Spores and vegetative cells of phenotypically and genetically diverse *Bacillus cereus sensu lato* are common bacteria in fresh water of Northeastern Poland

Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2017-0337.R2
Manuscript Type:	Article
Date Submitted by the Author:	15-Sep-2017
Complete List of Authors:	Bartoszewicz, Marek; Uniwersytet w Białymstoku Wydział Biologiczno-Chemiczny, Czyżewska, Urszula; Uniwersytet w Białymstoku Wydział Biologiczno-Chemiczny
Is the invited manuscript for consideration in a Special Issue? :	N/A
Keyword:	Bacillus cereus sensu lato, psychrotolerance, MLST, vegetative cells and spores, toxigenic isolate

SCHOLARONE™
Manuscripts

Title: Spores and vegetative cells of phenotypically and genetically diverse *Bacillus cereus sensu lato* are common bacteria in fresh water of Northeastern Poland

Author: Marek Bartoszewicz¹⁾, Urszula Czyżewska²⁾

- 1) Department of Microbiology, Institute of Biology, Faculty of Biology and Chemistry,
University of Białystok
1J Ciołkowski Street, 15-245 Białystok, Poland
Phone: +48 85 738 8414; Fax +48 85 738 8224
E-mail: mbartosz@uwb.edu.pl
- 2) Department of Cytobiochemistry, Institute of Biology, Faculty of Biology and Chemistry,
University of Białystok
1J Ciołkowski Street, 15-245 Białystok, Poland
Phone: +48 85 738 8414; Fax +48 85 738 8224
E-mail: urszula.czyzewska@uwb.edu.pl

Correspondence: Marek Bartoszewicz, E-mail: mbartosz@uwb.edu.pl, phone: +48 85 738 8384

Running title: *Bacillus cereus sensu lato* in fresh water

Conflict of interests: Authors declare no conflict of interest.

Abstract

Gram-positive rods named *B. cereus sensu lato* (*sl*), are common in natural habitats and food products. It is believed that they are restricted to spores, however their ecology in aquatic habitats is still poorly investigated. Thus, the aim of the study was to assess the rain-dependent fluctuations of the concentration of *B. cereus sl* vegetative cells and spores with evaluation of their phylogenetic and population structure in relation to the toxicity and psychrotolerance. We proved that vegetative cells of *B. cereus sl* are widely distributed in fresh water of rivers and lakes being as common as spores. Moreover, heavy rain shows huge impact on their concentration in undisturbed environments. The diversity of *B. cereus sl* reflects multiple sources of bacteria and differences between distinct environments. Next, their diverse genetic structure and phenotypes better fit to their ecological properties than to the taxonomic affiliation.

Keywords: *Bacillus cereus sensu lato*, psychrotolerance, MLST, toxigenic isolate, vegetative cells and spores

1. INTRODUCTION

Bacillus cereus sensu lato (*sl*) consists of at least nine species with diverse ecology and virulence. *B. cereus* is responsible for food poisonings (Dierick et al. 2005), *B. thuringiensis* synthesizes insecticidal proteins (Swiecicka 2008), *B. anthracis* causes anthrax (Jensen et al. 2003), *B. weihenstephanensis* is cold-adapted (Lechner et al. 1998), *B. cytotoxicus* is a thermotolerant and cytotoxic pathogen (Guinebretiere et al. 2013), while *B. toyonensis* exhibits both probiotic and toxic features (Jimenez et al. 2013), *B. wiedmannii* shows psychrotolerance and cytotoxicity (Miller et al. 2016), and finally *B. mycoides* and *B. pseudomycoides* form rhizoidal colonies on solid media (Jensen et al. 2003). All these Gram-positive sporeformers, excluding *B. anthracis*, are also motile and able to cause hemolysis and precipitate lecithin (Jensen et al. 2003). Traditionally they have been distinguished in the rank of species (Vilas-Boas et al. 2007). However, they cannot be always differentiated according to Bergey's Manual of Determinative Bacteriology (Schleifer and Whitman 2009). For example, MLST profiling or 16S rRNA sequencing do not provide sufficient data for the reliable distinguishing between *B. cereus*, *B. thuringiensis* or *B. anthracis* (Caamano-Antelo et al. 2015; Miller et al. 2016). Moreover, their genetic structure often fits better to ecological properties than taxonomic affiliation (Drewnowska and Swiecicka 2013). Thus, environmental studies employing genetic structure of natural population can be helpful in explanation of the difficulties in the *B. cereus sl* taxonomy. Screening for the presence of genes responsible for their virulence or drug resistance may provide an alternative scheme for differentiation of these bacilli into pathotypes or ecotypes (Gao et al. 2012). Moreover, this dividing scheme often fits better to phylogenetic data (Bartoszewicz and Marjanska 2017).

