefore reduction processes on the surface of plant roots take place very slowly; reduction in further stages of metabolic processes proceed much easier. Reduction and oxidation can be carried out over a full range of pH and different aerobic conditions by autotrophic and heterotrophic micro-organisms (serving as catalysts) or without them. For example, colloidal iron(III) hydroxide precipitates even at pH>2.28 when the concentration of iron(III) in the solution is 19.4 mg L^{-1}, which corresponds to a Knop medium. When the environment becomes neutral or slightly alkaline, co-precipitation of iron and manganese occurs (Graham and Copper 1959).

Manganese is one of the trace elements necessary for the survival of plants and animals. In plants it is crucial for photosynthesis as an essential part of photosystem II (water oxidation center) which catalyzes the release of oxygen. This center is a cluster of manganese incorporated in one of the ends of the protein and is the basic unit of the enzyme catalyzing the oxygen release reaction (Fraústo de Silva and Wiliams 1991). Manganese deficiency inhibits growth and photosynthetic activity in *Chlamydomonas* (Allen et al. 2007). Manganese is a trace metal for 'special tasks', because it accumulates in enzymes such as superoxide dismutase or acid phosphatase glycoside. It also influences the reduction of nitrate ions(V) in plants, the hydrolysis of peptides, amides (peptidases) and urea (arginase). Plants absorb manganese passively and metabolically. Passive absorption concerns dissolved manganese(II); its transport in the plant occurs primarily in a Mn^{2+} form or as dissolved organic matter (Van Goor and Wiersma 1976).

Manganese deficiency in plants can be induced by the antagonistic effect of other elements, including iron. Manganese, similar to iron, undergoes multiple oxidation-reduction processes either involving or without the participation of microorganisms. For example, *Metelloglinium personatum* produces catalase that induces oxidation of manganese(II), while *Escherichia coli* produce formic acid which is able to reduce manganese(IV) oxide. Goto et al. (1999) found that algae also secrete numerous metabolites in the environment, for example sugars or amino acids, which are a source of the electrons used for the reduction of manganese(IV).

Metabolites of algae, like algae themselves, are the cause of increasing eutrophication in the world. Intensive growth of algal biomass, resulting in algal blooms, leads to the inhibition of photosynthesis, and at a later stage a significant deterioration in water aerobic conditions. This extremely important problem may be dealt with by modifying iron and manganese concentrations in the environment, among other things. Previous research has dealt with the impact

of these ions on the development of higher plants, and algae, but there is a lack of information on the impact of these metals on the basic metabolic processes of *Trebouxiophyceae* in aquatic environments especially *Chlorella vulgaris*. In recent years, there have been reports concerning only the effect of iron on the synthesis of lipids in green algae, including *C. vulgaris* (Liu et al. 2008; Ruangsomboon 2012; Ruangsomboon et al. 2013; Concas et al. 2014; JinShui et al. 2015).

C. vulgaris (*Trebouxiophyceae*) is a crucial component of natural phytocenoses and it is a model experimental object in plant biochemistry because it can be cultivated on a simple mineral medium and is characterized by rapid cell division. Moreover, perception of the signalling molecule and biochemical response take place within the same cell.

Experimental procedures

Plant Material and Growth Conditions

C. vulgaris culture was sourced from the Department of Plant Physiology collection at the University of Bialystok (Poland). Microalgae were cultivated in stable conditions; humidity 45±5% and temperature 25±1 ºC. Illumination was supplied over a 16 hour photoperiod (8 hours dark period) by a bank of fluorescent lights, yielding a photon flux density of 50 μmol $m^{-2} s^{-1}$ of photosynthetically active radiation (PAR) at the surface of the tubes. PAR was measured with an FF-01 phytophotometer (SOMOPAN, Poland). Permanent synchronous growth was established according to the method by Pirson and Lorenzen (1966). The culture medium used was Knop medium with the following components: 500 mg KNO_3, 500 mg $Ca(NO_3)_2 \cdot 4H_2O$, 200 mg KH_2PO_4, 150 mg $MgSO_4 \cdot 7H_2O$, 10 mg $FeCl_3 \cdot 6H_2O$, 3 mg H_3BO_3, 2 mg $MnCl_2 \cdot 4H_2O$, 0.3 mg NH_4VO_3, 0.2 mg $ZnSO_4 \cdot 7H_2O$, 0.1 mg $(NH_4)_6Mo_7O_{24} \cdot 7H_2O$ per liter of distilled water (Bajguz and Asami 2004). The culture medium was sterilized by autoclaving at 125 ºC for 25 minutes. Besides this, glassware and bacteriological stoppers were sterilized in a thermal chamber at 105 ºC for 4 hours. The pH of the medium was adjusted to 6.8 with 1 mol L^{-1} NaOH (Pietryczuk et al. 2014). The pH of the culture medium was based on literature data on the optimal pH for the growth of *C. vulgaris*. Algae were cultured in Erlenmeyer flasks containing 250 mL of appropriate medium and shaken at 150 rpm in a rotary shaker. The culture from which the inoculum was taken was in a logarithmic growth phase. The initial cell density was about $12 \cdot 10^4$ cells per ml in all experiments.

In the present work, the effects of Mn(II) and Fe(III) concentrations applied at 0.1, 0.5, 2, 5, 10, 20 and 50 mg L^{-1} were analyzed. For this purpose, appropriate amounts of $FeCl_3$ and $MnCl_2$ were added to the Erlenmeyer flasks with Knop medium to obtain the desired concentrations of the tested ions. The experimental and control cultures were performed on Knop medium deficient in manganese(II) and/or iron(III). Control cultures contained either no manganese(II) or no iron(III). Cultures of the activity of manganese(II) were examined in the medium free of manganese(II), and in the medium without

manganese(II) and iron(III). Similarly, algae cells treated with various amounts of exogenous iron(III) were cultured in a medium devoid of iron(III) and in a medium containing neither iron (III) nor manganese(II). Cell number and proteins, monosaccharides, photosynthetic pigment level as well as antioxidant enzyme activity in response to manganese(II), iron(III) were analyzed. The cell number and biochemical parameters were determined at 24, 72 and 120 hours of cultivation. Cultures were conducted in three replicates.

Determination of cell number

The number of algae cells was determined by direct counting in a Bűrker chamber.

Determination of proteins

Protein concentrations were determined by spectrophotometric method according to Lowry et al. (1951). Folin's reagent was used in determinations. Bovine serum albumin of a known concentration was used as a standard. The absorbance of the extract was measured on a Beckman DU-650 spectrophotometer.

Determination of monosaccharides

The concentration of monosaccharides was determined by spectrophotometric method according to Somogyi (1954). Sugars were extracted in ethanol for 24 hours. Arsenic-molybdenum reagent was used for the assay. Pure glucose standard of a known concentration was used as a standard. The absorbance of the extract was measured on a Beckman DU-650 spectrophotometer.

Determination of photosynthetic pigments

The concentration of chlorophyll was determined by spectrophotometric method according to Wellburn (1994). Chlorophylls were extracted in 99.9% methanol at 70 °C for 30 minutes. The absorbance of the extract was measured on a Beckman DU-650 spectrophotometer. Chlorophyll concentrations were calculated using the equation proposed by Wellburn (1994).

Determination of superoxide dismutase (SOD) activity

For the extraction of superoxide dismutase, a fresh sample of *C. vulgaris* was passed through a paper filter under pressure and was homogenized using liquid nitrogen and subsequently a lysis buffer containing 0.1 mol L^{-1} phosphate buffer

(pH=7.8), 3 mmol L^{-1} $MgSO_4$, 1 mmol L^{-1} dithiotreitol (DTT) and 3 mmol L^{-1} EDTA. The homogenate was centrifuged at 12000 G for 10 minutes and the resulting supernatant was used to determine enzyme activity. SOD (EC 1.15.1.1) activity was measured based on inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) according to Beauchamp and Fridovich (1971). The reaction mixture consisted of 2.2 mL of 0.1 mol L^{-1} phosphate buffer (pH=7.8), 0.25 mL of 0.156 mmol L^{-1} riboflavin, 1.1 mL of 0.156 mmol L^{-1} methionine, 0.25 mL of 0.756 mmol L^{-1} NBT and 50 μL of enzymatic extract. Samples were incubated for 20 minutes at room temperature. The absorbance of the extracts was measured spectrophotometrically at 560 nm. One unit of SOD activity was defined as the enzyme concentration required to inhibit the reduction of NBT by 50%.

Determination of glutathione reductase (GR) activity

For the extraction of glutathione reductase, a fresh weight of *C. vulgaris* was passed through filter paper under pressure and homogenized using liquid nitrogen and subsequently a lysis buffer containing 0.02 mol L^{-1} phosphate buffer (pH=7.0). The homogenate was centrifuged at 12000 G for 10 minutes and the resulting supernatant was used to determine enzyme activity. The entire extraction procedure was carried out at 4 °C. Glutathione reductase (EC 1.6.4.2) activity was determined according to Foyer and Halliwel (1976). The reaction mixture consisted of 0.25 mol L^{-1} KCl in 1 mL of 0.02 mol L^{-1} phosphate buffer (pH=7.0), 0.25 mL of 7 mmol L^{-1} glutathione disulfide and 0.1 mL of ezymatic extract. The reaction was initiated by adding 0.5 mL of 0.7 mol L^{-1} NADPH. NADPH concentration was defined as the optical density at 340 nm, and activity was calculated using the extinction coefficient e=62.2 mmol L^{-1} cm^{-1} for NADPH. One unit of glutathione reductase was defined as the amount of enzymes needed to break down 1 μmol L^{-1} of NADPH per 1 mg soluble protein per minute.

Replication and statistical analysis

Each treatment consisted of 3 replicates and each experiment was carried out on at least two different occasions (N=6). The results were subjected to statistical analysis in SPSS 19. Kruskal-Wallis tests were used to estimate the difference between means. The standard deviations from the means of all tested parameters were not greater than 5%.

Results

Variant B – where iron and manganese were absent in the Knop medium and different amounts of iron were then added to the experimental cultures – was characterized by very low changes in all biochemical parameters (fig. 1B-6B) and number of cells (fig. 7).

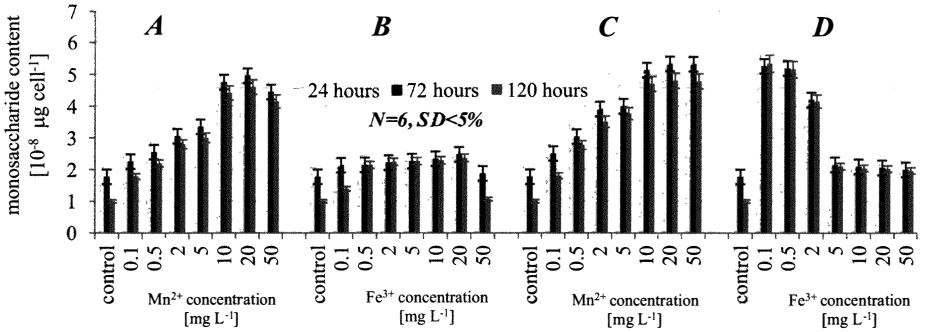

Figure 1. Change in monosaccharide concentrations in time induced by various concentrations of an introduced analyte, in a situation when ***A*** – iron and manganese were absent in the medium and manganese(II) was introduced; ***B*** – iron and manganese were absent in the medium a d iron was introduced (III); ***C*** – manganese was absent in the medium and manganese(II) was introduced; ***D*** – iron was absent in the medium and iron(III) was introduced; ***control*** – both metals were absent in the medium.

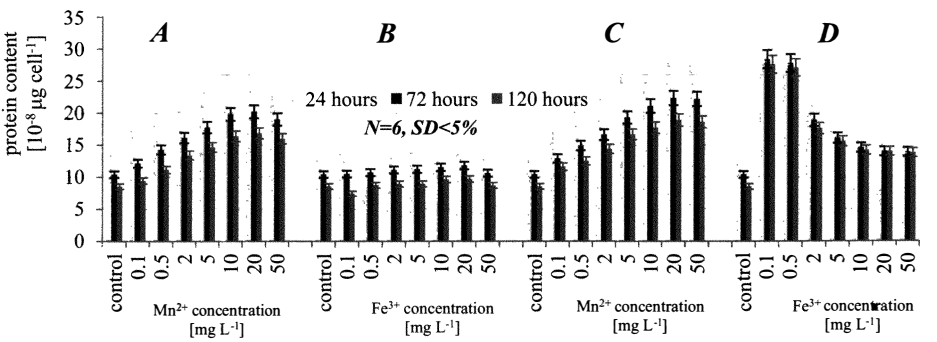

Figure 2. Change in protein concentrations in time induced by various concentrations of an introduced analyte, in a situation when ***A*** – iron and manganese were absent in the medium and manganese(II) was introduced; ***B*** – iron and manganese were absent in the medium and iron was introduced (III); ***C*** – manganese was absent in the medium and manganese(II) was introduced; ***D*** – iron was absent in the medium and iron(III) was introduced; ***control*** – both metals were absent in the medium.

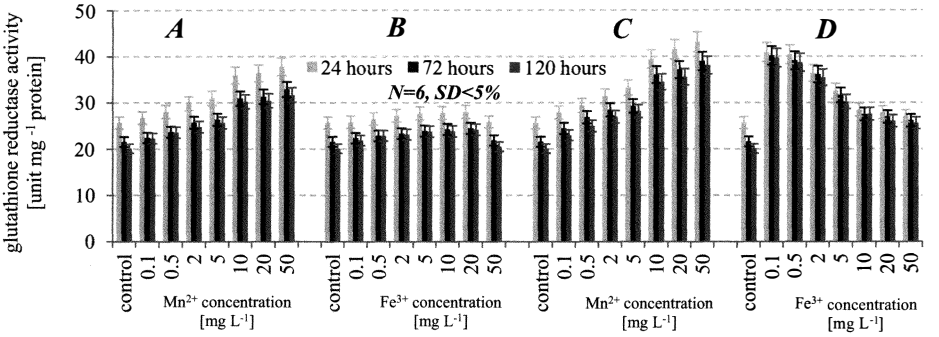

Figure 3. Change in GR activity in time induced by various concentrations of an introduced analyte, in a situation when *A* – iron and manganese were absent in the medium and manganese(II) was introduced; *B* – iron and manganese were absent in the medium and iron was introduced (III); *C* – manganese was absent in the medium and manganese(II) was introduced; *D* – iron was absent in the medium and iron(III) was introduced; ***control*** – both metals were absent in the medium.

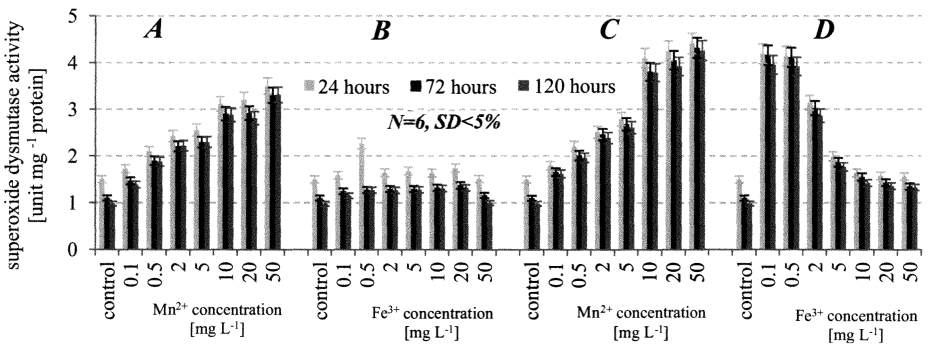

Figure 4. Change in SOD activity in time induced by various concentrations of an introduced analyte, in a situation when *A* – iron and manganese were absent in the medium and manganese(II) was introduced; *B* – iron and manganese were absent in the medium and iron was introduced (III); *C* – manganese was absent in the medium and manganese(II) was introduced; *D* – iron was absent in the medium and iron(III) was introduced; ***control*** – both metals were absent in the medium.

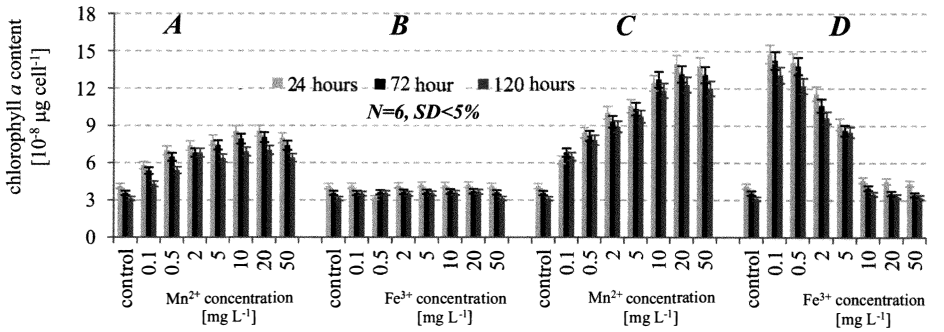

Figure 5. Change in chlorophyll *a* concentrations in time induced by various concentrations of an introduced analyte, in a situation when ***A*** – iron and manganese were absent in the medium and manganese(II) was introduced; ***B*** – iron and manganese were absent in the medium and iron was introduced (III); ***C*** – manganese was absent in the medium and manganese(II) was introduced; ***D*** – iron was absent in the medium and iron(III) was introduced; ***control*** – both metals were absent in the medium.

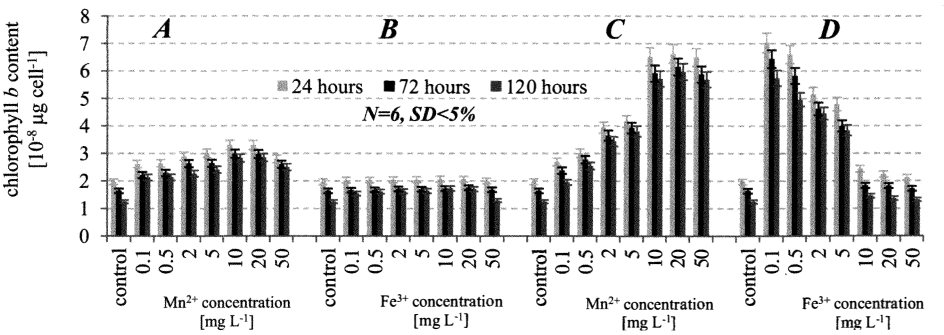

Figure 6. Change in chlorophyll *b* concentrations in time induced by various concentrations of an introduced analyte, in a situation when ***A*** – iron and manganese were absent in the medium and manganese(II) was introduced; ***B*** – iron and manganese were absent in the medium and iron was introduced (III); ***C*** – manganese was absent in the medium and manganese(II) was introduced; ***D*** – iron was absent in the medium and iron(III) was introduced; ***control*** – both metals were absent in the medium.

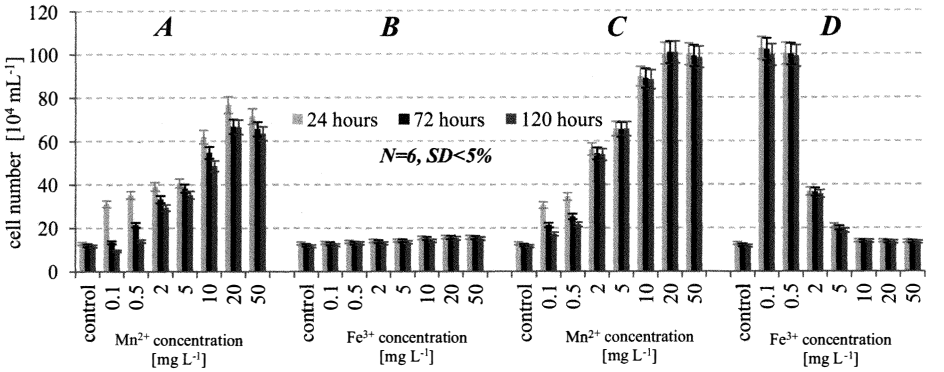

Figure 7. Change in the number of *chlorella vulgaris* cells in time induced by an introduced analyte, in a situation when ***A*** – iron and manganese were absent in the medium and manganese(II) was introduced; ***B*** – iron and manganese were absent in the medium and iron was introduced (III); ***C*** – manganese was absent in the medium and manganese(II) was introduced; ***D*** – iron was absent in the medium and iron(III) was introduced; ***control*** – both metals were absent in the medium.

Introducing iron to the medium at 0.1-20 mg L^{-1} resulted in a very slight increase in the concentrations of all the biochemical parameters in *C. vulgaris* cells, but the increase was not statistically significant relative to the control. When the algae were treated with 50 mg Fe L^{-1} all parameters increased slightly in the cells, including the number of cells (fig. 7) relative to the 20 mg Fe L^{-1}. The maximum values of concentrations of all the biochemical parameters examined were observed when iron was introduced to the medium at 20 mg Fe L^{-1}, regardless of the age of the culture (fig. 1B-6B). These regularities also applied to the activity of the studied enzymes, glutathione reductase and superoxide dismutase.

Variant C, in which only manganese was absent in the Knop medium and varying amounts of manganese were added to the solution, showed the highest variation in concentrations of all parameters and activity of the enzymes in the cells of *C. vulgaris* (fig. 1C-6C), and in the number of cells (fig. 7C). Similar to variant B, the introduction of manganese into the solution at 0.1-20 mg L^{-1} caused an increase in all parameters and concentrations of enzyme activity in the algae cells, and was statistically significant ($p<0.005$) compared to the control. The addition of 50 mg Mn L^{-1} to the solution caused, as in variant B, concentration decreases (not significant) in proteins and in chlorophylls a and b compared to when iron was introduced to the medium at 20 mg L^{-1} (fig. 2C, 5C, 6C). The solution of 50 mg Mn L^{-1} resulted in a further increase in the activity of glutathione reductase and superoxide dismutase (fig. 3C-4C), while monosaccharide levels in the algae cells did not differ from the culture grown with 20 mg Mn L^{-1} (fig. 1C). The average increase in concentration of monosaccharides when the solution of 50 mg Mn L^{-1} was introduced was more than 3-fold higher relative to the control (fig. 1C), while proteins increased more than 2 times (fig. 2C) and chlorophyll *a* and *b* more than 3.5 times (fig. 5C-6C). The activity of

glutathione reductase increased more than 2 times compared to the control (fig. 3C), while the activity of superoxide dismutase increased more than 3.5 times (fig. 4C).