B. cereus causes mainly food poisonings. The diarrheal type of this illness is linked to enterotoxins: non-hemolytic enterotoxin (Nhe) encoded by the operon *nhe* (*nheABC* genes), and hemolysin BL (Hbl) encoded by the *hbl* operon (*hblCDA* genes). One-element cytotoxin K (CytK) may form two types of toxin (Stenfors Arnesen et al. 2008). CytK-1 is synthesized by *B. cytotoxicus* and CytK-2 formed by other *B. cereus* group members (Guinebretiere et al. 2013). However, the presence of enterotoxins genes does not prove bacterial toxicity.

Several *B. cereus* *sl* virulence factors are also plasmid-encoded and may undergo horizontal transfer in different environments (Furlaneto et al. 2000; Thomas et al. 2001). Among them cereulide is linked to emetic syndrome with occasional fatal consequences (Dierick et al. 2005). Next, genes *cya*, *lef*, *pag* necessary for anthrax toxin formation and *cap* for the capsule are enabling the development of anthrax symptoms (Zwick et al. 2012). Finally crystalline inclusions (Cry) produced by *B. thuringiensis* are pathogenic to insects with no impact on health of humans (Jensen et al. 2003). This feature is also fundamental for preparation of commercial biopesticides that are safely sprayed over fields and forests for many years.

Until now, the presence of *B. cereus* *sl* has been confirmed in soil, plant material, water, food and activated sludge (Ichimatsu et al. 2000; Iriarte et al. 2000; Mizuki et al. 2001; Jensen et al. 2002; Ostensvik et al. 2004; Hendriksen and Hansen 2006; Bizzarri and Bishop 2007; Bartoszewicz et al. 2008; Bartoszewicz et al. 2009). However, pasteurization during bacteria isolation lead to perishing of vegetative *B. cereus* or *B. thuringiensis* cells and consequently, their underestimation. Another approach based on the isolation of nucleic acid directly from water and subsequent genotyping (Douville et al. 2007), unfortunately does not allow to specify the precise number of dormant spores and active cells. Thus the development of the PTA⁺ medium containing germination inhibitors (Bizzarri and Bishop 2007) enabled the isolation of vegetative cells only, while traditional procedure with sample pasteurization leads to isolation of spores. The presence of high number of vegetative *B. cereus* *sl* cells might indicate that in this environment they occur not only incidentally, but probably that they can carry on their normal life processes. Thus, the goals of this study were: (i) to estimate the level of fresh water contamination by the spores and vegetative cells of selected *B. cereus* *sl*; (ii) to assess their toxicity and psychrotolerance; (iii) to investigate their genetic diversity and population structure; and finally (iv) to search for any correlations between genomic profiles and phenotypic properties or between heavy rainfall and concentration of these bacilli.