In variant A, with a Knop medium deficient in both iron and manganese and where manganese was introduced to the medium in different amounts, concentrations of all biochemical parameters and enzyme activity (fig. 1A-6A) as well as cell number (fig. 7A) showed greater differences than in variant B and lower differences than in variant C. As in the case of variant B, there was an increase in the activity of glutathione reductase and superoxide dismutase over the whole range of concentrations of manganese introduced to the solution (fig. 3A-4A). The average increase in glutathione reductase activity was 1.5 times higher relative to the control (fig. 3A), and superoxide dismutase was almost 3 times higher (fig. 4A). Introduction of 50 mg Mn L^{-1} decreased ($p<0.01$) the levels of monosaccharides, proteins, chlorophylls a and b in *C. vulgaris* cells compared to the culture treated with 20 mgMn·L^{-1} (fig. 1A, 2A, 5A, 6A) and also cell number (fig. 7A). The average concentration of chlorophyll *a* in the cells in variant A was 30% lower than in variant C (fig. 5A, 5C), and in the case of chlorophyll *b* the difference was greater – 40% (fig. 6A, 6C).

All the biochemical parameters in variant D, where iron was completely absent in the Knop medium and iron was added in various concentrations, behaved completely differently than the other variants. Introduction of 0.1 mg Fe L^{-1} to the solutions resulted in a sharp increase in all the biochemical parameters in the algae and the number of cells, while the introduction of larger amounts of iron to the culture medium resulted in a decrease in the concentrations of all the biochemical parameters in *C. vulgaris* (fig. 1D-6D) and in cell number (fig. 7D). In the case of monosaccharides, chlorophylls *a* and *b*, and superoxide dismutase, 5 mg Fe L^{-1} resulted in a sharp decline in the concentrations of the indicators and activity of enzymes (fig. 1D, 4D-6D). For all the biochemical parameters there was a statistically significant difference ($p<0.001$) between the control and experimental cultures treated with iron at 0.1-2.0 mg Fe L^{-1}. In the case of the protein concentration and activity of the antioxidant enzymes tested, a statistically significant difference ($p<0.05$) was demonstrated between the control and the various cultures compared to the corresponding iron introduced into the solution at 5-50 mg L^{-1}, and in the case of both monosaccharides and the statistical significance of chlorophylls, a difference did not exist.

The described changes in the concentrations of biochemical parameters, the activities of enzymes, and the numbers of cells, were identical regardless of the age of cultures.

Discussion

There is a good number of literature data concerning the effect of manganese(II) and iron(III) on the growth of higher plants and basic metabolic processes such as photosynthesis, respiration, or antioxidant defense. It is also known that these elements are involved in the regulation of the growth and

metabolism of lower plants, especially algae (Pramod et al. 2014). However, there is a lack of detailed information on their impact on changes in the content of basic metabolites in the *Trebouxiophyceae* cells. Therefore, understanding the biochemical mechanisms behind the action of iron and manganese in the algae seems to be extremely important for the control of phytoplankton growth in water bodies; modifying the concentrations of these elements in the aquatic environment may have a beneficial effect. Previous studies of surface water showed that there is a strong relationship between the concentration of manganese in the water and algal biomass (Górniak and Cudowski 2006; Cudowski and Górniak 2008; Cudowski 2015). Intensive growth of algal biomass, resulting in their `blooms`, leads to inhibition of photosynthesis and in a further step to a significant deterioration of water oxidation, which is the first stage of eutrophication. This process has become a global problem, as it is recorded in lakes, seas, and rivers around the world (Imai et al. 2006; Selaman et al. 2008).

In our study, manganese(II) and iron(III) induced the growth of *C. vulgaris* cell numbers compared with the control culture. Manganese(II) is a stimulator of the growth of algae (Rousch and Sommerfeld 1999, Chen et al. 2010), and similar to other reports, in our study resulted in a much larger increase in the number of *C. vulgaris* cells than under the influence of iron(III). Treatment of seedlings of *Vicia faba L.* with manganese(II) at a concentration of 4 g L^{-1} by about 9% compared to control, while iron(III) at a concentration of 4 g L^{-1} caused an increase in angular length of 4% (Abd El-Razek et al. 2013). Also in the sunflower there was more growth, increased biomass and leaf surface as compared to the control plants after treatment with manganese(II) (Jabeen and Ahmad 2011). In contrast, the addition of exogenous manganese(II) and iron(III) to the culture medium, at a concentration of 4 g L^{-1} resulted in the largest increase in the length of the stem relative to control, by as much as 12% (Abd El-Razek et al. 2013), which confirms the results of our study on a single-celled algae. The greatest increase in the number of cells was observed in *C. vulgaris* growing on medium containing iron(III), into which the various concentrations of manganese(II) were introduced. In a reverse situation, when the culture was growing on a medium with manganese(II) and iron(III) added in the concentration range of 0.1-50 mg L^{-1}, a gradual decrease was observed in the number of cells up to the level of control, which is probably associated with the co-precipitation of iron(III) and manganese(II) in the form of iron-manganese concretions, which make manganese(II) unavailable to plants. Silveira et al. (2007) noted that the concentration of manganese in the dry weight of stem and root of rice fell below control when the plants were treated with 500 mg L^{-1} iron(III). The available literature data shows that iron(III) is also a stimulator of plant (Silveira et al. 2007) and algae (Chen et al. 2010) growth. However, compared to manganese(II), iron stimulates growth in the number of algal cells to a much lesser extent than manganese. In addition, treating *C. vulgaris* with iron(III) at a concentration of 50 mg L^{-1} caused a decrease in the number of algal cells. Silveira et al. (2007) observed that Fe(III) at a concentration of 500 mg L^{-1} induced a slight decrease in the dry weight of rice stems with respect to rice treated with 6.5 mg L^{-1}. It seems, therefore, that a much lower concentration of iron is needed to

inhibit the growth of single-celled algae, which is also confirmed by our results on changes in the content of other biochemical parameters under the influence of various concentrations of iron(III).

Our results show that manganese(II) and iron(III) not only activate cellular division in *C. vulgaris*, but also increase the content of chlorophyll *a* and *b*, and monosaccharides. Literature data show that manganese(II) is a trace element essential in the biosynthesis of chlorophylls, and its deficiency causes a disturbance of photosynthesis, contributing to the disintegration of photosynthetic pigments in terrestrial plants, such as *Cassia grandis* (Li et al. 2010), *Pisum sativum* (Gangwar et al. 2010) and also aquatic plants such as *Salvinia minima* and *Spirodela polyrhiza* (Lizieri et al. 2011), *Lemna gibba* (Doganlar et al. 2012), which is confirmed by our results. In the cells of algae *C. vulgaris*, manganese(II) at a concentration of 50 mg L^{-1} caused a decrease in chlorophyll *a* and *b* and monosaccharides. This is probably due to the fact that high concentrations of manganese displace cellular magnesium, essential for the biosynthesis of chlorophyll (due to competitive reaction between the metals). Furthermore, manganese may induce iron deficiency in plants (Hauck and Spribille 2002). It can be assumed that manganese(II) at a concentration of 50 mg L^{-1} will result in a gradual displacement of magnesium from cells of *C. vulgaris* and induce iron deficiency causing a decrease in the concentration of chlorophylls. However, this decrease is small, and the content of chlorophylls does not fall below the control, suggesting that a much higher concentration of manganese is required to cause the complete inhibition of photosynthesis and biosynthesis of photosynthetic pigments in *C. vulgaris*. For comparison, in the cells of *V. faba* a decrease in chlorophyll content was reported in cells treated with a much lower manganese concentration (4.4 mg Mn L^{-1}).

A manganese(II)-induced increase in chlorophyll *a* and *b*, on average by 40% compared to control, has been reported in studies on *V. faba*. Furthermore, that study revealed an iron-induced increase in the content of chlorophyll in cells, but only by 18-20% relative to the control culture. A similar increase in concentration of photosynthetic pigments (by about 18%) occurred after treatment of plants with manganese(II) and iron(III) simultaneously (Abd El-Razek et al. 2013). Our research showed that the content of chlorophylls *a* and *b* in algae cells increased nearly 3 times when iron(III)-containing medium was added to different concentrations of manganese(II). In contrast, manganese(II) added to the culture medium devoid of iron(III) resulted in only a 2-fold increase in the concentrations of these pigments. This indicates that iron is an essential micronutrient for *C. vulgaris*, required for the proper synthesis and function of chlorophylls in its cells. Moreover, in higher plants iron causes a 2-3-fold increase in the content of photosynthetic pigments, for example in *Citrullus lanatus*, and its deficiency induces a significant decrease in the concentration of chlorophylls, carotenoids, proteins, and the rate of net photosynthesis, primarily through disrupting the electron transport in the PSII system (Abadia et al. 1999). In turn, manganese deficiency inhibits the growth and photosynthetic activity in *Chlamydomonas* (Allen et al. 2007). It has been shown that manganese induces PSII in *Euglena gracilis* (Ferroni et al. 2004), *Amphidinium sp.* (Chunhui et al. 2011).

The crucial role of manganese and iron in photosynthesis is demonstrated in our study where algae cells treated with exogenous manganese(II) and iron(III) had an increased concentration of monosaccharides. *C. vulgaris* cells growing on an iron(III)-containing medium into which manganese(II) was added, had a much higher increased content of sugars in relation to the control than the cells treated with manganese(II) and growing on a medium without iron(III). In addition, *C. vulgaris* cells experienced a slight decline in photosynthetic pigments and monosaccharides when treated with iron (III) at a concentration of 50 mg L^{-1}. According to literature data, excess iron causes the degradation of chlorophylls and a decline in monosaccharides, which is probably a consequence of a reduction in the rate of net photosynthesis. Xing et al. (2010) shows that even at 10 mg L^{-1} iron causes a significant decrease in the content of chlorophylls (by 20% compared to control) and in monosaccharides (down to 0) in the cells of *S. polyrhiza*. Such a drastic decline in these biochemical parameters was not recorded in our study, even when the algae cells treated with exogenous iron grew on the medium without manganese(II).

Manganese is an essential element for the proper course of redox processes in plant cells, such as electron transport in photosynthesis. It is also involved in the biosynthesis of chlorophyll – the presence of which is necessary for the proper functioning of the PSII photosystem (Mousavi et al. 2011, Nusrat and Rafiq 2011). Increased photosynthesis efficiency may result in an increase in the concentration of monosaccharides in plant cells. The literature on higher plants confirms that manganese causes an increase in the content of reducing sugars, as observed, for example, in *V. faba* (Abd El-Razek et al. 2013). An increase in monosaccharide content compared to control was also observed in algal cells treated with iron(III) concentrations in the range of 0.1-50 mg L^{-1}, although small compared to the effect of manganese(II). This is consistent with literature data on higher plants (Abd El-Razek et al. 2013) and is probably due to the fact that iron is one of the factors which regulate the activity of enzymes involved in the metabolism of sugars. It is known that during the first 48 hours iron(III) reduces the activity of one of the Krebs cycle enzymes – malate dehydrogenase, which inhibits the breakdown of glucose in cells (Russo et al. 2010). In addition, in the first day of culture, iron(III) stimulates the activity of phosphoenolpyruvate carboxylase (PEPCase) activity in *Zea mays* (Russo et al. 2010). An increase in the activity of this enzyme can lead to increased levels of sugars in the cell, which could also have occurred in *C. vulgaris*, in which the first day of culture was marked by the highest levels of monosaccharides in cells treated with various concentrations of iron(III).

In the present study manganese(II) and to a lesser extent iron(III) stimulated the overall growth in protein content in *C. vulgaris*. A significant increase in the concentration of these metabolites in *L. gibba* cells induced by 0.25-16 mg L^{-1} manganese(II) was also observed by Doganlar et al. (2012). Literature data show that iron(III) is a factor that induces an increase in the accumulation of proteins in plant cells. An increase in this parameter induced by biochemical iron(III) was also observed in *S. polyrhiza*. Higher concentrations of iron(III) (higher than 10 mg L^{-1}) caused a gradual decline in these metabolites in the cells of *S. polyrhiza*

(Xing et al. 2010) and *Brassica napus* (Pourgholam et al. 2013). An increase in protein content in algal cells treated with exogenous manganese(II) may be associated with stimulation of RNA polymerase activity (Mousavi et al. 2011), or an induced increase in the concentration of simple sugars, essential components of DNA and RNA. Furthermore, the addition to the culture medium of manganese at 50 mg L^{-1} did not result in a statistically significant decrease in protein in algae as compared with other biochemical parameters, which may have been due to the synthesis of stress proteins in response to excess manganese in cells.

It is known that iron(III) (Sajedi et al. 2011, Jucoski et al. 2013) and manganese(II) (Li et al. 2010, Arya and Roy 2011, Sajedi et al. 2011) at physiological concentrations and higher enhance the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX). Moreover, both elements respond to oxidative stress by increasing the activities of the antioxidant enzyme superoxide dismutase (SOD) in *Thalassiosira pseudonana* and *T. oceanica* (Peers and Price 2004). In this study cells of *C. vulgaris* treated with manganese(II) in a range of 0.1-50 mg L^{-1} had a significantly increased activity of antioxidant enzymes, in particular superoxide dismutase (EC 1.15.1.1), compared to control. However, in algal cells treated with various concentrations of iron(III) an increase in the activity of superoxide dismutase and glutathione reductase (EC 1.6.4.2) was not statistically significant and was maintained at a control level. The largest increase in the activity of both enzymes was observed under the influence of manganese at 50 mg L^{-1} (II), which indicates that this concentration may already be toxic and induce oxidative stress in *C. vulgaris*. This is supported by the observation that manganese(II) in the aforementioned concentration caused a decrease of the content of chlorophylls, monosaccharides, and algae cell number. It seems that the increase in the number of algae cells and sugars under physiological concentrations of manganese(II) (0.1-20 mg L^{-1}) is indirectly caused by an increased activity of SOD which activates cell division and inhibits the catabolism of glucose via the inhibition of Krebs cycle enzymes (Morgan et al. 2008). Furthermore, stimulation of the activity of SOD by manganese(II) may be positively correlated with an increase in GR activity. It is known that manganese (Arya and Roy 2011) and iron (Abadia and Zaharieva 2003) significantly increase the amount of reduced glutathione. Therefore, it cannot be excluded that a similar relationship exists in *C. vulgaris.* Reduced glutathione is a cell division-inducing factor via stimulation of the transition of cells from G1 to S phase (Kawano 2003) and protects plants against chlorosis (Ramirez et al. 2013), which may explain the persistence of the control level of chlorophyll under all iron(III) concentrations or an increase above control when the cells were treated with manganese(II) over the range of concentrations used in *C. vulgaris* in this study.

The study confirmed the important role of the concentration of manganese(II) in the growth of algae in water bodies (Górniak and Cudowski 2006, Cudowski and Górniak 2008, Grabowska 2012), because the higher the concentration of manganese in the solution the stronger the growth of algae. All the results indicate that the introduction of an appropriate amount of iron(III) helps control the level of manganese(II) by the co-precipitation of the metals. In this way it

may help control eutrophication, observed in lakes, seas, and rivers around the world (Selaman et al. 2008).

Conclusion

The obtained results clearly indicate that both manganese(II) and iron(III) induce significant changes at a cellular level not only in higher plants but also in unicellular algae. The conducted research shows that the largest increase of concentrations of biochemical parameters and enzyme activity took place in a situation where manganese was removed from the medium and then was re-introduced in different portions to the solution. In the situation in which iron was completely removed from the medium and then re-introduced in various amounts, co-precipitation of iron and manganese occurred, since iron in the acidic medium precipitates in the form of colloidal $Fe(OH)_3$. As a result of the aforementioned reactions, both metals were not available, hence the observed decrease in the concentrations of studied biochemical parameters and enzyme activity. The experiment also showed that an appropriate amount of iron(III) helped control the level of manganese(II) thanks to the co-precipitation of metals, and so could help in tackling the worldwide problem of eutrophication. Our results also showed a greater impact of manganese(II) than iron(III) on the growth of *C. vulgaris*. When both metals were completely removed from the medium, the introduction of manganese(II) resulted in a rapid increase of concentrations of biochemical parameters and enzyme activity, while when iron(III) was added, the increase was low.

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FREE AMINO ACIDS AND THEIR DERIVATIVES IN ONCOLOGY

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Abstract

The purpose of this work is a theoretical and practical justification for the creation of effective compositions of highly purified free amino acids and specialized mini-aminosols for metabolic therapy of malignant growth.

On the basis of the experimental data we suggest that the differences discovered in certain amino acids concentrations in blood plasma, red blood cells, and tumors are criteria in early diagnostics of primary cancerous growth as well as in estimation of the efficacy of specific cancer treatments. Clinical studies on biological fluids and tumors of patients with cancer of the mammary gland, lungs, prostate, ovaries, bladder or digestive tract showed significant changes in physiological concentrations of amino acids which either directly or indirectly regulate processes of antitumor response, oncogenesis, immunogenesis and apoptosis were shown. Our strategy of application of amino acids as medicinal preparations includes a targeted effect on the functional and metabolic relationships which are changed in specific pathology through the effect on the regulatory mechanisms of intermediate metabolic reactions, limiting stages of metabolic flows, utilization of energy substrates and transport systems restricting the processes of amino acids pool formation. The creation methodology of pathogenetic compositions of amino acids and their derivatives on the basis of their physiological concentration for practical application of their regulatory effects in oncology was discussed.

Key words: Free amino acids, regulatory effects, oncology.

Introduction

Tumor, the formation of amino acid imbalances and metabolic processes in the background of malignant growth: the levels and nature of the interaction.

The appearance of a malignant neoplasm is accompanied by changes in the intermediate metabolism, which manifest themselves at the level of the whole organism, individual organs, tissues, cells and enzymatic reactions. These changes are determined primarily by the properties of the tumor as a structure consisting of relatively poorly differentiated cells, characterized by unregulated growth, metastasis and necrosis [1-11].

Active tumor growth against the background of the relative reduction of individual chemical reactions (in particular, low enzyme activity and the rate of enzymatic amino acid metabolism) and the lack of perfect regulatory mechanisms

to maintain the metabolic balance in malignant cells leads to a competitive relationship between the tumor and the host organism in metabolism and energy.

These relationships are manifested in the ability of tumor cells to use the plastic material of the tumor carrier for the generation of energy and their own growth. Thereby, a high activity of metabolic processes is ensured in the tumor, in particular, accompanied by the formation of a significant amount of energy. Thus, the substrate provision of tumor cells becomes the limiting factor of their growth.This is particularly pronounced in cases where compounds that are absolutely or relatively essential (for example, some amino acids, essential lipids) for a macroorganism are used as an energy or plastic material. In addition, as is well known, a significant amount of cofactors of key enzymatic reactions (vitamins, microelements) are also irreplaceable nutrient factors to varying degrees.

The degree of indispensability of the above compounds against the background of malignant growth, thus, increases, inducing the formation of their functional deficiency both in the body of the tumor carrier and in the tumor itself.

Obviously, with their additional exogenous intake, the elimination of this deficiency will be determined by the activity of the transport systems of such compounds into host cells or tumors. Therefore, for example, anorexia at the level of the microorganism, on the one hand, and the ability of the tumor to actively "capture" endogenous compounds and nutrients, on the other hand, should be considered adaptive responses that provide in these conditions the principle of maximum survival of each system.

Such a kind of "metabolic competition" of a tumor and a macroorganism significantly limits the possibilities of metabolic correction and treatment of cancer patients in cases where it is associated with the need to introduce additional quantities of biologically active compounds of natural origin.

The next most important element that determines the tumor-tumor carrier relationship at the biochemical level is the phase growth of the tumor, in particular, periods of massive necrosis of cancer cells, accompanied by the appearance in the host fluids and tissues of significant amounts of a wide range of non-oxidized products and highly active prooxidants that induce a redox potential and metabolic imbalance in normal cells.

The described trends in cancer diseases are especially pronounced in the metabolism of amino acids. Our particular interest in studying the patterns of formation of a free amino acid pool is justified by the fact that malignant growth:

1. Against the background of anorexia, causes quantitative insufficiency of essential and relatively essential amino acids in the body – simultaneously, as a result of the activation of the degradation processes of endogenous proteins and amino acids, inducing a negative nitrogen balance;
2. Violates the processes of absorption and transport of free amino acids;
3. Shifting the redox potential of cells disrupts the relationship between anabolic and catabolic reactions in the exchange of amino acids;
4. The toxic effect of the decay products of the tumor is blocked by amino acids directly interacting with them (cystine, lysine) or their derivatives (glutathione), which leads to a deficiency of these compounds;

5. Changes the ratio and activity of metabolic processes associated with intermediate metabolism of amino acids (glycolysis, gluconeogenesis), as well as the processes of formation and energy consumption;
6. In addition, as mentioned above, amino acids and their derivatives are natural regulators of the activity of the processes of proliferation, differentiation and apoptosis of malignant cells [1-11].

Amino acids and their derivatives are mostly universal natural regulators and endogenous modifiers of biological reactions. However, numerous biological properties of these compounds as drugs have been used for correction of deficiencies or realization of pharmacological and immediate metabolic effects, disregarding any regulatory action. Amino acid profile indices that allow early detection of diseases, which would provide time for intervention before irreversible damage occurs, are being created. Thus, amino acid profiles represent biomarkers for diseases or deviations from a normal state of health. Our array technology will play an important role in metabolomics in biomarker discovery, clinical medicine, including cancer, as well as at other stages of drug discovery and development (for example, target discovery, mechanism of action, or predicting toxicity) [1-7].

Changes in the amino acid pool in liquids and tissues of patients specifically characterize the development of cancer. Correction of the intermediate metabolic changes in cancer can be reached by the use of certain amino acids or their combinations [8-10].

To understand the metabolic processes and vital functions of the regulatory effect of amino acids which manifests itself under natural or near concentrations of these compounds in body fluids and tissues, it is obvious that the effective use of L-amino acids or their derivatives for metabolic correction and directional changes in metabolism under pathological or extreme conditions is limited by insufficient accumulation of information about the key mechanisms of regulatory effects of the compounds tested at concentrations comparable to their physiological (endogenous) levels [1-3,7,10-13].

Amino acids and their derivatives are mostly universal natural regulators and endogenous modifiers of biological reactions. However, numerous biological properties of these compounds as drugs have been used for correction of deficiencies or realization of pharmacological and immediate metabolic effects, disregarding the regulatory action. Amino acid profile indices that allow early detection of diseases, which would provide time for intervention before irreversible damage occurs, are being created. Thus, amino acid profiles represent biomarkers for diseases or deviations from a normal state of health. Our array technology will play an important role in metabolomics in biomarker discovery, clinical medicine, including cancer, as well as at other stages of drug discovery and development (for example, target discovery, mechanism of action or predicting toxicity) [1-5].

Changes in the amino acid pool in liquids and tissues of patients specifically characterize the development of cancer. Correction of the intermediate metabolic changes in cancer can be reached by the use of certain amino acids or their combinations [7-8].

At present, there are the following main lines in exploitation of the biochemical (metabolic) properties of amino acids and their derivatives in clinical practice:

- Use of amino acids or multicomponent mixtures of amino acids (mainly, essential elements combined with vitamins and trace elements) for replacement therapy or deficiency of essential nutrients and proteins.
- Use of drugs containing individual amino acids or their compositions, designed on the basis of their additive functional and metabolic action, which "exploits" pharmacological activity (the effects of activation of redox processes, reactions of energy metabolism and neutralization of xenobiotics compounds) of this class [4-6,9].

However, the use of certain levels of L-amino acids, or their compositions or their deficiency, implements direct pharmacological effects that practically ignore their regulatory effects on metabolic processes and key metabolic reactions.

To understand the metabolic processes and vital functions of the regulatory effect of amino acids which manifests itself under natural or near concentrations of these compounds in body fluids and tissues, it is obvious that the effective use of L-amino acids or their derivatives for metabolic correction and directional changes in metabolism under pathological or extreme conditions is limited by insufficient accumulation of information about the key mechanisms of the regulatory effects of the compounds tested at concentrations comparable to their physiological (endogenous) levels [1-9].

Materials and methods

The results and our concept are based on research into the formation of free amino acids found in the biological liquids and tissues of about 1500 patients with cancer of the mammary gland, lungs, prostate, ovaries, bladder or digestive tract.

Our development methodology is based on:

- Studies on physiological concentrations of free amino acids, their derivatives, precursors and metabolites, as well as biochemical marker parameters in healthy donors and patients with various pathologies.
- Creation of a unified database for the parameters investigated, construction of an empirical mathematical model consisting of pathogenic markers of specific pathology and amino acid profiles.
- Specialized development of new formulations of the compositions of infusion solutions of amino acids and their derivatives [4].

The results and our concept are based on research into the formation of free amino acid pools in biological liquids and tissues of patients with cancer of the mammary gland, lungs, prostate, ovaries, bladder or digestive tract.

Results and discussion

Numerous results determining amino acids and their derivatives in human body fluids and tissues [3] allowed systematization of the accumulated data and identified the areas for exploitation of their metabolic effects, primarily in laboratory diagnostics, and application in clinical practice as drugs [4].

In view of the fact that free amino acids are represented by a wide range of related chemical structures and metabolic transformations of compounds that are formed in the body fluids and tissues, the findings obtained proved that quantification of the amino acid pool contributes to the diagnosis of various diseases, including hepatobiliary pathology, cardiovascular and immune systems, oncological cases, cerebrovascular pathology, as well as alcoholism and diabetes [1,2,4,9-11]. It turned out that the vast majority of the diagnostic values of the group had alterations in the levels of functionally and metabolically related amino acids and their derivatives while no specific changes, as such, were observed in the concentrations of individual compounds of this class.

Metabolically related amino acids and their derivatives had the nature of amino acid profiles of the body fluids and tissues of animals and humans when compared with the use of a multivariate analysis and mathematical modeling. At the same time, it was convincingly demonstrated that removal or correction of intermediate metabolic changes can be achieved using individual amino acids and their derivatives, or a combination of them as universal natural bioregulators – compounds that at physiological concentrations have a direct effect on the mechanisms of cellular metabolism [5,6,12].

By now, there is some evidence for the importance of not only amino acids as building blocks for protein synthesis, but also regulators of gene expression at the level of mRNA translation by an mTOR-dependent mechanism, signaling molecules and biological response modifiers, as well as precursors of a wide range of bioregulators, which play a key role in the integration of major metabolic fluxes [10-15].

Based on the positions of metabolomics, the free amino acid pool in biological fluids and tissues is regarded as a single information unit which is a kind of "chemical projection" of the genome; the proteome realized through this approach not only develops ideas about the pool of amino acids as a dynamic system-generated supply of them from outside, but also due to endogenous synthesis, transport, degradation and excretion, allows the identification of "key points" in the intermediate metabolic equilibrium shift that may reflect ratios at the individual levels of endogenous amino acids and related species (metabolically- related) compounds [4].

We were the first to demonstrate that endogenous levels of free amino acids in fluids and tissues are the most important integral indicators and regulators of metabolism.

This enables us to prove the use of individual amino acids or their combinations for guided correction of metabolism under specific human diseases, and significantly expands the area of practical application of these compounds as blood substitutes. The regulatory effects of amino acids contribute

to understanding their influence on biochemical processes and vital functions which is manifested at natural (endogenous) or close to natural concentrations of these compounds. The regulatory effect observed after administration of amino acids can be achieved by using either individual amino acids, or a combination of their small sets. The structure of these compositions in specific ratios may include almost any amino acids, their structural analogs, or derivatives with a known mechanism of action.

The trend developed is notable since it allows assessment of the pool of amino acids in biological fluids and tissues as a single information unit and while analyzing it, it makes possible to estimate the segments which disturb the metabolic balance (changes in the metabolic flow ratio and their balance shifts).

Our methodology proposed for development of new formulations of multicomponent infusion solutions based on amino acids and related compounds for the correction of metabolic imbalance occurring in various diseases relies on the application of research results to the regularities of formation of the amino acid pool in biological fluids and tissues under various pathological conditions. The composition and amount of highly purified amino acids in these infusion solutions should be determined primarily by their physiological (regulatory) concentrations, which distinguishes them from traditionally used amino acid solutions for parenteral nutrition, where the content of their components is calculated from the daily requirements of the human body for them without due consideration for the regulatory actions of the compounds administered.

In oncological practice, individual amino acids or their compositions should be applied according to their physiological concentrations and changes in the structure of the amino acid pool in patients. Amino acids with anti-carcinogenic effects may be leucine, tryptophane, and taurine.

Conclusion

Changes in the amino acid pool of liquids and their fund tissues of patients specifically characterize a cancer illness. To a large extent, they arise as a result of metabolic competition for common substrates for the plastic needs of the body cells and tumors. Correction of intermediate metabolic changes at a cancer can be reached by the use of separate amino acids or their combination.

The methodology of development of new constituents of multicomponent infusion solutions offered by us on the basis of amino acids and their related connections is intended for correction of the metabolic imbalance arising in various diseases and is based on application of the results on research regularities of formation of the amino acid pool in biological liquids and tissues of individuals under most various pathological states.

In oncological practice, certain amino acids or their compositions should be applied according to their physiological concentrations and changes in the structure of amino acids pools in patients. As a result of numerous experimental and clinical studies, we proved that diagnostically significant in oncology and as an "anticarcinogenic" are such amino acids as leucine, tryptophan, and taurine.

The new methodology of developing minicomponent amino acid infusion solutions offered by us intended for correction of the metabolic imbalance arising in the case of various localization and stages of malignant growth is based on the results of research into the regularities of formation of amino-acid pools in biological liquids and tissues.

The ratios of the individual components and their amount in such mixtures should comply with their physiological (endogenous) concentrations in normal blood plasma and tissues.

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DR. JEKYLL AND MR. HYDE: THE DUAL NATURE OF *MALASSEZIA PACHYDERMATIS*

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Abstract

Malassezia pachydermatis is a part of the physiological biota of the skin and mucous membranes of most mammals and birds. This opportunistic species causes mainly surface skin infections. Since *M. pachydermatis* is isolated almost equally from diseased animals and those without clinical symptoms of the disease, accurate diagnosis of infections caused by this yeast is a problem of great importance in veterinary and medicine. Therefore, the isolation of these fungi from patients is not equivalent to the identification of the etiologic agent of infection. According to the current state of our knowledge, the problem of diseases associated with *M. pachydermatis* is correlated with the metabolic, hormonal and immunological state of the host. However, in the light of a review of the literature, a significant question arises, whether the pathogenicity of *M. pachydermatis* is solely conditioned by host-dependent factors, or maybe the pathogenic strains have independent virulence mechanisms while the other factors only trigger their expression.

Key words: yeast, pathogen, commensal, pathogenicity markers

Introduction

The most common disease caused by *Malassezia pachydermatis* diagnosed in dogs is *otitis externa* (inflammation of the external auditory canal; Patterson and Frank 2002). However, the etiology factors predisposing to this disease and the role of mentioned microorganisms in the pathogenesis of the disease are not fully explained. Precise and early diagnosis determining the causes of the disease is very important for proper and effective treatment. According to the literature data, *M. pachydermatis* is isolated with almost the same frequency from sick and healthy animals (Dworecka-Kaszak and Adamski 2005, Girao *et al.* 2006, Prado *et al.* 2008, Campbell *et al.* 2010). This suggests that the development of disease symptoms depends on the immune as well as the metabolic and hormonal status of the host (Patterson and Frank 2002). On the other hand, it may indicate the presence of certain features of fungi, by which they regulate their impact on the host organism, which results in inhibition or stimulation of the immune response. Therefore, *M. pachydermatis* is referred to as the immune paradox (Dworecka-Kaszak and Adamski 2005), and the issue of a closer understanding

of their biology and mutual relationship with the host organism is still valid and current.

Although fungi of the *M. pachydermatis* species are commonly found in the microbiota of animals, especially in dogs and cats (Prado *et al.* 2008, Duarte *et al.* 2009), the methods of their influence on the host organism are still only partly explained. Most authors suggest that *M. pachydermatis* plays the role of commensal and opportunist (Cafarchia *et al.* 2008, Bond 2010, Shokri *et al.* 2010, Saunders *et al.* 2012). Nevertheless, there are data on the comparison of the characteristics of *M. pachydermatis* strains originating from animals without disease symptoms and suffering from *otitis externa*, which indicate some substantial differences between the strains representing these two groups (Kobayashi *et al.* 2011, Czyżewska *et al.* 2016, Czyżewska *et al.* 2018, Czyżewska *et al.* 2019). The existence of a correlation between the properties of strains and their potential virulence leads to the question – is it possible that different strains of *M. pachydermatis* have a distinct nature: commensal or pathogenic? In the case of a positive answer, we should also search for the discriminatory features characteristic of each particular ecological property. Consequently, there are two possibilities: either *M. pachydermatis* is (i) a typical opportunist and the infection develops as a result of host homeostasis disorders, or (ii) typically commensal strains that have specific characteristics which, in combination with conditions related to the physiological condition of the host, allow them to induce infection.

Genetic findings and properties

If we assume that certain biological features of the fungus affect its virulence, we should expect that potentially pathogenic strains should be more closely related to other virulent isolates, not to commensal strains. Data obtained by the nucleotide sequencing of the intergenic transcribed spacer (ITS1) fragment allow us to trace and reconstruct the phylogeny of the studied microorganisms. Moreover, the lack of natural selection effects on these fragments means that all their variability reflects phylogenetic relationships and the population structure of *M. pachydermatis*.

The results of the comparison of the nucleotide sequence of 65 strains of *M. pachydermatis* (Czyżewska *et al.* 2018) enabled us to identify 11 different genotypes that could be grouped into 3 major clades (Figure 1). The distinction of 3 separate genotypic groups is consistent with other available data in the literature in which *M. pachydermatis* strains originating from the skin and ears of dogs in Korea were analyzed (Han *et al.* 2013). What is more, a few authors have pointed out the relationship between the genetic profile of the studied fungi and their geographical origin (Machado *et al.* 2010), while others did not see any correlation at the genetic level, but noted relationships at the phenotypic level regarding the presence and activity of selected enzymes (Teramoto *et al.* 2015). Nevertheless, further research contributing to a deeper understanding of *M. pachydermatis* biology will help us to understand better the existing relationships between host and pathogen or commensal. Nevertheless, data from

various sources clearly indicate that we are dealing with a dualistic microorganism, and the genesis of *M. pachydermatis*-associated skin infections are at least partly dependent on the fungus properties.

The diversity of the nucleotide sequence of the ITS1 fragment indicates that the virulence of *M. pachydermatis* isolates may be correlated with their phylogenesis (Czyżewska *et al.* 2018). It should be noted that phylogenetic clade II (Figure 1) is composed of about 80% of the isolates from clinical cases. What is more, they represent two slightly different genotypes. Despite the definite dominance of potentially virulent strains in this clade, about 1/5 of the strains were isolated from animals without any signs of infection. On the other hand, in clades I and III, we observed the domination of isolates obtained from healthy dogs, while strains from *otitis externa* cases were constituting 35% and 31%, respectively. These observations are consistent with previous data on the great importance of the host animal's condition for the risk of disease development (Chang *et al.* 1998, Masuda *et al.* 2000, Tragiannidis *et al.* 2010, Gaitanis *et al.* 2012, Al-Sweih *et al.* 2014). Nevertheless, the close kinship of strains derived from sick animals supports the supposition of the participation of specific properties of the fungus in the development of infection, which may indicate the validity of the hypothesis about the occurrence of strains displaying both a pathogenic and commensal nature within the studied species. In addition, some premises from the literature also indicate the importance of features (which correlates with the genetic profile) of the fungus in the genesis of inflammation in animals. For example, it has been observed that specific subtypes of *M. pachydermatis*, established on the basis of the sequencing of the intergenic segment (IGS-1) correlate with excessive development of the fungal population and the synthesis of phospholipases that are a factor of its virulence (Kobayashi *et al.* 2011). Also Han *et al.* (2013), analyzing the IGS-1, ITS-1 fragments and the 26S rRNA gene found a different frequency of isolates representing different genotypes in healthy and sick animals. Kobayashi *et al.* (2011) noted the fact that strains grouped from sick animals regardless of strains isolated from healthy animals. However, there is no unambiguous data to answer the question whether the clear separation of strains involved in clinical cases is not the result of a speciation process associated with, for example, lack of gene flow (isolation) and natural selection. On the basis of present knowledge, there is no clear evidence of speciation (of virulent strains) among *M. pachydermatis*.

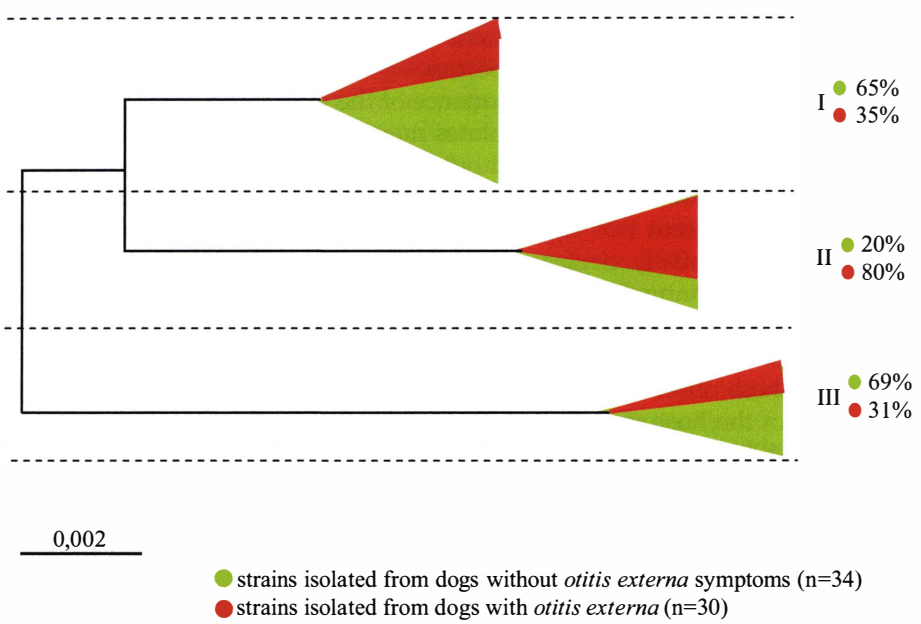

Figure 1. Dendrogram depicting the genetic relationships between *M. pachydermatis* isolates from healthy dogs and dogs with *otitis externa* (Czyżewska *et al.* 2018).

Comparison of lipid profiles

Data obtained from genetic analyses indicate the high diversity of *M. pachydermatis* strains. As mentioned above, a relationship between the origin of the fungus (from a sick or healthy animal) and its genetic profile was found. Hence, the natural consequence is the search for potential, genetically conditioned features of the fungus enabling it to effectively overcome the host's defense barriers.

Lipid profiles are used as an easy, fast and highly selective method that allows the identification of fungal pathogens such as *H. capsulatum* (Zarnowski *et al.* 2007), *C. albicans* and *Candida dubliniensis* (Mahmoudabadi and Drucker 2006), *Cryptococcus neoformans*, as well as also non-pathogenic fungus, *S. cerevisiae* (El Menyawi *et al.* 2000). In 2012, the lipid profile of *M. pachydermatis* was also compared with profiles established for *C. albicans* and *S. cerevisiae*; however, the research concerned only the reference strain (Tylicki *et al.* 2012). In 2016, lipid profiles of *M. pachydermatis* strains derived from dogs were determined (Czyżewska *et al.* 2016). These studies indicated that strains derived from dogs without *otitis externa* symptoms contain much more fatty acids than those isolated from sick dogs. This difference is maintained regardless of culture conditions (medium supplemented with lipids and medium without the addition of lipids). Differences between both groups of tested strains were also observed in

the content of particular fatty acids. Thin layer chromatography also provided interesting information on the differences in lipid profiles between the strains studied. Strains isolated from dogs with *otitis externa* showed significantly lower levels of triacylglycerols and ergosterol esters. These results show that the strains from the two studied groups are different, which may be a basis for determining whether a given strain is potentially pathogenic.

If we assume that *M. pachydermatis* is a typical opportunist, it is possible that strains derived from animals without disease symptoms are in a dormant phase (Brand 2012). *M. pachydermatis*, in addition to the ability to synthesize myristic acid, also has the ability to obtain lipids from the environment. Lower lipid content in strains derived from dogs with *otitis externa* may be due to the rapid proliferation of cells correlated with the appearance of disease symptoms. This process requires large amounts of energy, which could be also provided by lipids previously accumulated in the cells. Therefore, it can be assumed that the ability to quickly activate lipid reserves is necessary to recover *M. pachydermatis* cells from dormancy.

However, looking at the problem from a different perspective, the question arises whether the differences observed in lipid profiles may indicate the pathogenic nature of *M. pachydermatis* strains? Triacylglycerols are the primary source and storage form of energy in fungal cells, as well as a source of fatty acids necessary for the biosynthesis of membrane phospholipids (Rajakumari *et al.* 2010). Triacylglycerols are hydrolyzed to diacylglycerols and fatty acid residues, which can then be used in the β-oxidation process to release energy. Diacylglycerols, resulting from this hydrolysis, can serve as a scaffold for the biosynthesis of phosphatidylcholine or phosphatidylethanolamine in the Kennedy cycle (Czabany *et al.* 2007, Rajakumari *et al.* 2008). Sterols, which are released from sterol esters, can be introduced into the membrane, where they modulate the properties of the protein-lipid bilayer (Czabany *et al.* 2007). It was also observed that some nutrient components stimulate *in vitro* mycelium formation by *Malassezia* sp. Porro *et al.* (1977) obtained the growth of the mycelium of *Malassezia* sp. by adding cholesterol and its esters to the medium. Perhaps potentially pathogenic strains of *M. pachydermatis*, having a lower concentration of triacylglycerols and ergosterol esters, must compensate the deficiencies of these compounds using the resources of the host organism. This strategy combines potentially pathogenic strains with causing clinical manifestations of the disease.

Activity of extracellular phospholipases

M. pachydermatis is capable of extracellular secretion of many enzymes, including phospholipases. These enzymes are considered to be one of the factors leading to skin lesions (Juntachai *et al.* 2009). Phospholipases are active against the cell membrane composed of phospholipids and proteins. This naturally leads to the formation of pores and disruption of membrane function. In this situation, the pathogen has easier access to the cell, which further results in tissue invasion and the use of host resources to maintain fungal metabolic

transformations (Coutinho and Paula 2000). Inflammatory processes of the host skin are also associated with the release of arachidonic acid from cells, under the influence of fungal phospholipases (Buommino *et al.* 2016). In their research, Cafarchia and Otranto (2008), Vlachos *et al.* (2013) and Teramoto *et al.* (2015) found that *M. pachydermatis* strains isolated from dogs suffering from *otitis externa* have higher overall phospholipase activity compared to strains from dogs without clinical signs of disease. However, the method for determining the overall phospholipase activity is not completely specific and is considered prescreening (Pini and Faggi 2011). In 2015, Teramoto *et al.* determined phospholipase C activity. It turned out to be higher in strains derived from sick dogs. The increased activity of phospholipases in strains obtained from dogs with *otitis externa* in comparison with strains isolated from healthy animals indicates that these enzymes play an important role in the pathogenesis of diseases caused by *M. pachydermatis*. Due to the significant differences in phospholipase activity between strains from different sources (infected and healthy animals), phospholipase activity can be used as a marker for distinguishing between typically commensal and potentially pathogenic strains. By using such an additional criterion, it would be possible to improve the effectiveness of diagnostics of diseases caused by *Malassezia* fungi.

Differences in protein profiles

One of the tasks of proteomic research is to look for differences that can be used to improve and facilitate clinical diagnosis, choose an appropriate therapy, and constantly monitor the course of the disease (Kossowska *et al.* 2009). Currently, proteomic tests can be used in clinical diagnostics. They make it possible to indicate possible differences between the protein profiles of sick and healthy people, because the cause or consequence of a given disease may be the presence of a specific protein or proteins (Nahaczewska *et al.* 2014). Nowakiewicz and Ziółkowska (2013) indicated significant differences in the presence or concentrations of 8 proteins produced by *M. pachydermatis* by comparing isolates from infected and unaffected dogs. There are also reports in the literature that strains of *M. pachydermatis* from sick dogs have proteins that induce a strong immune response in animals (Bond and Lloyd 2002, Chen *et al.* 2002, Kim *et al.* 2010). In 2019, Czyżewska *et al.* (2019) compared the protein profiles of *M. pachydermatis* strains isolated from dogs suffering from *otitis externa* and from dogs without signs of disease. The performed analyses were based on electrophoretic separation of proteins by two-dimensional electrophoresis, followed by their identification by liquid chromatography coupled with tandem mass spectrometry (LC-MS / MS). As a result of spectroscopic studies, the authors found the presence of some proteins that occur only in one of the groups of strains studied and other proteins that differ between both groups in terms of quantity. The obtained results indicate differences in protein profiles between *M. pachydermatis* strains of different origins (Table 1). Naturally, we should assess if the identified proteins could be considered as a marker of pathogenicity.

In this context, the presence of NADP-dependent mannitol dehydrogenase protein in dog strains is of greatest interest. This enzyme is involved in the mannitol cycle, catalyzing the oxidation of D-mannitol to D-fructose, which is involved in the regeneration of NADPH in the cells of many fungi. This enzyme has also been shown to exist as a single isoform. Characterization of this protein in *Aspergillus parasiticus* was done by Niehaus and Dilts in 1982. This protein may be associated with the pathogenicity of microorganisms. Simon-Nobbe *et al.* (2006) showed that this protein is the main allergen of *Cladosporium herbarum*, which is considered to be the cause of allergic diseases in almost all climate zones. They found that NADP-dependent mannitol dehydrogenase is recognized by IgE antibodies in approximately 57% of all patients suffering from allergies associated with *C. herbarum*.

It is also interesting that the presence of ketol-acid reductoisomerase was also found in strains from clinical cases of *otitis externa*, but not in strains obtained from uninfected dogs. This enzyme is involved in the branched-chain amino acid biosynthesis pathway that occurs in various organisms from bacteria to fungi and higher plants, except for animals. Liu *et al.* (2014) characterized the homologue of *S. cerevisiae* ketol-acid reductoisomerase from *Fusarium graminearum*, which is an optional parasite of many plant species from cereals to tropical crops. In their studies using mutants with a deletion of the gene encoding this protein, they showed that this enzyme is necessary for the complete development of infections caused by *F. graminearum*. Therefore, there are premises to conclude that ketol-acid reductoisomerase may be of significant importance in the process of infections caused by *M. pachydermatis*.

Table 1. Proteins identified in selected spots in *Malassezia pachydermatis* strains (Czyżewska *et al.* 2019).

Proteins	**The name of the protein**
characteristic for strains isolated from dogs with *otitis externa*	– NADP–dependent mannitol dehydrogenase – cytochrome–b5 reductase – atpase v1 a1 complex subunit e – ketol–acid reductoisomerase – phosphoglycerate kinase
common for strains isolated from dogs	– cytochrome c – hypothetical protein Malapachy_0291 – aldo keto reductase – fructose–bisphosphate aldolase – ubiquinol–cytochrome c reductase complex core protein 2 – NAD–malate dehydrogenase – glyceraldehyde–3–phosphate dehydrogenase
characteristic for strains isolated from dogs without *otitis externa* symptoms	– guanine nucleotide binding protein beta subunit – ATP synthase f1 gamma – polyadenylate–binding protein – glycyl–tRNA synthetase

Conclusion

In summary, we should try to evaluate if all the mentioned facts are significant enough to be used as criteria for differentiation between pathogen and commensal. It is not easy to provide a clear answer. However, taking into account the presented research results, it cannot be completely excluded that *M. pachydermatis* comprises two, partly independent groups with different ecological properties. Furthermore, there are some pieces of evidence that support this hypothesis at the level of lipid profiles, phospholipase activity, and the protein profiles of *M. pachydermatis* strains isolated from dogs. These studies have shown that the lipid profiles of individual strains differ depending on the source of their origin. It is worth noting that some differences (reduced content of ergosterol esters, reduced total fatty acid content, presence of nervonic acid in strains isolated from sick dogs) are conservative and observed despite any changes in culture conditions. In addition, strains isolated from *otitis externa* cases showed significantly higher phospholipase C activity. These strains also showed the presence of characteristic proteins, the most important of which seem to be NADP-dependent mannitol dehydrogenase and ketol-acid reductoisomerase.

The situation observed in the case of *M. pachydermatis* appears to be similar to a recent finding in the field of bacteriology. For example, the *Bacillus cereus* sensu lato group of strains is also formed by strains with quite similar genetic properties and a common evolutionary history confirmed by multi-locus sequence typing and 16S rRNA gene sequencing (Bartoszewicz and Czyzewska, 2017a; Bartoszewicz and Czyzewska, 2017b; Bartoszewicz and Marjanska, 2017). Surprisingly, even among representatives of one species, there are often distinct virulence-associated strains with the ability to synthesize toxins. Thus, an approach that distinguishes different ecotypes (eg. pathotypes, thermotypes) in the entire species has recently become widely accepted and favored. Consequently, in the entire *M. pachydermatis* species, slight genetic differences result in small dissimilarities in the ecological properties of the fungus.

Considering the information presented above, it can be concluded that the results of all these studies can be used in practice to more accurately diagnose and determine whether we are dealing with a potentially pathogenic strain. If the host has no symptoms of the disease and is a carrier of *M. pachydermatis*, the lipid profile of this strain can be determined and, based on its analysis, it can be assessed whether the carrier is at risk of developing the disease. Therefore, preventative treatment can be given before the symptoms of the disease are revealed. To more accurately determine the risk of disease, a combination of several characteristics, eg. determination of the phospholipase activity or a search for characteristic proteins can be specified. This combination of traits may be the most effective marker of pathogenicity among clinically isolated strains.

In order to finally confirm this hypothesis, besides the necessary clinical experiments on animals, a description of the entire proteome of *M. pachydermatis* strains of different origins should also be carried out. Expression analysis of genes coding identified proteins can also be performed by the use of real-time RT-PCR in order to determine the level of expression of marker proteins

in clinical isolates. Summing up these facts, we strongly believe that *M. pachydermatis* consists of both ecotypes, pathogenic and commensal isolates, but still, host-associated factors are important for the development of the infection.

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AN ALL SOLID STATE ION-SELECTIVE ELECTRODE FOR LEAD MONITORING IN THE ENVIRONMENT

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Abstract

Cheap and simple, an all solid state lead ion selective electrode for lead determination is described. The PVC electrode membrane was modified by addition of multiwalled carbon nanotubes and ionic liquid 1-hexyl-3-methylimidazolium hexafluorophosphate. The electrode with a modified membrane was characterized by a lower detection limit and better selectivity in relation to sodium, potassium, and calcium ions than an electrode having membrane with a classic composition. It also exhibited good potential stability and reproducibility.

Key words: lead ion-selective electrode, solid contact, ionic liquid, multiwalled carbon nanotubes

Introduction

Lead is a very strong poison that is one of the environmental pollutants and tends to accumulate in organisms and ecosystems. Exposure to this element even at a low level causes adverse effects on human health. Lead is an inhibitor of hemoglobin synthesis and shortens the life of erythrocytes, resulting in anemia. It has a detrimental effect on the functioning of the central nervous system, it can lead to brain edema, degeneration of nerve cells and the death of nerve cells. Poisoning with this element can also cause so-called symptoms of psychopathic anxiety, irritability, mental numbness and memory loss, headache or muscle tremors, and in drastic cases even coma and death [1]. Due to the harmful effects of lead, it is very important to monitor its content in the natural environment. It is present in air, soils and natural waters. In the case of marine waters, it is about 10-60 ng/l, and inland surface waters - 200 ng/l [2]. In the environment, lead occurs primarily as inorganic Pb (II) which can be readily detected and quantified potentiometrically with ion-sensitive electrodes (ISEs) sensitive to lead ions. Among different types of construction, ISEs with a solid contact are become more and more popular due to their numerous advantages including small size, simple construction and operation, low cost of production and ability to operate in high pressure environments. Unfortunately in the case of direct connection between polymeric membrane and electronic

conductor, there are often problems with the stability and reproducibility of the potential. Therefore numerous modifications in the electrode construction were introduced including, conducting polymers [3], metal nanoparticles [4], self-assembled redox layer [5] and carbon based nanomaterials [6].

In this paper a new lead ion-selective electrode in all solid state mode is presented. For membrane preparation two additional components, multiwalled carbon nanotubes and ionic liquid, were used.

Materials and methods

Reagents: Lead ionophore(IV)- Fluka, bis(1-butyl)pentyl adipate (BBPA) – Fluka, polyvinyl chloride (PVC) –Aldrich, multiwalled carbon nanotubes (MWCNTs) –Aldrich, potassium tetrakis(p-chlophenyl)borate (KTpClB)- Fluka, tetrahydrofurane (THF)-POCH. Other reagents were obtained from Fluka.

The ion-selective electrodes were prepared using a glassy carbon (GC) disc of 3 mm diameter as an internal electrode. Before membrane deposition GCEs were polished with alumina powder and rinsed carefully with water and THF. The composition of the membrane mixture was, for electrode 1, 3% (w/w) HMImPF6, 1% (w/w) MWCNT 63% (w/w) BBPA, 33% (w/w) PVC; for electrode 2, 1% (w/w) ionophore, 0,5% (w/w) KTpClPB, 65,75% (w/w) BBPA, 33% (w/w) PVC; for electrode 3, as in electrode 2; for electrode 4, 1% (w/w) ionophore, 3% (w/w) HMImPF6, 1% (w/w) MWCNT, 62% (w/w) BBPA, 33% (w/w) PVC. The membrane components were weighed, dissolved in THF and the mixture was homogenized using an ultrasonic bath for ca. 40 min. Next 50 µL of membrane cocktail was applied on the top of GCE. In the case of electrode 3 the MWCNTs were introduced as an intermediate layer between the membrane and internal GC electrode. The obtained solid contact electrodes were conditioned in $1x10^{-3}$ mol L^{-1} $Pb(NO_3)_2$ for at least 24 h to saturate the PVC membrane with lead ions. All conditioning and sample solutions have the same background $5x10^{-3}$ mol L^{-1} acetate buffer pH=4.8.

All potentiometric measurements were performed at room temperature in a solution stirred with magnetic stirrer using a measuring system consisting of a multichannel data potentiometric acquisition system (Lawson Labs. Inc., USA) connected to a PC computer. As a reference electrode the Ag/AgCl electrode Metrohm 6.0750.100 was used.

Results

The effect of membrane composition on electrode performance was evaluated on the basis of calibration curves determined in $Pb(NO_3)_2$ solution in the range $1x10^{-7}$ – $1x10^{-2}$ mol L^{-1}. The obtained results are shown in Figure 1.

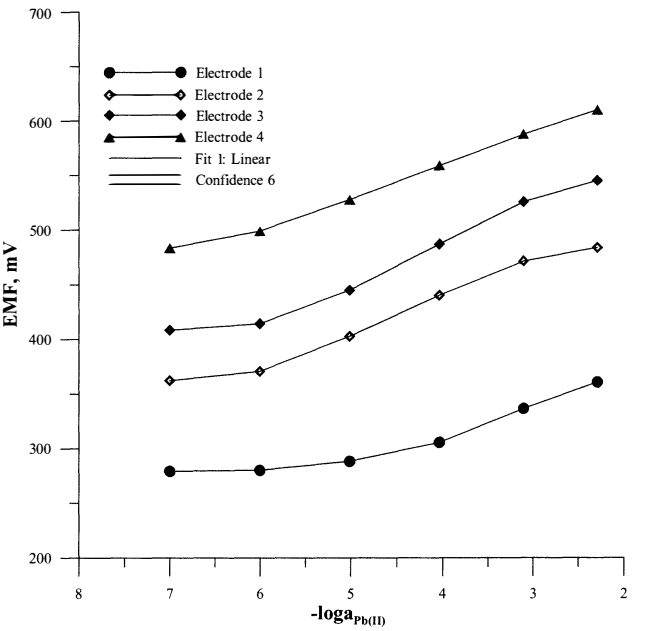

Figure 1. Potentiometric response of lead ion-selective electrodes.

On the basis of course the calibration curves basic analytical parameters, such as detection limit, linear range, and characteristic slope were determined. They are summarized in Table 1.

Table 1.

Parameter	Electrode 1	Electrode 2	Electrode 3	Electrode 4
Characteristic slope mV/ $pa_{Pb(II)}$	31.6	31.4	36.5	30.1
Detection limit, mol L^{-1}	$1.6x10^{-5}$	$5.6x10^{-7}$	$4.2x10^{-7}$	$1.0x10^{-7}$
Linear range, mol L^{-1}	$1x10^{-4}$ $-1x10^{-2}$	$1x10^{-6}$ $-1x10^{-3}$	$1x10^{-6}$ $-1x10^{-3}$	$1x10^{-6}$ $-1x10^{-2}$
Potential drift, mV/day	0.28	3.02	2.86	0.13
Potential reversibility, mV	2.39	1.54	1.32	0.78
Potential reproducibility, mV	7.34	42.93	55.13	1.09

The selectivity of studied electrodes toward interfering ions such as: (Na^+, K^+, Ca^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}) was evaluated. To this purpose the selectivity coefficients values were determined using a separate solution method [7]. The obtained results are listed in Table 2.

Table 2.

Ion	Electrode 1	Electrode 2	Electrode 3	Electrode 4
Na^+	-2,2	-1,5	-2,5	-4,3
K^+	-1,1	-3,3	-3,8	-5,3
Ca^{2+}	-3,7	-2,2	-3,7	-3,8
Mg^{2+}	-3,6	-6,9	-6,8	-6,5
Co^{2+}	-3,4	-6,9	-6,8	-5,9
Ni^{2+}	-1,4	-6,7	-6,6	-5,6
Cu^{2+}	0,39	-6,7	-6,6	-5,3
Zn^{2+}	-1,5	-6,9	-6,8	-5,9
Cd^{2+}	-2,8	-3,5	-4,4	-3,4

Discussion

In this research the effect of membrane modification on electrode properties was studied. Four kinds of ion selective electrodes were constructed and studied. As can be seen in Figure 1 all electrodes exhibited theoretical sensitivity to lead ions but the detection limit and the linear range were different for particular electrodes. Electrode 2 with membrane containing commonly used components responds linear to Pb^{2+} ions in the activity range $1x10^{-6}$ – $1x10^{-3}$ mol L^{-1}. The linear range and the detection limit were improved by introduction to the membrane phase IL and MWCNTs. Electrode 4 whose membrane contained these components exhibited the lowest detection limit $1x10^{-7}$ and a linear response in the activity $1x10^{-6}$ -$1x10^{-2}$ mol L^{-1}. Electrode 4 also had better parameters in comparison to electrode 3 with classic membrane composition and carbon nanotubes used as a separate inner layer between the ion-selective membrane and internal electrode. The modification of the membrane by MWCNTs and IL results in essential improvement of potential stability, reversibility, and reproducibility. These parameters determined for electrode 4 were significantly better than for the remaining electrodes (Table 1) .

Selectivity is one of the most important electrode parameters. From the analysis of the values of selectivity coefficients given in Table 2 it can be concluded that all electrodes were characterized by good selectivity coefficients towards interfering ions. Electrode 4 with modified membrane exhibited better selectivity in relation to sodium, potassium, and calcium ions in comparison to electrodes 2 and 3. This is a beneficial effect, from a practical point of view in the case of sample analysis, for instance of natural water where the content of these ions is higher than the lead concentration. On the other hand slightly worse values of selectivity coefficients were observed in relation to cobalt, nickel, copper and zinc ions. This change is not important because the values obtained were still very good and interference from these ions can be negligible.

Conclusions

As a result of the conducted tests, it was found that modification of the membrane with carbon nanotubes and ionic liquid has a positive effect on the parameters of the electrode. Electrode no 4 with a modified membrane exhibits better parameters than electrode 2 with a classic membrane composition and electrode 3 with classic membrane composition, carbon nanotubes used as a separate inner layer between the ion-selective membrane and an internal electrode. The electrode is characterized by a lower limit of detection and better selectivity in relation to sodium, potassium and calcium ions. It also demonstrates good stability and reproducibility of the potential. An additional advantage is the fact that its preparation, usage and service are very simple.

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ECOTYPES OF *BACILLUS CEREUS* SENSU LATO

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Abstract

Bacillus cereus sensu lato is a group of bacteria common in the environment, which have an important impact on the activity of humans. Their taxonomy is still in doubt, mainly because of their properties encoded by plasmid-borne genes that may potentially undergo horizontal gene transfer. Here we present genetic and phenotypic data that support the idea of a bacterial group (named *B. cereus* sensu lato) instead of several independent taxa. We conclude that *B. cereus* sensu lato is composed of distinct, but closely related ecotypes.

Key words: cold-adaptation, 16S rRNA, sequencing, real-time RT-PCR, phylogeny

Introduction

Bacillus cereus sensu lato is a group of bacterial species that are common in the natural environment as well as in plant tissues, the intestines of animals and food matrices (Bartoszewicz and Czyżewska, 2017b). The special interest focused on these bacilli is associated with their properties. For instance, *B. cereus* and related species are able to biosynthesize several enterotoxins. Among them, two three-component enterotoxins (Nhe, HBL) and one cytotoxin (CytK) are involved in human food poisoning. On the other hand, rare isolates of *B. cereus* may also form a cereulide called an emetic toxin due to the list of symptoms caused after intoxication (Bartoszewicz et al., 2006). The next interesting property of *B. cereus* sensu lato is their ability to form spores. Apart from their resistance to heat or a lack of nutrients and water, they may also stand conditions that occur in food processing lines (high pressure, low concentration of oxygen, rapid temperature changes), which directly leads to contamination of ready-to-eat products, especially those made from milk and cereals, but not only. The next threat is linked to *Bacillus anthracis*, as this bacterium produces a complex toxin (composed of a protective antigen and lethal toxin or, in another variant, of protective antigen and edema factor), which is responsible for anthrax. Moreover, these bacilli also form a capsule that is not recognized by the host's immune system, so the infection course is usually rapid, and without proper treatment leads to death. Naturally, the mentioned bacteria also display positive properties. For instance, *B. thuringiensis* is used for the production of

bioinsecticides used worldwide in order to protect crops. Next, *B. toyonensis* shows some probiotic activities, so there is a hope to use it in the production of probiotics for cattle (Bartoszewicz and Czyżewska, 2017b).

Apart from numerous studies on the biology and ecology of *B. cereus* sensu lato, the taxonomy of this group is not coherent and it is still being discussed. It is suggested that due to high homogeneity in the 161S rRNA gene sequence (often >99% identity) and similarities in total DNA-DNA hybridization, all the rods pertaining to *B. cereus* sensu lato should be treated as one polymorphic species. However, there are some important phenotypic properties typical only for a small group of isolates. Only representatives of *B. thuringiensis* form crystal parasporal inclusions, which can be visualized by a specific staining technique or by the application of phase-contrast microscopy. In turn, *B. mycoides* forms colonies with rhizoidal morphology. Isolates of *B. anthracis* are non-motile (in opposition to the rest of the group), do not cause hemolysis on sheep blood agar, and are susceptible to penicillin. Finally, the still discussed *B. weihenstephanensis* differs in the sequence of the major cold-shock protein gene (*cspA*) and has a unique motif in its 16S rRNA gene. In the case of *B. weihenstephanensis*, mentioned features are associated with its psychrotolerant characteristics. What is more confusing, several discriminatory properties of particular taxa in the *B. cereus* group are encoded by genes located on large, potentially mobile plasmids and could be transferred during conjugation or lost during intensive proliferation in optimal conditions (Bartoszewicz and Czyżewska, 2017b). Regarding these facts, the hypothesis of ecotypes instead of species in the *B. cereus* sensu lato has been proposed. Thus, we decided to evaluate the impact of the average temperature on the frequency of different ecotypes in samples from distinct climate zones. We also tried to compare ecological properties with data provided by nucleotide sequencing of the 16S rRNA of randomly selected isolates representing different *B. cereus* sensu lato taxa.

Materials and methods

Bacteria isolation. Thirteen different sampling sites in distinct locations were chosen in order to obtain samples. Each sample of soil (20 g) was aseptically collected using falcon tubes from a layer located approximately 2-5 cm below the ground surface after all the plants were removed. Next, soil samples were suspended (10% w/v) in LB broth, incubated for 10 minutes with vigorous shaking and subsequently pasteurized (72°C for 10 minutes) in order to eliminate competitive gram-negative microflora. Finally, 100 µl of the soil suspension was transferred onto the surface of MYP agar (mannitol-egg yolk-polymyxin agar, Oxoid), which is a selective medium for *B. cereus* sensu lato. After two days of incubation at 30°C (this temperature supports the growth of both psychrotolerant and mesophilic isolates), matt purple colonies with a visible zone of lecithin precipitation were selected and bacteria were transferred onto Columbia 5% Sheep-Blood agar (Oxoid) to investigate their hemolytic activities. Finally, after

purification by passages on LB agar using the streak plate technique, bacteria were frozen in LB with glycerol (v/v 1:1) at -80°C.

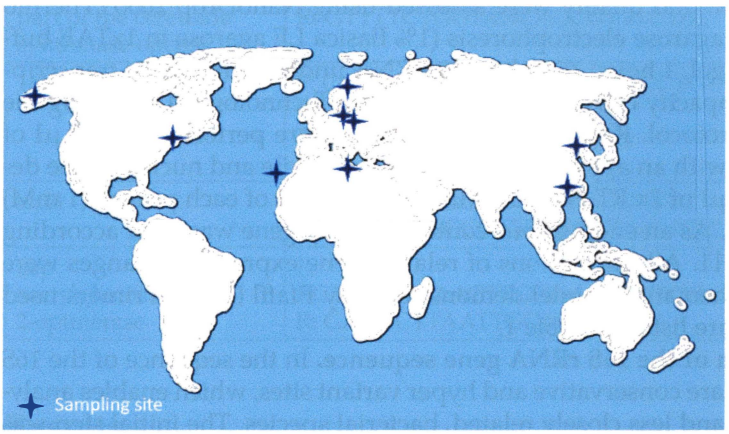

Figure 1. Approximate localization of sampling sites.

Identification of *B. cereus* sensu lato. Isolates were identified according to the procedure described elsewhere. Bacteria with rhizoidal colonies were identified as *B. mycoides/B. pseudomycoides* without further discrimination. Isolates with typical colonies were visualized under phase-contrast microscopy (Olympus BX-61). The presence of any parasporal inclusions (in case of sporulated cells incubated for 72 hours at 30°C on T3 sporulating agar) was interpreted as confirming *B. thuringiensis*, while a lack of protein crystals suggested *B. cereus* or *B. mycoides*. Next, visible growth of isolate at 7°C on LB agar after 9 days of incubation indicated psychrotolerance. Such isolates were tested for the presence of the specific sequence in the *cspA* gene (what is typical for *B. weihenstephanensis*). The lack of this genetic feature combined with adaptation for growth at cold conditions indicated psychrotolerant *B. cereus*. All isolates were also screened for the *pag*, *lef* and *cap* genes characteristic for *B. anthracis*; however, none of the samples gave a positive result.

Characterization of isolates. Bacteria were tested for their adaptation to different temperatures and growth rates, next their putative toxicity was assessed by the use of real-time RT-PCR technique (in relation to Nhe, HBL and CytK synthesis) or PCR (for investigation of cereulide synthetase gene complex). Psychrotolerance was assessed initially as described above; however, for growth rate at cold and optimal temperatures, liquid LB cultures were applied. In this case, chilled LB broth (5 ml in sterile glass tubes) was inoculated with 50 µl of a fresh culture of a particular strain (optical density OD_{600}=1,5). Immediately, the optical density of this sample was assessed by spectrophotometer (Jasko V 670) and incubation was started. Independent samples of each tested strain were incubated at 4°C (10 days), 7°C (10 days), 30°C (2 days), 37°C (2 days), 43°C (2 days) and 54°C (2 days). Next, every 24 hours, each sample density was measured to assess the proliferation dynamics. All cultures and measurements were repeated twice. In order to evaluate potential toxicity, total RNA was isolated

from randomly selected strains by the use of Total RNA Mini Plus Kit (A&A Biotechnology) according to the protocol provided by the manufacturer. Next, RNA concentration and quality were assessed using NanoDrop 2000 (Thermo Scientific) and by agarose electrophoresis (1% Basica LE agarose in 1xTAE buffer, 5V/cm of the gel, 1 hour). Good quality RNA underwent reverse transcription with High Capacity cDNA Synthesis Kit (Life Technologies) following the manufacturer's protocol. Real-time amplifications were performed in 20 μl of total volume and with an application of the 6 μl of sterile and nuclease-free deionized water, 10 μl of 2x RT HS-PCR Mix SYBR A, 1 μl of each primer (1 mM) and 2 μl of cDNA. As an endogenous control, the *udp* gene was used according to Reiter et al., 2011. All calculations of relative gene expression changes were based on the mathematical model demonstrated by Pfaffl (2001). Primers used for amplification are listed in Table 1.

Polymorphism of the 16S rRNA gene sequence. In the sequence of the 16S rRNA gene, there are conservative and hyper variant sites, which enables analyses of both, more and less closely related, bacterial species. The initial step was DNA isolation from overnight cultures using Blood and Tissue DNA Isolation Kit (Qiagen) and automatic station for nucleic acid isolation (QiaCube, Qiagen) with the protocol for gram-positive bacteria. Obtained DNA was tested using NanoDrop 2000 and then was used as a template for amplification as described elsewhere (Bartoszewicz et al. 2009). Amplified samples were purified using Clean-up kit in order to remove any residual nucleotides, primers, and enzymes, according to the protocol provided with the kit. Next, samples were amplified in GeneAmp 9700 thermocycler (Applied Biosystems) using BigDye Terminator v4.1. kit (Life Technologies), and residual terminators were removed by the use of ExTerminator Kit (A&A Biotechnology) with the typical procedure recommended by the manufacturer. Finally, samples were sequenced in DNA sequencer ABI 3150 (Life Technologies). Obtained sequences were visually examined in Chromas Lite software and imported to BioEdit software for performing sequence alignment. In the next step, sequences were exported to Mega 6 software. Before the reconstruction of *B. cereus* sensu lato phylogeny, we performed tests for the optimal evolutionary model. On the basis of the BIC scores, the maximal likelihood method with general time reversible model (GTR+G+I) was applied to construct a dendrogram.

Table 1. Sequences of primers used in real-time PCR and PCR assessment of *B. cereus* sensu lato.

Gene	Sequence 5'-> 3'	Reference
nheA part of the Nhe operon	F: TTTAATTGCGGGGTTATTGG R: ACTACTCATCGCGCTCACC	Melnick et al. (2012)
hblA part of the HBL operon	F: CCTTGCAAAAGGCTGGATTA R: TCGTGTCCCAAGTAACAGC	Melnick et al. (2012)
cytK	F: GATAATATGACAATGTCTTTAAA R: GGAGAGAAACCGCTATTTGT	Świecicka et al. (2006)

Gene	Sequence 5'-> 3'	Reference
cesA part of the cereulide synthetase operon	F: GGTGACACATTATCATATAAGGTG R: GTTTTCTGGTAACAGCGTTTCTAC	Ehling-Schulz et al. (2005)
16S rRNA	F: CGCGGGATCCGAGTTTGATCCTG-GCT R: GGCCGTCGACACGG(A/C) TACCTTGTTACGACT	Lechner et al. (1998)
cspA	F: CGAATTTGATAATGTGTGGATTC R: CCCGGATCCGGTTACGTTA(G/C)C	Lechner et al. (1998)
udp acetylglucosamine 2-epimerase	F: ACTAGAGAAACTTGGAAATGATCG R: GACGCTTAATTGCACGGAAC	Reiter et al. (2011)

Results

During the entire study, 400 *B. cereus* sensu lato isolates were obtained from soil samples collected during two years in China (2 sampling sites, N=84 isolates), Poland (3 sampling sites, N=71), Germany (1 sampling site, N=59), Egypt (2 sampling sites, N=66), Madera (2 sampling sites, N=56), USA (Alaska, 1 sampling site, N=15) Sweden (1 sampling site, N=20), and finally Rhode Island (1 sampling site, N=39).

The distribution of particular taxa pertaining to the *B. cereus* group is presented in Figure 2.

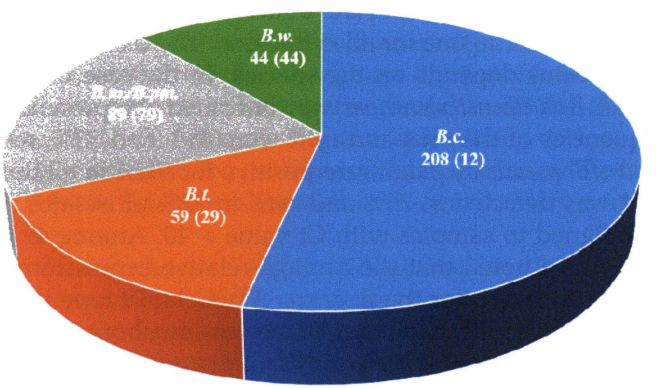

Figure 2. Distribution of *B. cereus (B.c.)*, *B. thuringiensis (B.t.)*, *B. mycpides/B. pseudomycoides (B.m./B.pm.)* and *B. weihenstephanensis (B.w.)* in soil samples. Presented values indicate total number of isolates and (in brackets) number of cold-adapted isolates.

As shown, the dominant species in all samples was *B. cereus* representing more than half of the isolates. However, only 12 of them (5.7%) shared cold-adapted

characteristics but did not possess the *cspA* gene or specific motif in the 16S rRNA gene characteristic to *B. weihenstephanensis*. The frequencies of remaining species were quite similar, but psychrotolerance was more common in *B. weihenstephanensis* (all isolates) and *B. mycoides/B. pseudomycoides* (89%), while surprisingly half of the *B. thuringiensis* isolates proliferated at 7°C and in most cases also possessed the *cspA* gene detected in the PCR screening. Apart from the fact that the presence of each species in samples from different climate zones were similar, samples were significantly different in the percentage of psychrotolerant strains. In the Alaskan soil, as well as in samples from Sweden, we noted 55% of all psychrotolerant *B. thuringiensis* isolates. Moreover, samples contained significantly more *B. mycoides/B. pseudomycoides* and *B. weihenstephanensis* than samples from the remaining locations. On the other hand, samples obtained from Egypt, Madera and one of the Chinese sampling sites (Shenzhen) were devoid of psychrotolerant representatives of *B. cereus* sensu lato. Finally, soil obtained from Rhode Island (USA), Poland, Germany and China (approx. 70 km from Pekin) showed a moderate frequency of cold-adapted bacilli. For instance, both samples from Poland contained totally 39 *B. cereus* isolates (among them 3 were psychrotolerant), 5 psychrotolerant *B. thuringiensis* isolates (as well as 9 mesophilic ones), 7 *B. weihenstephanensis* and 11 *B. mycoides/B. pseudomycoides* (all psychrotolerant). Similarly, in soil from Germany, we found 31 *B. cereus* (1 psychrotolerant), 11 *B. thuringiensis* (including 3 psychrotolerant ones), 12 *B. mycoides/B. pseudomycoides* (among them 11 were cold-adapted) and finally 5 *B. weihenstephanensis*.

The toxicity of *B. cereus* sensu lato was assessed using real-time RT-PCR in the case of 50 randomly chosen strains representing each species proportionally. As reference strains, we used the *B. cereus* ATCC 14579 type strain (for *nheA*, *hblA*, and *cytK*). The value of the relative level of expression for the reference strain was arbitrarily set to one for all cytotoxins. The distribution of the genes encoding enterotoxins depends on the species. In the case of all *B. cereus, B. thuringiensis* and *B. weihenstephanensis* we confirmed the presence of the *nheA* gene; the frequencies of the *hblA* and *cytK* were 48% and 37%, respectively. In turn, *B. mycoides/B. pseudomycoides* were positive for *nheA* in all but one isolate. However, *hblA* was detected in 76% and *cytK* in 22% of isolates. The negative results were assigned to samples with Ct value > 40. Among 49 isolates positive for the *nheA*, we noted that the average relative expression rate was 0.45 of the reference expression for the *B. cereus* ATCC 14579 type strain. However, *B. mycoides/B. pseudomycoides* exhibited an expression about 25% lower in comparison to *B. cereus, B. thuringiensis* and *B. weihenstephanensis*. In turn, the *hblA* gene was expressed more efficiently in *B. mycoides/B. pseudomycoides* (its average rate was 1.54 times higher than in the case of reference *B. cereus* ATCC 14579 strain) and the remaining taxa showed expression rates ranging 0.47 (*B. thuringiensis*) to 0.67 (*B. cereus*). Finally, the *cytK* gene was expressed efficiently only in three *B. cereus* isolates (average expression rate 1.13 of the reference expression), while in the case of *B. thuringiensis* we noted only 0.44 of the reference, and in *B. mycoides/B. pseudomycoides* or in *B. weihenstephanensis* it did not exceed 0.32. None of the strains was positive in the case of the *cesA* gene necessary for the biosynthesis of cereulide.

Tests of growth proved that *B. cereus* sensu lato can multiply at a wide range of temperatures. *B. mycoides/B. pseudomycoides* were able to grow at 4ºC (33% of isolates) and at 7ºC 89% were multiplicated on solid and liquid media. Next, 50% of *B. weihenstephanensis* grew well at 4ºC and all isolates were proliferating at 7ºC. However, none of *B. mycoides/B. pseudomycoides* and *B. weihenstephanensis* grew at or above 43ºC. Among *B. thuringiensis* one psychrotolerant isolate grew at 4ºC, while the rest of the cold-adapted strains multiplicated at 7ºC and at 30-37ºC, but not at higher temperatures. *B. cereus* isolates were not able to grow at 4ºC, but almost 6% were proliferating at 7ºC. Those psychrotolerant isolates were also able to multiply at 30ºC, 37ºC, and 43ºC, but not at 54ºC. In general, no isolates were growing at 54ºC (the temperature, which is a diagnostic feature for thermotolerant *B. cytotoxicus*).

The polymorphism of the isolates was assessed with the aid of 16S rRNA gene sequencing from 147 randomly selected isolates. Obtained data were used for the phylogeny reconstruction of the isolates in the context of their origin, taxonomy, toxicity, and temperatures of growth. A dendrogram depicting relationships between isolates belonging to the *B. cereus* sensu lato group is presented in Figure 3. On the basis of all presented data we can conclude that *B. cereus* and related taxa exhibited a high level of genetic polymorphism in the 16S rRNA gene sequence. However, the number of variable sites suggests that variant fragments of this gene constitute up to 1% of the entire examined fragment (about 1420 kbp). Thus, all the members of the *B. cereus* sensu lato have their 16S rRNA gene sequence with an identity above 99%. The next important outcome of the study is linked to the group signed as 'cold-adapted' bacilli. Among them are members of *B. weihenstephanensis, B. mycoides/B. pseudomycoides*, but also representatives of *B. thuringiensis* and *B. cereus*. Interestingly, apart from typically psychrotolerant *B. mycoides/B. pseudomycoides* or *B. weihenstephanensis*, cold-adapted strains comprised also both *cspA*-positive *B. thuringiensis* and *cspA*-negative *B. cereus*. Deeper analysis showed that psychrotolerant bacteria constitute two branches on the phylogenetic tree. On the first one (clade C2 in Figure 3), there were strains with psychrotolerance that could be easily linked to the presence of the *cspA* gene, while the second one, named clade C1 (with the domination of *B. cereus* and *B. thuringiensis*), was devoid of this genetic feature.

Apart from those findings related to cold-adaptation, the 16S rRNA gene does not allow for differentiation between particular *B. cereus* sensu lato. In some cases, isolates of *B. cereus* are much more similar to *B. thuringiensis* than to other representatives of *B. cereus*. For example, strains pertaining to clade A were representing *B. cereus* and *B. thuringiensis*. However, we did not find any case where the identical nucleotide sequence of the 16S rRNA gene could be observed for more than one species. Clonal *B. cereus* sensu lato (the biggest group consisted of seven *B. mycoides/B. pseudomycoides* isolates from clade C2 and a few other groups composed of four isolates identified as *B. cereus* from clade D), *B. weihenstephanensis* (from clade C2) or *B. mycoides* (also from C2), respectively were tested to assess if they share the same phenotypic characteristics. According to the results, the range of growth temperatures was the same and the ratios of expressions of the *nheA, hblA* and *cytK* were not different significantly within the group of species with

an identical 16S rRNA gene sequence. What could be added (data not shown), clonal isolates were also identical when examined using REP-PCR fingerprinting technique. Surprisingly, we did not observe any correlation between the sequence of the 16S rRNA and the origin of the strains. For example, the mentioned clonal group of seven identical *B. mycoides/B. pseudomycoides* isolates was composed of isolates from Poland, both Chinese sampling sites, and from Rhode Island (USA).

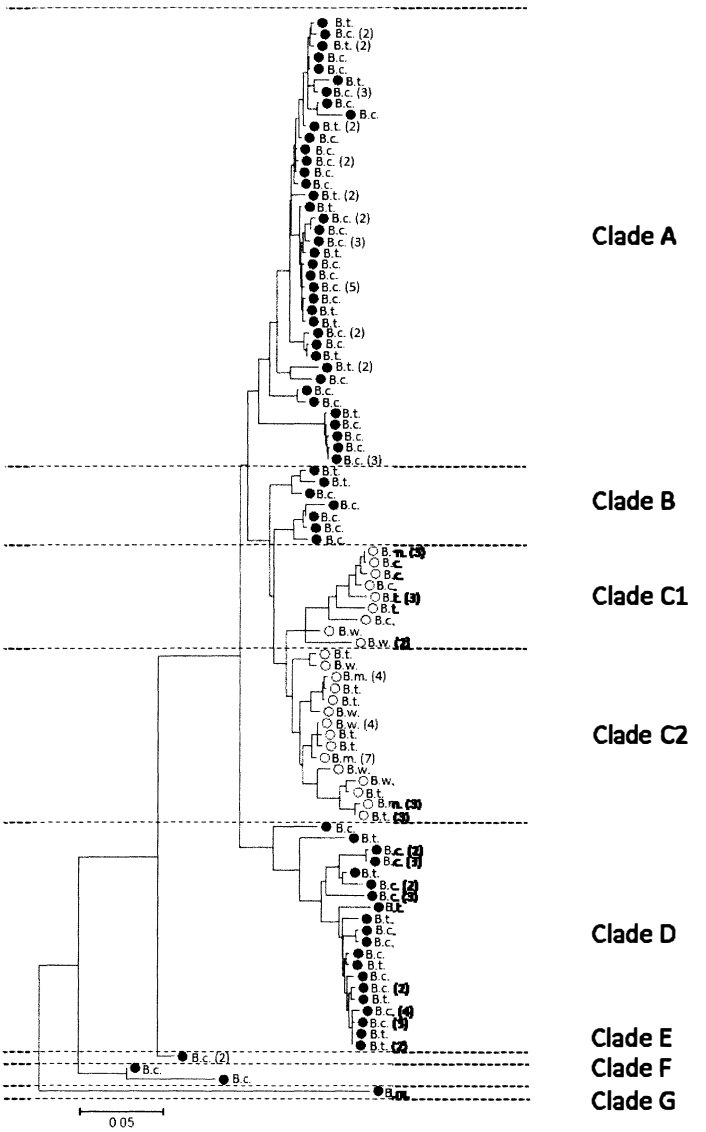

Figure 3. Dendrogram depicting phylogenetic relationships between selected isolates of *B. cereus* sensu lato. Phylogeny reconstruction using minimal evolution method and general time reversible model (GTR+G+I)

using Mega 6.0 computer software. Cold-adapted bacilli are marked with an empty circle, while mesophilic isolates are marked with black wheels. Species affiliation is as follows: B.c. – *B. cereus*, B.t. – *B. thuringiensis*, B.w. – *B. weihenstephanensis*, B.m. – *B. mycoides/B. pseudomycoides*. Numbers in brackets display the number of isolates with identical 16S rRNA gene sequence. Scale at the bottom corresponds to the genetic distance.

Discussion

B. cereus sensu lato is an interesting object for intensive study on the border of ecology, medicine, veterinary science and industry (especially the dairy industry). Their huge impact on human activities and the health status of people and animals cannot be omitted. However, still there are some important gaps in the general knowledge about these bacteria. Their taxonomy is doubtful. Some evidence supports the hypothesis of one polymorphic species while other evidence suggests that we are dealing with different taxa (Bartoszewicz and Czyżewska, 2017). Recently, a few authors, basing on different and independent results have suggested that we should change our perception of *B. cereus* sensu lato. The approach that leads to the recognition of distinct species has practical advantages (eg in medical diagnostics), but it is not sufficiently supported by experimental data. In turn, treating all diverse *B. cereus* sensu lato as one species with several unique properties characteristic to only one taxon is also wrong. Thus, we should rather treat *B. cereus* sensu lato as a bacterial group (with a rank between genus and species) and focus our attention on the properties of particular, interesting strains.

The percentage of psychrotolerant and mesophilic *B. cereus* sensu lato varies between samples; however, we see a strong correlation between climate at the sampling site and the range of temperatures of growth. In previous studies, the highest rate of cold-adapted bacilli was noted in the temperate climate zone (Bartoszewicz et al., 2009), because it could give an advantage in competition with soil microflora at these conditions. For example, the average temperature in Poland (on the basis of data from 1981-2010) is 7.3ºC with the hottest month (July) with 18.0ºC and the coldest one (January) with -3.1ºC. For comparison, in Cairo (Egypt) the coldest month is also January with 14.0ºC, while the highest temperatures are observed in July (28.4ºC), so these conditions much better fit to mesophiles. Interesting questions are often asked about the development of cold-adaptation. This feature, initially addressed only to *B. weihenstephanensis* (Lechner et al., 1998) and *B. mycoides* (Jensen et al., 2003) appears to be more common. Bartoszewicz et al. (2009) proved the existence of cold-adapted *B. thuringiensis* and suggested that they appeared as a result of a conjugation event between *B. weihenstephanensis* (as a recipient) and *B. thuringiensis* (as a plasmid with *cry* genes donor). However, MLST studies (Bartoszewicz and Marjańska, 2017; Bartoszewicz and Czyżewska, 2017) showed another subgroup among

B. cereus sensu lato with a psychrotolerant phenotype. In combination with current results (the lack of the *cspA* gene in some of the psychrotolerants) it may indicate the independent origin of cold-adaptation and possible different pathways for achieving this feature.

The lack of any correlation between climate and toxicity could also be explained. The ability of toxin biosynthesis is useful in different conditions. *B. thuringiensis* uses its insecticidal toxins to break the intestinal barrier of larvae, which results in a leak of the hemolymph into the intestine and provides nutrients for bacteria (Bartoszewicz and Czyżewska, 2017). On the other hand, food-poisoning toxins that were examined in the present study may have a quite different practical application for bacteria. One of the theories assumes that those toxins are used for quorum sensing by the bacteria (Raymond et al., 2013). In this context, their synthesis may be favored in habitats with high concentrations of *B. cereus* sensu lato, for example in food matrices and in the dairy industry, where biofilms containing *B. cereus* and related species could be formed.

Conclusion

Summing up all the mentioned findings, we should note that *B. cereus* sensu lato is rather one polymorphic species with several distinct ecotypes. Natural selection for the properties which lead to increased fitness in specific conditions is the force which forms closely related clades more typical to occupied habitats than to taxonomic affiliations. Moreover, horizontal gene transfer may complicate the taxonomy of *B. cereus* sensu lato leading to the acquisition of properties typical to the different taxa, as was suggested for *B. weihenstephanensis* with features of *B. thuringiensis*. Finally, climate conditions appear to be the most powerful force for the selection of psychrotolerant members of *B. cereus* sensu lato; however, cold-adaptation is probably characteristic to two different evolutionary lineages of the entire group.

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IT'S COMPLICATED: DIFFICULT RELATIONSHIPS BETWEEN MICROBIAL ECOLOGY AND TAXONOMY

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Abstract

Taxonomy is a branch of the sciences that is focused on the classification of organisms. A crucial problem for microbial taxonomy is elaboration of a coherent definition of bacterial species. With the aid of *Bacillus cereus* sensu lato, we discuss the different ideas for this definition and demonstrate the role of ecology and the ecological niche occupied by the bacterium in its evolution. We conclude that direct application of an actual definition of a bacterial species is not always possible.

Keywords: *Bacillus cereus* sensu lato, natural selection, species, the phylo-phenetic definition of species

Introduction

Taxonomy is a branch of science that encompasses the identification of particular organisms as well as their classification with suitable nomenclature and descriptive properties. Among distinct approaches important in taxonomy, especially evolutionary taxonomy based on Darwin's findings and post-Darwinian classification, phenetic taxonomy (that requires comparison of overall similarities) and phylogenetic conception (based on features derived from common ancestors) are widely discussed and used in scientific practice (Cohan, 2002b; Małek et al, 2005). Ecology is focused on any interactions between different organisms (including intraspecific and interspecific relations) or between organisms and their environment. Those relations may have both positive and negative impacts on the studied life forms, but if they are not neutral, they will play an important role in their evolution (Bartoszewicz, 2018). Consequently, we cannot analyze that evolution without paying attention to the habitats and ecological niches occupied by each particular species, because no organism is completely isolated from other life forms and its natural environment.

Naturally, the entire relationship between ecology and taxonomy has been widely studied. However, most findings are based on animals and plants. In opposition to them, knowledge about microorganisms and their evolution in

the context of their ecology is not so wide. Moreover, we still do not have a precise definition of bacterial species, and the criteria used for identification of microorganisms are often far from being perfect. Thus, the first step on the way to understanding the interesting relation between ecology and other sciences in microbiology must start with elaboration of a coherent definition of the bacterial species (Konstantinidis and Tiedje, 2005; Konstantinidis et al., 2006; Wiedenbeck and Cohan, 2011). So we have decided to discuss different approaches to this problem and the criteria used for precise identification and differentiation between microbes by the aid of model organisms.

Why does the definition of a eukaryotic species not fit bacteria?

For several years, scientists have tried to elaborate a coherent definition of species as it is fundamental for taxonomy and systematics. Actually, following Mayr's ideas, species are defined as a basic unit of biological classification which groups genetically-related individuals capable of producing fertile offspring as a result of sexual reproduction. Organisms classified into different species should be also reproductively isolated from others, which naturally do not preserve formation of sterile hybrids. Following these criteria, we can conclude that organisms from one species usually occupy similar ecological niches (but their range depends on their ecological tolerance, which is different for distinct species) and should originate from a common ancestor. Such a definition reflects the evolutionary traits and fits very well with the natural systematics. Naturally, as in most cases with living organisms involved, here we can also expect some doubts and challenges. For example, it is difficult to assess where the border of a species is if a ring species is found. One of the popular examples (however also recently discussed) are herring gulls (*Larus argentatus* and *Larus fuscus*) – for example, by Liebers et al. (2004) or by Martins and Packert (2007). Nevertheless, ring species are found to be a bridge between the micro- and speciation process (Irwin et al., 2001). They form distinct populations which do not interbreed, but they are connected by a geographical ring of populations that may interbreed. Next, common shrews (*Sorex araneus*) are represented by numerous chromosomal races (races with different karyotypes formed in the process of Robertsonian fusions of chromosomes). However, representatives of different races are able to have offspring whose fertility is reduced (but often not blocked) due to severe complications in meiosis (eg during the conjugation of chromosomes, prophase I).

Unlike the research focused on eukaryotes, studies on bacteria have a much shorter history. First reports on the existence of microscopic forms, yet unknown, were provided by Antoine van Leeuwenhoek in the seventeenth century. The next microscopic observations, description of bacterial shapes, and even Pasteur's theory of fermentation caused by microbes were only initial studies. Finally, Robert Koch formed and elaborated a method for isolation of pure bacterial cultures. This finding enabled precise description of the properties of

known microbes and, with Koch's postulates, facilitated differentiation between commensal and pathogenic bacteria. However, still, the describing of bacterial species was a case of artificial taxonomy because it reflected only phenotypic and/or ecological similarities.

Even later, after Mayr published his definition of the species, bacterial taxonomy was based on different criteria (Mayr, 1942). The most important problem was related to the lack of sexual reproduction among prokaryotes (Cohan, 2002b). Bacteria proliferate by cell division, and as a result of this process, two daughter cells are formed. Apart from spontaneous mutations that may occur during chromosome replication, those daughter cells are identical, so bacterial reproduction leads to the formation of a population of clonal cells. Next, the diversity observed among microbes is much higher than among eukaryotes. A high polymorphism rate is an effect of multiple factors. First of all, bacteria are haploids, so mutations (besides neutral and silent) are visible in their phenotype. The lack of nuclear membrane makes bacterial chromosomes much more exposed to different damaging factors, like bacteriophages or free radicals. This, combined with the weaker repair capabilities (weak correctional activity of bacterial DNA polymerase) and lower fidelity of DNA replication, results in high diversity, even among a group of closely related microorganisms (Bartoszewicz, 2018). We also should be aware that most bacteria (especially at optimal environmental conditions) multiply very rapidly and quickly, often with the generation time below half an hour. This leads to successive generations in a short period of time. Even if the mutation rate is constant, with each of the numerous generations the chance of mutation also increases. Next, which remains in connection with ecology, bacteria, even pertaining to one species, occupy different niches. Even species typically associated with animals, like *Escherichia coli* (its primary habitat is the colon, a part of the large intestine) can be found in soil samples or as a form of municipal pollution in water. Finally, this bacterium can be found in food products as a consequence of poor hygiene standards and selected isolates may lead to the development of infections (eg enterohaemorrhagic isolates, EHEC). Such a wide range of potentially isolated habitats requires different adaptations, but it results in independent accumulation of mutations and polymorphism (Cohan, 2002a). As mentioned before, bacteria are not able to reproduce by a sexual process. Instead, they have developed a few parasexual processes, which allow them to exchange their genetic information. Conjugation, the process of transferring (usually) plasmid DNA between a donor (a cell that has already replicated its plasmid or replicates it during the conjugation event) and a recipient (a cell that obtains DNA from the donor), results in propagation of some genetically encoded features in the bacterial population. Some of these features might be evolutionarily beneficial, like antibiotic resistance for clinical isolates. Another kind of horizontal gene transfer (HGT) is transformation, where bacteria obtain DNA from the environment and may incorporate such a fragment into its own chromosome and express the genetic information. The effect of this process, described for streptococci, is phenotype change, from apathogenic to pathogenic. Finally, transduction, another case of HGT but involving bacteriophages, may cause formation of a stable recombinant form.

What is interesting, the kind of environment is important here. For example, conjugation is much more frequent and effective in habitats rich in nutrients, probably because of easier to achieve higher concentrations of microbes. Thus, food matrices may be endangered by this phenomenon. Another surprising fact that complicates the taxonomy of microbes is the need to establish a pure culture prior to the describing of a new species. The percentage of viable but non-culturable microorganisms (VBNC) is not precisely established, but we could expect their high frequency, especially in environments where mineral compounds dominate, and among autotrophic bacteria (Oliver, 2005).

Naturally, we completely omit another unique form placed on the border between life forms and complex chemical particles, viruses. On the one hand, they do not behave like living organisms outside of its host, so there is no replication, metabolism, or motility observed in the environment. On the other hand, while present in cells, they are replicated and released; moreover they undergo phenomenon typical to organisms, like inheritance of their properties, mutation, or evolution. What is more, different viruses may have distinct genetic material (dsDNA, ssDNA, ssRNA, dsRNA) with unusual organization (segmented, circular, linear, covalently closed). In the present paper, the problems of taxonomy of viruses will therefore not be included.

Phylo-phenetic definition of bacterial species

According to the facts provided above, Mayr's definition does not suit the prokaryotes. However, we could expect that similar processes, like in the case of eukaryotes, will lead to speciation. Speciation is in general a result of some kind of isolation and subsequent accumulation of distinctive features. So, the gene flow between representatives of different species should be prohibited or at least reduced. Next, natural selection should favor beneficial properties (in the context of actual environmental conditions) and eliminate unfavorable features. Here we meet an important correlation between ecology and evolution. Bacteria with wide distribution in the environment are often more diverse. This is a result of the need to adapt to different conditions present in the natural state. Complex niches (like soil) with its different structure and nutrients, require the ability for multiple metabolic processes necessary for obtaining energy from distinct substrates. Typical pathogens with a narrow range of hosts must be, in turn, adapted to one niche. Their requirements include mechanisms of avoiding host immune system mechanisms, rapid proliferation, and resistance to the most common antibacterial therapeutic agents. This may be observed in the case of *Klebsiella pneumoniae*, which is quite common, but the 'hospital environment' pressures resulted in selection for the resistant isolates and finally development of *K. pneumoniae* NDM-1 (also known as *K. pneumoniae* New Delhi strain) producing metallo-β-lactamases and being resistant to plenty of antibiotics used in therapy (Khan et al. 2017).

All these facts were considered in order to elaborate a suitable definition of a bacterial species. At the early beginning of microbiology, based on bacterial

shape, colony morphology, virulence, and substantial biochemical properties, an artificial taxonomy was constructed. It was useful in medicine and veterinary research, however this approach did not reflect evolutionary relationships between organisms. Next, deeper and more detailed biochemical analyses became important and they enabled a first attempt to reconstruct phylogeny on the basis of similarities that might be genetically encoded. Moreover, numerical methods could be applied for biochemical data. However, the most important data were provided by molecular techniques. Similarities in G+C content, total DNA-DNA hybridization (DDH) reflecting the overall similarities between genomes of compared bacteria, gained importance. Next, sequencing of housekeeping genes (in MLST technique, multi-locus sequence typing) or comparison of nucleotide sequences of ribosomal DNA (eg 16S rRNA gene) depicted provided data that were suitable for reconstruction of phylogeny. Finally, the recent approach is based on the comparison of complete genomes. This method is getting cheaper and the special equipment necessary for this procedure becoming more accessible. Unfortunately, even recent molecular techniques are not ideal. 16S rRNA genes may be present in several copies in the bacterial chromosome and there is no guarantee of their identity in one isolate. Next, conservative sites display a low level of variability and only short fragments represent hyper-variable sites, so the comparison of closely related microbes could be difficult and hard to note. In turn, new generation sequencing, a powerful tool for the assessment of complete genomes, detects all the variability and provides huge pieces of data that might be difficult to interpret.

Summing up all these troubles and limitations, a phylo-phenetic concept of species in bacteriology was proposed (Rossello-Mora and Amann, 2001). It assumes that bacteria pertaining to one species must have at least 97% of identity in the 16S rRNA gene sequence, not less than 70% of similarity in the DNA-DNA hybridization, and one or more phenotypic distinctive features. This definition, accepted by most microbiologists, meets some restrictions, especially in relation to groups of related bacteria that differ mainly in their phenotype. Thus, we decided to verify how a phylo-phenetic definition of bacterial species suits *Bacillus cereus* sensu lato rods.

What is *Bacillus cereus* sensu lato?

B. cereus sensu lato (often named the *B. cereus* group) comprises several bacterial taxa, which are common in the natural environment and play important roles in ecology, medicine, and in the dairy industry. The best-known representative of the whole group is *Bacillus cereus* (also named *B. cereus* sensu stricto). This bacterium is found in soil, however it easily penetrates into the food processing chain, mainly on dairy farms (Bartoszewicz and Czyżewska, 2017). Consequently, it is commonly found in raw and pasteurized milk, salads, spices, pasta, and vegetables. Unfortunately, *B. cereus* is able to produce several food-poisoning toxins that create a health hazard to consumers. Among the toxins, hemolytic enterotoxin (HBL), non-hemolytic enterotoxin (Nhe), or cytotoxin K

(CytK) are dangerous even when only a small piece of contaminated food undergoes consumption because they are produced in the small intestine by vegetative forms of the mentioned bacteria. After reaching sufficient concentrations, they interact with the intestinal epithelium, leading to the development of symptoms of diarrheal syndrome. In turn, cereulide, a dodecadepsipeptide ring structure toxin (with important resemblances to the ionophore antibiotic, valinomycin) is produced in food prior to its consumption (Bartoszewicz and Czyżewska, 2017; Kroteń et al. 2010). It is a quite stable peptide, so even heat-treatment and denaturing chemical factors do not inactivate cereulide. Its intoxication causes disturbance of ATP synthesis in mitochondria, which leads to acute liver and kidney failure, which might be fatal. Like the other representatives of *B. cereus* sensu lato, this species is able to form spores (called also endospores) which are resistant to UV light, drought, lack of nutrients, as well as high temperature or freezing. Dormant spores may survive in food processing lines and germinate even after several years of unfavorable environmental conditions. Spores can be easily dispersed by the air (wind) or water. For many years it was thought that spores are common in the natural environment, but vegetative cells with metabolic activity are typical only to restricted environments, like the digestive tracts of invertebrates or mammals. Since recent findings have proved the presence of vegetative cells in plant material (eg cabbage) or in the freshwater of Poland, this assumption has had to be revised. Consequently, if vegetative cells are more common than previously thought, they may undergo horizontal gene transfer and interactions with the environment. Moreover, natural selection in wild-type habitats could favor not only strains forming extremely resistant spores but also those with cells able to use nutrients present in the environment.

Another important representative of the mentioned group is *Bacillus anthracis,* which causes anthrax, a dangerous disease of mammals including humans. This species synthesizes a unique capsule and toxins responsible for the development of the symptoms. Its virulence is directly linked to two mega plasmids, pXO1 and pXO2. It is puzzling that similar plasmids (but often devoid of the pathogenicity island) have been found in numerous isolates of *B. cereus.* The next species, *Bacillus thuringiensis* is known due to its insecticidal activities. *B. thuringiensis* owes its properties mainly to *cry* genes located on plasmids that potentially could be mobile and undergo horizontal transfer. Finally, psychrotolerant *B. mycoides* with rhizoidal colonies on solid media and *B. weihenstephanensis* (recently recognized only as a variant of *B. mycoides*) are able to proliferate at low temperatures (4-7ºC), but their multiplication completely stops above 43ºC. Other species classified into the *B. cereus* sensu lato (eg *B. toyonensis, B. cytotoxicus, B. wiedmannii*) have little importance or are not commonly accepted and described on the basis of digital DNA-DNA hybridization (like *B. manliponensis, B. bingmayongensis, B. gaemokensis*). For more details, please see the review by Bartoszewicz and Czyżewska (2017).

Phylo-phenetic species definition vs. *Bacillus cereus* group

In order to assess the correctness of the phylo-phenetic species definition, we should follow all the criteria and try to apply them for the mentioned bacteria. So, the first point is the similarity level of the 16S rRNA gene sequence. For bacteria in one species, we expect values >97%, while members of one genus should display at least 95% identity. Numerous studies using sequencing of this gene, as well as alignments of sequences available in public databases (eg NCBI, National Centre for Biotechnology Information) clearly show that *B. cereus* sensu lato has less than 1% of polymorphic sites in the 16S rRNA gene. This directly leads to the conclusion that we are dealing with one species. However, there is an important rule. If the similarity of the 16S rRNA gene sequence is below the threshold value, this is evidence for a distinct species. However, values above 97% should be interpreted as the lack of evidence for different species, not as evidence for one species.

The next criterion in modern taxonomy is the level of similarity in DDH. This method is based on spectrophotometrical measurement of the hybridization of DNA isolated from two compared isolates. This method is informative; however, recently its variant, dDDH (digital DDH) has become more common and useful. If strains display more than 70% similarity, they should be assigned to one species. Consequently, lower values confirm two different species. However, even from our studies performed in 2012-2016, we found two isolates representing *B. cereus* and *B. thuringiensis* that shared 76% similarity in DDH (which indicates one species) and surprisingly we also found isolates from distinct sources (soil, milk, freshwater) that were phenotypically and genetically identified as *B. cereus* (case 1) and *B. weihenstephanensis* (case 2), though they showed only 37% (case 1) and 43% (case 2) similarity, respectively. Moreover, dDDH is believed to be too sensitive, which could result in description of a new species that in fact does not yet exist.

Finally, as mentioned above, bacteria belonging to one species must have at least one, common among them but distinctive from others, phenotypic property. This is a seemingly simple situation here. For example, *B. cereus* differs from *B. thuringiensis* by the lack of parasporal crystal inclusions. They are quite easy to find under phase-contrast microscopy and their presence clearly indicates that we are dealing with *B. thuringiensis*. However, one can ask what would happen, if during rapid proliferation this property is lost in the process called curing? Without plasmids containing *cry* genes, it is impossible to synthesize crystal proteins and *B. thuringiensis* becomes indistinguishable from *B. cereus*. Another problem was shown by Bartoszewicz et al. (2009). Authors isolated *B. thuringiensis* from soil (NE Poland) that shared psychrotolerant characteristics of *B. weihenstephanensis*. A proposed explanation of this fact is horizontal transfer of the *cry* genes from *B. thuringiensis* to recipient cells of *B. weihenstephanensis*. However, the taxonomic affiliation of these strains is confusing and problematic. They represent features typical for two different taxa and meet the criteria enabling identification of them as both *B. thuringiensis* and *B. weihenstephanensis*. Further experiments with conjugation proved that HGT may occur within *B. cereus* sensu lato leading to the formation of cold-adapted strains with protein

crystals in the described way, but the efficiency of this process is extremely low (Bartoszewicz, unpublished data). Another example is *B. mycoides* and *B. weihenstephanensis*, which both, in fact, are psychrotolerant. In this context, the only way for differentiating between them is to compare their colonies on solid media: *B. mycoides* forms rhizoidal forms in opposition to the typically round colonies of *B. weihenstephanensis*. Finally, typical properties of *B. anthracis* are linked to its virulence associated with two large plasmids: pXO1 and pXO2. Genes from these extrachromosomal DNA encode toxins and capsule, both necessary for causing symptoms of anthrax. Actually, we know that the same type of plasmids (called pXO1-like and pXO2-like) are common in the entire *B. cereus* group and do not always result in virulence (Bartoszewicz and Marjańska, 2017). Apart from distribution of the pXO1 and pXO2-like plasmids, *B. anthracis* has its own properties, like susceptibility to penicillin or lack of motility. Moreover, probably because of the need to adapt to a narrow niche and the activity of purifying selection, this pathogen is much more homogenous and may represent an independent evolutionary line within the group.

Thus, several authors have suggested that species within *B. cereus* group may only play a role in applied microbiology and be useful in clinical diagnostics due to the properties traditionally associated with particular taxa. Nevertheless, an approach that recommends remaining on the bacterial group level or even genus level, without distinguishing individual species, can definitely reflect our current knowledge better than strictly following and applying the phylophenetic definition of species. The enormous role of environmental pressures affects bacterial characteristics, including their stability and diversity, proliferation rate, occurrence of mutations, specific adaptations, and the frequency and efficiency of horizontal gene transfer.

Conclusion

Summing up these facts, we have to say that the current criteria for bacterial species play their role for most microbes; however in some cases, they are still problematic. Thus, it is necessary to elaborate a brand-new approach for bacterial taxonomy: an approach that includes the difference of microorganisms, their high rate of polymorphism, problems associated with horizontal gene transfer and data obtained from well-performed studies including the entire genome sequencing. And until that moment, we have to remain aware that the systematics and taxonomy of bacteria are partly artificial and based on arbitrarily set criteria. An approach based on ecotypes (ecological types of bacteria within a species, eg pathotypes, thermotypes) much better reflect their characteristics and effects of natural selection, so they should be also considered in further bacterial taxonomy.

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SEQUENCE DIVERSITY OF MHC CLASS II DRB GENES IN THE MOOSE *ALCES ALCES* FROM ITS CONTINUOUS RANGE IN POLAND

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Abstract

Many vertebrate species show a very high degree of polymorphism of the genes of the major histocompatibility complex (MHC), which is associated with its key role in their immune response. However, studies on MHC class II DRB gene diversity in moose populations in Europe and North America have shown a low level of polymorphism, with clear differences between populations. In our study we examined the diversity in a fragment of an MHC class II DRB gene with 226 base pairs in 196 moose from Poland from seven populations. We identified eight (from 5 to 8 in particular populations) MHC II DRB alleles, of which *DRB1*11* was unique to the moose population in the Biebrza Valley. This population has experienced a series of numerous significant reductions in historical times and has been largely isolated from other moose populations. Although genetic differentiation among populations for the MHC II DRB gene was low, PCA analysis clearly showed the distinct character of the Biebrza population. This may confirm the relict character of this moose population.

Key words: *Alces alces*, DRB gene, MHC, moose, relict

Introduction

Major histocompatibility complex (MHC) genes play a key role in vertebrate immune response (Janeway et al., 2004). They are characterized by significant allele diversity, high durability, and high heterozygosity (Klein et al., 1993). The high diversity of MHC loci potentially contributes to increased resistance to environmental pathogens and thus to the survival of the species. Many studies conducted on wild mammals show a correlation between low MHC gene diversity and decreased adaptation (Yasukochi et al., 2012). Reduced MHC diversity has been observed in populations or species at risk of extinction or those that had previously experienced a bottleneck effect (Babik et al., 2005, 2009; Mainguy et al., 2007; Radwan et al., 2007). However, although many studies do show correlations between MHC diversity and resistance to infectious diseases, it does not seem to be a universal principle. MHC diversity in the California sea lion (*Zalophus californianus*) – a non-threatened species with a healthy level of

genetic diversity – is much smaller than in other mammalian species (Bowen et al., 2004). On the other hand, a very low level or even lack of MHC diversity, including the DRB locus, in the snow goat (*Oreamnos americanus*), most probably does not increase its susceptibility to diseases (Mainguy et al., 2007). Reduced diversity in the MHC loci may be the result of population reductions followed by postglacial expansion in temperate and boreal areas (Babik et al., 2009). MHC genes are potentially influenced by natural selection, with the pressure of parasites being the likely main factor influencing their diversity (Hedrick et al., 2000; Lachish et al., 2007). Another significant evolutionary force playing a role in MHC evolution is genetic drift (Garrigan and Hedrick, 2003). When population size is small, MHC diversity is shaped mainly by demographic processes and genetic drift, as indicated by a number of studies comparing the diversity of MHC and neutral loci (Radwan et al., 2010).

The moose (*Alces alces*, Linnaeus, 1758) is the second largest living representative of the terrestrial mammals of Europe, smaller only than the European bison (*Bison bonasus*). It was an important element of Polish fauna from the end of the ice age to the early Middle Ages (Gumiński, 2003). However, at the beginning of the 19th century the moose population in Poland collapsed to such an extent that only the forests near Rajgród in the Biebrza Valley were the westernmost range of this species in Poland (Brincken, 1826). That population survived World War II as a group of only several to a dozen individuals in the Biebrza Valley, which then gave rise to a herd consisting of the descendants of the native population that has lived in the area since the original settlement (Dzięciołowski and Pielowski, 1993; Raczyński, 2006). The total moose population in Poland peaked in 1981 at nearly 6,200 between various populations around the country (Dzięciołowski and Pielowski, 1993; Gębczyńska and Raczyński, 2001). An important role in that recovery was played by the immigration of moose through the northern and eastern borders of Poland and the dynamic development of the reintroduced moose in the Kampinos National Park in the 1950s. However, excessive hunting in the 1980s and 1990s soon led to a significant reduction in the population size and range. That is why in 2001, the Polish Minister of the Environment announced a hunting ban on moose, which helped rebuild the population of this *Cervidae* species.

In this study, we examined variations at the MHC II DRB locus in the moose, a Cervidae species that has undergone an extreme bottleneck in Poland after World War II.

Material and methods

Sample collection and DNA extraction. A total of 229 moose samples were collected in 2010 – 2011 from seven moose populations in Poland (Fig. 1).

The 70 samples of muscle and skin were taken from animals killed in road accidents, found dead or poached. Additionally, 159 stool samples were collected in winter. Samples were stored at –20°C pending DNA extraction, using a DNeasy Blood and Tissue Kit or, for stool samples, the QIAamp DNA Stool

Mini Kit (Qiagen, Hiden, Germany), in accordance with the manufacturer's instruction.

Molecular and statistical analyses. Each tissue and stool sample was examined according to 11 microsatellite loci (Świsłocka et al., 2015), to identify multilocus genotypes of moose individuals. In total, 229 samples from 196 individuals were examined and included in further analyses. The sample size from the analyzed populations ranged from 7 to 68 individuals (Table 1).

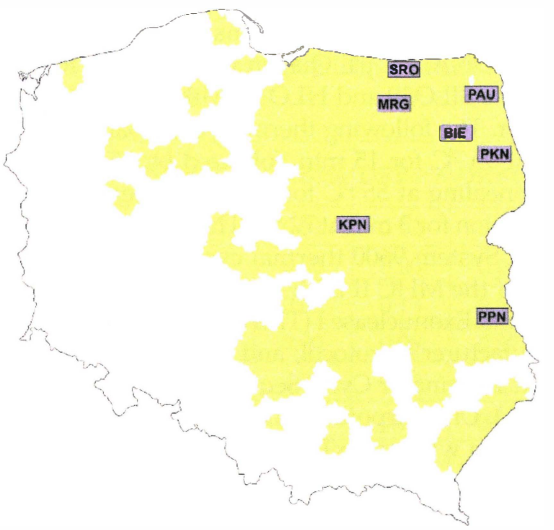

Figure 1. Study area and moose sampling in Poland. BIE – Biebrza Valley, PAU – Augustów Forest, PKN – Knyszyn Forest, MRG – Mrągowo State Forest, SRO – Srokowo State Forest, KPN – Kampinos National Park, PPN – Polesie National Park. Green color – moose range in Poland.

Table 1. MHC II DRB loci diversity indices for seven moose populations studied in Poland. N – sample size; Nh – number of alleles; h – alleles diversity; π – nucleotide diversity (%); S – number of segregating sites; PD – mean number of pairwise differences; SE – standard error. BIE – Biebrza Valley, PAU – Augustów Forest, PKN – Knyszyn Forest, MRG – Mrągowo State Forest, SRO – Srokowo State Forest, KPN – Kampinos National Park, PPN – Polesie National Park.

Population	N	Nh	h ± S.E.	π ± S.E. (%)	S	PD ± S.E.
BIE	68	8	0.759 ± 0.018	1.616 ± 0.912	8	3.653 ± 1.861
PAU	20	5	0.787 ± 0.026	1.637 ± 0.939	8	3.700 ± 1.910
PKN	15	5	0.775 ± 0.041	1.574 ± 0.916	7	3.556 ± 1.859
MRG	7	5	0.725 ± 0.104	1.391 ± 0.860	8	3.143 ± 1.732
SRO	19	6	0.751 ± 0.041	1.416 ± 0.831	8	3.200 ± 1.691
KPN	35	6	0.745 ± 0.030	1.475 ± 0.850	8	3.334 ± 1.732

Population	N	Nh	h ± S.E.	π ± S.E. (%)	S	PD ± S.E.
PPN	32	6	0.728 ± 0.033	1.426 ± 0.827	8	3.224 ± 1.686
TOTAL	196	8	0.7268 ± 0.009	1.549 ± 0.875	8	3.501 ± 1.788

The largest sample (68 individuals) was from the relict moose population in the Biebrza Valley, the largest natural wetland area in Central Europe. PCR amplification of the MHC II DRB locus was performed in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems) at a final volume of 5 μL containing ~25 ng genomic DNA, 1.7 μL Qiagen multiplex PCR Master Mix (1×), 0.3 μL mix of the primers HLO30 and HLO32 (Mikko and Andersson, 1995) and 1 μL RNase-free water. The following thermocycling parameters were used: initial denaturation step at 95 °C for 15 min, followed by 35 cycles with denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, followed by final elongation for 3 min at 72 °C. The multiplex PCRs were performed in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems). Amplified PCR products of the MHC II DRB locus were purified with shrimp alkaline phosphatase (SAP) and Exonuclease I (Thermo Scientific) in an enzymatic reaction following the manufacturer's protocol, and then processed for cycle sequencing PCR with a BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA). Unincorporated dideoxynucleotides were eliminated from the sequencing reaction with an ExTerminator Kit (A&A Biotechnology, Gdynia, Poland). The sequencing products were run on an automated capillary sequencer ABI 3130 (Applied Biosystems). The resulting 226-bp-long sequences of the MHC II DRB locus were aligned in BioEdit v.7.0.4 (Hall, 1999) and reviewed manually. UNPHASE option in DnaSP v.5 (Librado and Rozas, 2009) was used to identify different haplotypes (alleles). The levels of gene diversity of the MHC II DRB locus were calculated within seven moose populations as the number (Nh) and frequency of alleles, alleles diversity (h), nucleotide diversity (π, in percent), the number of segregating sites (S) and the mean number of pairwise differences (PD), using the software Arlequin v3.5.1.2 (Excoffier and Lischer, 2010) and DnaSP v.5 (Librado and Rozas, 2009). Genetic differentiation between moose populations was described as pairwise Φ_{ST} and F_{ST} values for the seven populations using Arlequin v3.5.1.2 (Excoffier and Lischer, 2010) and statistically tested in Arlequin. Principal component analysis (PCA) was performed on MHC II DRB F_{ST} data using GenAlEx v.6 (Peakall and Smouse, 2006).

Results

In a sample of 196 moose from Poland, out of the eight identified alleles of the MHC II DRB, seven had previously been described by Mikko and Andersson (1995) and by Udina et al. (2002) in moose from Europe, Far East and North America: *DRB1*1* (GenBank X82398), *DRB1*2* (GenBank X83278), *DRB1*3* (GenBank X83279), *DRB1*4* (GenBank X83280), *DRB1*5* (GenBank X83281), *DRB1*8* (GenBank X83284) and *DRB1*9* (GenBank X83285). One allele *DRB1*11*, had

not been described before. The number of alleles in the studied moose populations was generally low and similar, and ranged from 5 (pop. PAU, PKN and MRG) to 8 (pop. BIE; Table 1), despite the fact that sample size varied from 7 to 68 individuals. Alleles *DRB1*4* and *DRB1*11* were found only in the Biebrza Valley moose population. The most common alleles were *DRB1*1*, *DRB1*3* and *DRB1*9*, which were identified in all studied populations, with a frequency ranging from 14% to 50%. Frequencies of particular alleles were generally similar among the studied populations (Table 2).

Table 2. Frequency of eight MHC II DRB alleles found in the moose populations studied in Poland.

Allel / Population	DRB1*1	DRB1*2	DRB1*3	DRB1*4	DRB1*5	DRB1*8	DRB1*9	DRB1*11
BIE	0.32	0.04	0.15	0.01	0.02	0.13	0.32	0.01
PAU	0.30	0.17	0.17	0.00	0.00	0.06	0.30	0.00
PKN	0.35	0.07	0.21	0.00	0.13	0.00	0.24	0.00
MRG	0.14	0.07	0.50	0.00	0.00	0.07	0.22	0.00
SRO	0.18	0.03	0.40	0.00	0.08	0.05	0.26	0.00
KPN	0.39	0.06	0.28	0.00	0.07	0.04	0.16	0.00
PPN	0.41	0.07	0.30	0.00	0.02	0.06	0.14	0.00

The haplotype (alleles) diversity (h) in particular populations were remarkably similar and ranged from 0.725 ± 0.104 (pop. MRG) to 0.787 ± 0.026 (pop. PAU), while the nucleotide diversity (π) ranged from 1.391 (%) ± 0.860 (pop. MRG) to 1.637 ± 0.939 (pop. PAU; Table 1).

Genetic differentiation among populations was low (average $F_{ST} = 0.024$) and insignificant (Table 3). Only 10% (Φ_{ST}, range from 0.04 to 0.07) and 24% (F_{ST}, range from 0.03 to 0.07) of the pairwise comparisons significantly differed from zero ($p < 0.001$; Table 3). In the case of Φ_{ST}, significant values, according to the Wright scale (1978) mean small and moderate genetic differentiation, were obtained by comparing the population SRO with KPN and PPN populations (0.04 and 0.07, respectively). The values significant for F_{ST} were obtained mainly for the comparisons of the BIE population with MRG, SRO, KPN, PPN (0.07, 0.04 and 0.03 respectively) and for SRO *vs* PPN population pair (0.03).

Table 3. Genetic differentiation of MHC II DRB loci between moose population pairs in Poland, as measured by Φ_{ST} (below the diagonal) and F_{ST} (above the diagonal).

Population	BIE	PAU	PKN	MRG	SRO	KPN	PPN
BIE	-	0.00	0.01	**0.07**	**0.04**	**0.03**	**0.03**
PAU	0.00	-	0.00	0.04	0.03	0.02	0.02
PKN	0.00	0.00	-	0.05	0.02	0.00	0.00

Population	BIE	PAU	PKN	MRG	SRO	KPN	PPN
MRG	0.02	0.05	0.03	-	0.00	0.03	0.04
SRO	0.03	0.05	0.03	0.00	-	0.02	**0.03**
KPN	0.00	0.01	0.00	0.03	**0.04**	-	0.00
PPN	0.01	0.02	0.02	0.05	**0.07**	0.00	-

Significant values ($p < 0.05$) are indicated in bold.

PCA analysis based on the F_{ST} matrix showed that the main components accounted for 94.39% of the system's variation (PC1: 69.10%, PC2: 25.29%). This analysis confirmed that the relict population of the moose in the Biebrza Valley is separate from other moose populations from Poland (Fig. 2). The BIE and PAU populations differed significantly from SRO and MRG populations in terms of PC1 values, and additionally from the populations of KPN, PPN and PKN with respect to PC2 scores.

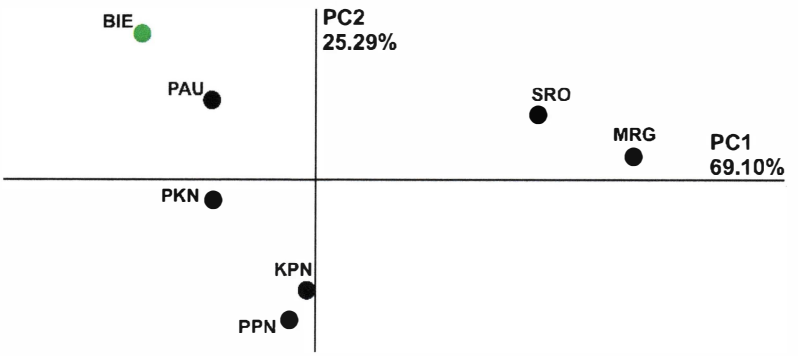

Figure 2. Principal component analysis (PCA) performed on pairwise F_{ST} values of the studied seven moose populations in Poland. The first and second axes of the PCA explained 69.10 and 25.29% of the total variability, respectively.

Discussion

In this paper we determined the diversity of the MHC II DRB, which is the most variable gene of MHC class II in humans, domestic cattle, and other ruminants (Marsh and Bodmer, 1990; Andersson et al., 1991). Previous research on MHC class I and II in moose (Mikko and Andersson, 1995; Ellegren et al., 1996; Udina et al., 2002; Wilson et al., 2003) has shown that both Eurasian and North American moose populations are characterized by low levels of polymorphism, with clear differences between populations. In our sample of 196 moose individuals, we identified eight MHC II DRB alleles, seven of which had been described in the moose throughout the range of the species (Mikko and Andersson, 1995;

Udina et al., 2002; Wilson et al., 2003). The *DRB1*11* allele found in the Biebrza Valley moose population is new to this species. At the same time we did not identify the *DRB1*6* allele present in the Swedish relict moose population and *DRB1*7*, and *DRB1*10* reported in Canadian and Far East moose populations (Mikko and Andersson, 1995; Mikko et al., 1999; Udina et al., 2002; Wilson et al., 2003). The most frequent allele in moose in Poland was *DRB1*1*, which is also one of the most frequent allele in moose in the Eurasian range of this species, carried by every four out of five individuals (Mikko and Andersson, 1995; Udina et al., 2002). The second most frequent *DRB1*9* allele in Poland is rare in Sweden and in the Far East, while the third *DRB1*3* allele is very rare in the Eurasian part of the range, but occurs in 20% of moose individuals from Canada (Wilson et al., 2003). Interestingly, the *DRB1*5* allele found in moose in Poland, is the dominant allele in North America, where it occurs in over half of all individuals (Mikko and Andersson, 1995; Mikko et al., 1999; Wilson et al., 2003).

The highest number of MHC II DRB alleles was identified in the Biebrza moose population (Table 2). Out of eight alleles, two – *DRB1*4* and *DRB1*11* – were not detected anywhere in Poland outside the Biebrza Valley. *DRB1*11* was unique for the Biebrza population. In the light of previous analyses of the MHC II DRB gene, the relict population in Sweden has the unique *DRB1*6* allele (Mikko and Andersson, 1995), while the relict population in the Biebrza Valley has the *DRB1*11* allele. Interestingly, the *DRB1*4* allele, which occurred only in the Biebrza Valley moose populations, has so far been identified in the relict moose population in Sweden and in the Russian part of the moose range (Mikko and Andersson, 1995; Udina et al, 2002). In the relict Biebrza population a very low frequency was observed for as many as four alleles of the MHC II DRB gene: *DRB1*2*, *DRB1*4*, *DRB1*5* and *DRB1*11* showed, compared to three (*DRB1*2*, *DRB1*5* and *DRB1*8*) in the remaining populations of moose in Poland (Table 2). The distinctness and uniqueness of the Biebrza population in terms of the MHC II DRB sequence was further confirmed by PCA analysis based on population pairwise F_{ST} values and especially the fact that the Biebrza moose population was characterized by the largest number of significantly different from zero comparisons of F_{ST} values with other populations (Fig. 2).

Ungulate mammals are generally characterized by a high diversity of MHC loci, with the highest levels of polymorphism found in chamois (*Rupicapra rupicapra)*, bighorn sheep (*Ovis canadensis*), white-tailed deer (*Odocoileus virginianus*) and red deer (*Cervus elaphus*) (De et al., 2011). Low levels of MHC diversity in some ungulates may be largely due to Pleistocene glaciations (Mikko et al., 1999; Loehr et al., 2006), when gene pool depletion was caused by inbreeding combined with genetic drift (Mainguy et al., 2007). During Pleistocene glacial-interglacial cycles, moose – belonging to the group of species living in temperate and boreal regions – experienced a reduction in population size and a related reduction in genetic diversity. Babik et al. (2009) argue that the reduced genetic diversity was due to the founder effect in populations that underwent a bottleneck before the postglacial expansion.

According to Mikko and Andersson (1995), moose has lost most of its MHC diversity over its evolutionary history, which could have been caused by random

favouring of one or more MHC haplotypes, or a very drastic bottleneck effect that preceded the separation of individual phylogenetic groups. Given that European and North American moose share the same limited diversity of MHC, Mikko and Andersson (1995) suggest that the ability of the moose population to survive at this low diversity of MHC must have been kept in equilibrium for at least 100,000 years, allowing moose to colonize the circumpolar areas of the northern hemisphere.

The current MHC diversity which has evolved since the bottleneck is characterized by a small number of alleles differing mainly in non-synonymous substitutions (Mikko and Andersson, 1995) – the effect of positive selection. In addition, Mikko and Andersson also argue that the lower number of MHC II DRB alleles in ruminants, compared to primates, is due to the fact that they are evolutionarily younger.

It is also interesting in the context of the moose population studies to note that MHC diversity increases with temperature, as a result of which species living in higher latitudes may retain lower genetic diversity within MHC (Weber et al., 2013). Van Den Bussche et al. (2002) also suggest that Arctic ungulate mammalian species may be exposed to fewer pathogens and parasites than those living in areas close to the equator, and that we should therefore expect less variation in MHC in ungulates in northern areas than in lower latitudes. Interspecies studies confirm the hypothesis of Van Den Bussche et al. (2002) that latitude may influence the level of MHC DRB diversity in wild ungulates by the likely relationship between MHC diversity and pathogen diversity (Mainguy et al., 2007).

The low diversity in the DRB locus in moose and reindeer is also likely to be related to the limited exposure of these species to boreal parasites (Mikko and Andersson 1995; Ellegren et al., 1996; Mainguy et al., 2007). The limited diversity of MHC genes can also be explained to some extent by the impact of factors associated with the social organisation of the species (Mainguy et al., 2007). These factors include the lonely lifestyle of the moose, and its monogamy (Ellegren et al., 1996; Sommer et al., 2002), which reduce the incidence of intra-species contacts. Thus the reduced risk of transmitting infectious diseases may favour low levels of MHC polymorphism. Irrespective of the fact that the number of MHC DRB II alleles in moose is low, it should be noted that their frequencies in the studied moose populations were remarkably similar. Thus, it could not be ruled out that their frequencies within populations reflect selection pressures based on the heterozygote advantage principle (Ejsmond et al., 2010).

It should also be noted that hunting has a significant impact on the ecology and genetics of vertebrates. By reducing genetic diversity, it may lead to a short-term decrease in the levels of fitness components (Altizer et al., 2003; Fernández-de-Mera et al., 2009). In a bighorn sheep population, selective trophy hunting resulted in a significant decrease in average horn weight and size in males (Coltman et al., 2003). Analyses of moose in Canada have shown that the relatively higher diversity in the MHC II DRB gene in populations from protected areas (national parks) was due, among other things, to the lack of hunting in these areas (Wilson et al., 2003). Interestingly, three Polish moose

populations in national parks: Biebrza Valelly, Kampinos and Polesie National Parks, in our study also showed the highest number of MHC II DRB loci. Wilson et al. (2003) suggest that the higher density of moose in national parks creates unusual conditions for the definitely lonely lifestyle of this species (Ellegren et al., 1996). The increasing number of contacts and the associated increase in the number of transmitted pathogens increases the pressure of natural selection on populations living in protected areas.

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POLYENES IN THERAPY OF MYCOSIS – CHARACTERISTICS AND MECHANISMS OF RESISTANCE

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Abstract

Polyenes are one of the most commonly used drugs in the treatment of mycoses. In the polyenes group we can find amphotericin B, nystatin, and natamycin. The resistance of pathogenic fungi is rare, but more visible than a few years ago. Several mechanisms of resistance have been described, concerning ergosterol in cell membrane, biofilm formation, and others. The aim of this paper was to describe the polyenes and try to explain mechanisms which may lead to resistance of fungi.

Key words: antibiotics, pathogenic fungi, fungemia, superficial mycoses

Introduction

Mycoses are very common. Estimated data say that over one billion people suffer because of mycosis [1]. One of the most commonly used groups of antifungal drugs are polyenes, which have been present in medicine from the mid-20th century. Data from Centers for Medicare & Medicaid Services says that in 1991-2009 in the United States of America polyenes were the second most often prescribed group of antifungal drugs (generally azoles were first). They were on about 120 000 prescriptions. In the last 18 years patients in the USA paid for polyenes about 490 mln dollars [2]. Polyenes are effective against most of human's pathogenic fungi. They are used in the treatment of superficial mycosis (nystatin, natamycin), and systemic mycosis as well (amphotericin B) [3]. Amphotericin B is called the "gold standard" in the treatment of systemic mycosis and has the widest range of action among all available antifungal drugs [4].

The aim of this paper is to describe polyene drugs, including their structure and mechanism of action, and explain the processes which are the basis of antifungal resistance for this group of drugs.

Polyenes structure and their antifungal mechanism of action

Polyenes antibiotics are natural products of the aerobic metabolism of Gram-positive bacteria from the genus *Streptomyces spp.* [4, 5]. They are made of a macrocyclic lacton ring, which contains 4-7 conjugated double bonds. The ring is connected by a glycosidic bond with an aminosaccharide [6]. There are also many hydroxyl groups in the polyene structure [7]. These drugs are sensitive to light; that's why they are easily oxidized [6].

The antifungal mechanism of action of all polyenes is based on the disruption of the integrity of the fungal cell membrane. The amphiphilic structure of the molecule lets polyenes bind to ergosterol – the main sterol of fungal cell membranes – creating pores, thereby increasing permeability, for example for potassium and magnesium ions (Fig. 1). As a result, the membrane is destabilized and the cell dies [8, 9].

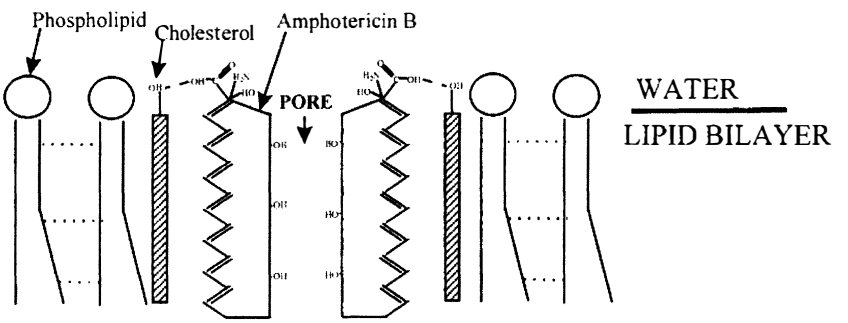

Fig. 1. Mechanism of action of polyenes [10]

Characteristic of selected polyenes

Amphotericin B. Amphotericin B (Fig. 2) has been present in medicine from 1959, and it's called the "gold standard" of mycosis treatment [11, 12]. Despite the development of many new antifungal agents, amphotericin B is still the most commonly used antifungal agent in hospitals due to its strong antifungal activity. It's a life-saving drug in the treatment of serious systemic fungal infections [7]. It's produced by *Streptomyces nodosus* [13]. It has a wide spectrum of activity against most species of the genus *Candida spp.* and *Aspergilus spp.* In addition, it exhibits antifungal activity against *Cryptococcus spp., Histoplasma capsulatum, Blastomyces dermatitidis, Coccidioides immitis and posadasii, Paracoccidioides spp.* [4, 6]. Despite many decades of clinical use, resistance to amphotericin B is very rare. It's exhibited by strains such as *Candida lusitanie, Candida guilliermondi, Aspergillus terreus, Trichosporon beigelii, Scedosporium apiospermum* [3, 4, 7,

9]. Amphotericin B is ineffective in the treatment of monosporiosis and trichosporiosis [14]. However, it's characterized by its effective action in the treatment of candidiasis: oral cavity, digestive system, urinary system, lungs, meninges, pancreas or spine. Amphotericin B is present in various pharmaceutical forms: lozenges, washing suspensions, suspensions for intravenous administration [15]. Due to its insolubility in water, it's practically not absorbed from the digestive system, therefore it's usually administered intravenously. Chemical modification of the antibiotic by combining it with sodium deoxycholate allowed obtaining a water-soluble pharmaceutical – Fungizone [13]. The daily dose of amphotericin B for the treatment of systemic fungal infections ranges from 0.5 to 1 mg / kg / day (in severe cases up to 1.5 mg / kg / day). In central nervous system infections, the antibiotic is given intrathecally due to its poor penetration into the cerebrospinal fluid [13, 14]. This antibiotic has a high toxicity associated with similarity in the structure of fungal and human cell membranes and with the mechanism of cytokine activation [3, 6]. During therapy it can cause many side effects, e.g. fever, chills, or shortness of breath, and in almost every patient after long-term therapy causes kidney damage [14]. It's characterized by a long half-life and high degree of spread in tissues [3]. Amphotericin B, despite a wide spectrum of activity, causes many serious side effects. It's the most toxic compound among polyene antibiotics. It's characterized by high neurotoxicity. During therapy, many patients have adverse reactions, such as fever, nausea, headache, muscle and joint pain, chills, hypotension, dyspnoea, hepatotoxicity, hypokalaemia, hypomagnesemia and anemia. Long-term therapy almost always ends in impaired renal function [6, 7, 14]. Too high doses can bind to the cholesterol of human cell membranes, disrupting their integrity and structure [6]. There are many contraindications regarding the use of amphotericin B, including renal failure, hypocalcaemia, or liver damage [3]. This limits the possibilities of its use in long-term therapies [14]. Research shows an antagonism between amphotericin B and azoles. It's likely that azoles inhibit the effectiveness of amphotericin B as a result of lowering the sterol content in fungal cell membranes [7]. In addition, the mechanism of amphotericin B and other toxicity may be associated with the activation of cytokines and the secretion of TNFα (tumor necrosis factor) by macrophages [3].

To reduce side effects, amphotericin B is often combined with other substances, such as flucytosine or fluconazole [14]. In addition, to minimize cytotoxicity – and increase its penetration into tissues – lipid forms of amphotericin B were introduced: AmBisome (liposomal amphotericin B), Amphocil (colloidal suspension, amphotericin B with cholesteryl sulfate), Abelcet (amphotericin B in the form of a lipid complex). Because of the use of those preparations, higher doses of the active substance (AmBisome – 3mg/kg bw/d, Amphocil – 3-6 mg/kg bw/d, Abelcet – 5 mg/kg bw/d) can be used [7, 14]. Research is also being carried out on nanoparticles as carriers of amphotericin B, which after interaction with macrophages would release the drug gradually, thereby reducing side effects [3].

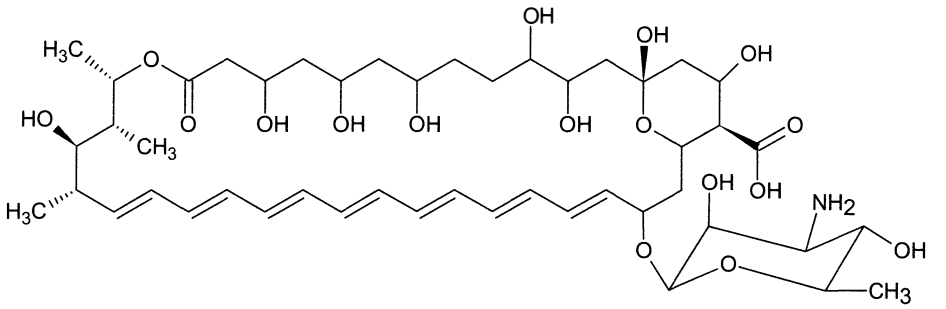

Fig. 2. Chemical structure of amphotericin B

Nystatin. Nystatin (Fig. 3) was the first antifungal drug from the polyenes group in treatment. It's been used since 1949 [7]. It's used in treatment of superficial infections of the skin, nails, vagina, mucosa of the digestive system, mouth and eyeball [3, 6, 15]. It's produced by *Streptomyces noursei* [8]. This antibiotic works mainly against fungi of the genus *Candida spp.*, *Cryptococcus spp.*, *Fusarium spp.*, *Aspergillus spp.* It's not effective against dermatophytes [7, 8]. Nystatin is insoluble in water, therefore it's used for prophylaxis after antibiotic therapy, in cancer patients, or before surgery [6]. This drug is present in different forms: gel, ointments, rinse suspensions, and vaginal balls. It has different trade names; it's sold e.g. as Moronal or Mycostatin. It's the only antifungal drug used in the treatment of vaginal and vulvar candidiasis in the first trimester of pregnancy [15, 16]. It has high toxicity after parenteral administration. The developed liposomal form of nystatin has higher vitro activity than standard nystatin [7].

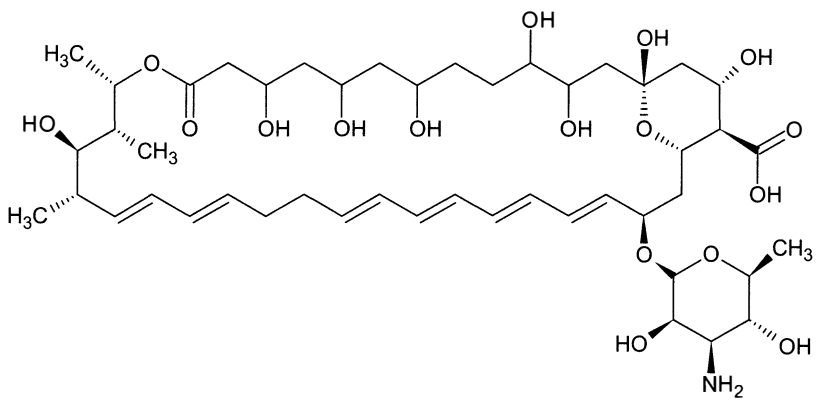

Fig. 3. Chemical structure of nystatin

Natamycin. Natamycin (Fig. 4) was first isolated in 1955 from the bacterium *Streptomyces natalensis* [8, 17]. It's used locally in vaginal and vulvar candidiasis, onychomycosis of the feet, nails, mucous mebranes, urinary and sex organs in men, yeast skin folds or keratitis [13, 16]. It has in vitro activity against fungi

from genus *Aspergillus* spp., *Candida* spp., *Penicilium* spp., *Cephalosporium spp., Fusarium spp., Cladosporium spp., Scopulariopsis spp.* [18]. Natamycin is insoluble in water and it can't be absorbed from the digestive system. It's applied in the form of creams (in combination with e.g. glucocorticosteroids), globules, ointments or solutions [13, 16]. Commercial preparation appears as Pimafucin. Natamycin is highly sensitive to ultraviolet light. The daily dose of this drug is 0.3 mg / kg / day. Besides in medicine, natamycin is used in the food industry as a preservative in food (such as cheese, yogurt, sausages, wine and juice). Maximum concentration of this antibiotic can be 52 mg / l, but the growth of fungi is inhibited for 16 weeks in four times lower concentrations [18].

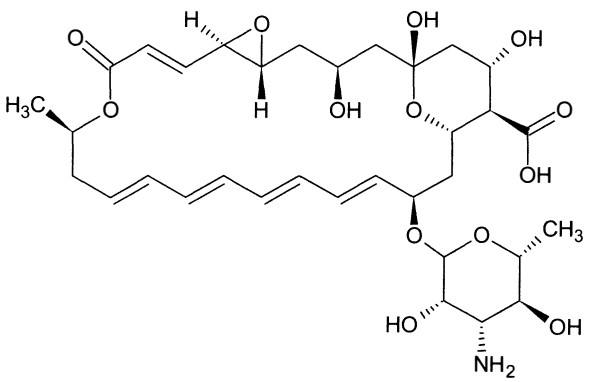

Fig. 4. Chemical structure of natamycin

Mechanisms of resistance of fungi to polyenes

Changes in ergosterol content in fungal cell membrane. The mechanism of the action of polyenes is combined with the content of ergosterol in cell membranes. That's why one of the basic mechanisms of resistance is connected with the content of this sterol in fungal cell membranes, or even its complete absence. In the results drugs can't be stuck to the compounds of a cell and make pores in it. It has been shown that ergosterol in cell membranes is replaced by episterol or fecosterol (intermediates of the ergosterol biosynthesis pathway) [19]. Extremely significant, genetic changes underlying the resistance of fungi to azoles, may also be appropriate for resistance to polyenes [20-22]. The most common mutations connected with resistance to polyenes are point mutations in the *ERG3, ERG6, ERG4* or *ERG11* genes [20, 23-24], coded enzymes compatible with the synthesis of some intermediates of the ergosterol biosynthesis pathway.

Research has been done that includes this correlation of fungal resistance levels and content of ergosterol and other sterols in cell membranes. Analysis of the content of *Cryptococcus neoformans* cell membrane before and after amphotericin B treatment in the analysis of infection in AIDS patients shows fungi

isolated before treatment had 75% ergosterol content in membranes, and after treatment - only 4%.

Furthermore, the accumulation of intermediates of the ergosterol biosynthesis pathway, e.g. fecosterol [23] has been demonstrated. Studies which were done on *Candida glabrata* with nonsense mutation in the *ERG6* gene showed that this kind of mutation leads to increased resistance and reduced content of ergosterol in cells [25]. Other studies concerned *S. cerevisiae* and *C. albicans*. Scientists have shown that changes in the composition of *ERG11* affect the reduced sensitivity to amphotericin B, as well as being needed in the increasing range of accumulate intermediates of the ergosterol biosynthesis pathway [23, 26]. Studies carried out on *C. albicans* in 2003 by Geraghty and Kavanagh showed that mutants that had changes at the level of the respiratory chain were also less sensitive to the effects of amphotericin B [27]. In addition, these mutants had a significantly lower ergosterol content in the cells than the other variants [27], which may be due to the association of this organelle with the ergosterol biosynthesis pathway [28]. A decrease in mitochondrial activity may also reduce the production of free radicals. Thanks to this, the damage they cause will be reduced [28].

Biofilm formation. A biofilm is a structure that is created not only by bacteria, but also by fungi. It is believed that fungal organisms are relatively easily organized into structures composed of many layers of cells and extracellular substances. The structure of individual biofilms as well as the chemical composition of the extracellular matrix determines the level of sensitivity of these structures to the effects of drugs [29]. They usually cover solid surfaces, but there are also biofilms appearing on the surface of the water (liquid-air interfaces) [30]. In studies on *Candida* fungi it has been shown that biofilms had about 8 times higher resistance to amphotericin B than cells that don't form these kinds of structures [31]. Unfortunately, biofilm-forming cells may be resistant to most of the currently used drugs (the exception among polyenes are amphotericin B lipid compounds) [32]. In addition, patient-safe drug levels do not guarantee sterilization, which is likely to cause biofilm regrowth [31]. Monitoring the development of resistance of these structures is also important for patient safety.

The biofilm structure determines the reduced availability of fungicidal compounds to cells. The extracellular matrix includes, among others, polymers of glucan, which is responsible for the retention of antimicotic molecules outside the cell, which completely or partially prevents them from entering the cells. As a result, it is necessary to use a higher concentration of the drug for biofilm-forming fungi than for cells that don't form them [31]. In studies conducted on biofilms of *Candida albicans* mutants, it was noted that changes in the expression of the *FKS1* gene affect changes in sensitivity, both to amphotericin B as well as to other antimicotics [33]. Increased expression of this gene reduces the sensitivity of these structures to the compounds used [33]. In addition, in the biofilm structure persisters are presented, which have high tolerance to antimicotics [34]. The reduced sensitivity of these cells to amphotericin B may be due to changes in the level of signaling pathways. In studies on *S. cerevisiae*, a correlation was observed in the increase in resistance to this compound and the inhibition of TORC1, EGO and RAS complexes [35]. In addition, it was shown

that mutants having mutations in the *ras1* and *ras2* genes had definitely more persisters than wild forms [35]. These cells can form the basis for a new biofilm that is being formed [36]. Due to their location, these cells receive a much lower dose of drugs than cells in the outer part of the biofilm. In addition, because of their presence, structures can survive in high concentrations of antifungal drugs [37]. Due to these features, biofilms are considered to be the main mechanisms of resistance of fungi in chronic infections [38].

In the case of biofilms and their resistance to polyenes, attention is also paid to the rate of its growth. It's believed that the low growth rate in relation to non-biofilm-forming cells may reduce the sensitivity of these structures to the effects of antimicotics [39]. Comparing cell density *of C. glabrata, C. tropicalis* and *C. parapsilosis* in biofilms, the first species usually shows higher resistance to amphotericin B than the others, which can be correlated with higher density in the structure being created [30].

Antioxidant protection. Polyenes have a significant effect on the level of oxidative stress within cells. In addition to forming pores in the cell membrane, polyenes also increase the lipid peroxidation in this structure, which increases the therapeutic effect of these compounds. Disorders within the cell membrane may be the result of the action of hydroxyl radicals or hydrogen peroxide [23]. The exact role of amphotericin B in cell oxidative stress is not entirely known. However, it is recognized that this compound may also have antioxidant activity [28]. It is currently believed that one of the mechanisms that may underlie polyene resistance is greater activity of antioxidant enzymes (for example catalase [19]). Studies on *C. albicans* have shown that resistant strains produced greater amounts of catalase, both intra- and extracellular, than control [23]. In addition, studies conducted on strains of *Candida* fungi resistant and sensitive to amphotericin B showed higher activity of both catalase and superoxide dismutase in strains resistant to the tested drug [40]. In studies on *Trichophyton rubrum*, induction of genes coding for GSS (glutathione synthetase) or AOX (alternative oxidase) proteins was observed in response to amphotericin B [41]. In the case of glutathione peroxidase (GPX), amphotericin B reduced the transcription of the gene encoding this protein [41].

Other mechanisms of resistance to polyenes. Studies carried out on *T. rubrum* have shown diverse expression of genes encoding enzymes involved in the metabolism of lipid compounds [41]. In response to this compound, both an increase (e.g. IVD gene) and a decrease in expression of these genes (e.g. ELO2 or MVD1 genes) has been observed [41]. Further analysis of the differentiation in expression of these genes will allow a broader look at the cellular response of fungi to polyenes as well as at the problem of resistance.

It turns out that the cell wall structure can affect the sensitivity of fungal cells to polyenes. Studies on *C. albicans* have shown that the low content of chitin in the cell wall was associated with increased resistance to amphotericin B [42-43]. Research on the chemical composition of the *A. flavus* cell wall has shown that strains sensitive to amphotericin B had a lower content of glucans than in resistant strains [44]. However, in studies carried out on *T. rubrum*, amphotericin B inhibited the expression of the *FKS1* gene encoding 1,3-glucan synthase [41].

These results may suggest that increased 1,3-glucan synthase activity may be one of the mechanisms of fungal resistance to polyenes, e.g. as a result of brief contact with amphotericin B. However, the effect of cell wall structure on the sensitivity of fungi to polyenes, as well as the role of the compounds, isn't entirely clear and requires further research. The role of heat shock proteins (HSPs) in building resistance to polyenes also requires additional analysis. Currently, attention is on the potential impact of their presence and synthesis on reduced sensitivity to drugs from this group [45]. Studies on *T. rubrum* have shown that amphotericin B induces a number of genes that are responsible for the response of cells to stress, including gene coding for HSP70 or HSP104 proteins [41]. In the case of the *HSP10* gene, a reduction in transcription was observed under the influence of this drug [41]. Researchers also point out the importance of yeast life cycle stages to their sensitivity to amphotericin B. Research shows that cells in the steady-state growth phase are more resistant than in the exponential phase [23].

Conclusion

Mycosis appears very often. In treatment, we can use only a few groups of antifungals. One of the most commonly used are polyenes. Those compounds work very well, but using them has many side effects. The aim of using polyenes is to disrupt the integrity of the fungal cell membrane (by binding molecules of the drug to ergosterol in cell membrane and creating pores). The membrane becames destabilized which causes the death of the cell. Nowadays, resistance of pathogenic fungi is rare, but many serious infections are treated by polyenes. Amphotericin B is called the "gold standard" in treatment of systemic mycosis and has the widest range of action among all available antifungal drugs. Polyenes are made of a macrocyclic lacton ring, which contains a few conjugated double bonds. The ring is connected with aminosaccharide. In the structure polyenes have many hydroxyl groups.

Knowledge about the possible mechanisms of resistance to polyenes is very narrow. However, few papers describe the several potential mechanisms of increased sensitivity of fungi to polyenes. Scientists underline the role of ergosterol content in cell membrane as one of the major mechanisms. Lower amount of ergosterol (or even lack of it) in cell membrane protects cells from creating pores in this structure. Polyenes binding becomes impossible or very unsuccessful. A second major mechanism of resistance of fungi is the formation of biofilms. The structure of biofilms has a big impact on the method of treatment. Unfortunately, resistance of fungal biofilms is higher than resistance of cells which don't make it. That's why it's necessary to use a higher concentration of the drug to fight with infection. Biofilms have the ability to regrow, which can be a basis for losing sensitivity to used drugs. These and other mechanisms have been basically described, but there is much more to do. Especially, because amphotericin B is often "the last chance drug". Creating new compounds, or using old ones

but in another way, may be a possible useful modification of treatment of fungal infections.

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