2. MATERIALS AND METHODS

2.1. Collection of environmental samples, bacteria isolation

Water samples were collected from four different locations (shown in Fig. 1S) in NE Poland: Garbas Lake (Suwalki Landscape Park) and Wigry Lake (Wigry National Park), Narew River (Narew National Park) and Biebrza River (Biebrza National Park) in June, July and August 2012 and immediately (within 2 hours) transported (on ice) to the Department of Microbiology for further analyses. Each month, the first sampling occasion was within 24 hours after intensive rainfall (>16 mm) and the second sampling was performed after at least five days with no rain over the sampling site and the catchment area. In general, 24 water samples were obtained. Detailed properties of rainfall events and water samples are presented in Table 1S (Supporting information).

Samples from each location underwent: (i) isolation and enumeration of the total *B. cereus* *sl* count and (ii) the determination of vegetative cells count (both in duplicates). To achieve these, in each repetition 50-150 ml of water from each sample underwent membrane filtration by the use of the sterile nitrocellulose filters (Sartorius 11404-50 ACN). To avoid cross contamination, the equipment used for filtration was sterilized each time and the entire procedure was carried out in the laminar flow hood. Afterwards each filter was washed with 100 ml of sterile water to remove undesirable residues (e.g. growth inhibitors or germination inductors). Then filters were transferred aseptically onto the surface of proper medium (described below) and incubated for 48 hours at 25°C, a temperature that supports growth of psychrotolerant and mesophilic members of the *B. cereus* group. For viable *B. cereus* *sl* count determination, PTA (peptone-tryptone agar) medium was used. In order to isolate vegetative forms, a selective medium named PTA+ containing inhibitors of germination (3.15 mM Na-p-tosyl-L-arginine methyl ester and 0.3 mM diphenylamine; both reagents from Sigma) was applied (Bizzarri and Bishop 2007). Each bacterial isolate was tested for growth and lecithin precipitation using MYP (mannitol egg-yolk polymixin) selective agar medium and for hemolytic properties on Columbia SB Agar (both from Oxoid, Basingstoke, UK).

2.2. Identification and enumeration

All isolates grown on the surface of nitrocellulose filters, and forming purple colonies with a zone of egg yolk precipitation on the MYP agar were initially classified as the members of the *B. cereus* group. Besides mesophilic isolates with typical colonies and the absence of parasporal bodies pertaining to *B. cereus*, isolates with crystal inclusions in sporulating cells detected under phase contrast microscopy (BX-61, Olympus) were identified as *B. thuringiensis*. Cold-adapted bacilli, growing at 7°C in a liquid Luria-Bertani broth (Oxoid) after 10 days incubation, with a psychrotolerant motif of the *cspA* gene (Francis et al. 1998) were assigned to *B. weihenstephanensis*. Next, all isolates were tested for the *cytK-I* gene and the growth at 50°C typical for *B. cytotoxicus* strains as well as for the *pag*, *cya*, *lef* and *cap* genes responsible for the pathogenicity of *B. anthracis*. During the study no isolates with rhizoidal morphology of their colonies corresponding to *B. mycoides* or *B. pseudomycoides* were found. The bacterial population density (CFU L⁻¹), independently for total bacterial count and vegetative cell count was calculated based on viable CFU count and grouping scheme used here.

2.3. The REP-PCR and MLST profiling

The genetic polymorphism of all the *B. cereus* *sl* isolates obtained here was initially assessed using REP-PCR, an easy and precise technique developed in order to compare *B. cereus* *sl* strains by Reyes-Ramirez and Ibarra (2005). For amplification (in GeneAmp 9700 thermocycler, Applied Biosystems) of repetitive palindromic sequences from *B. cereus* *sl* genomes, a 25 µl mixture of 2.5 U of Taq polymerase (Fermentas, Vilnius, Lithuania), 300 ng of both primers (listed in Table 2S, Supporting information), 200 mM dNTP and 5 mM of MgCl in 1x concentrated reaction buffer with KCl was applied. Products of amplification (ranging 50 bp – 3.5 kb) were electrophoresed in 1.3% agarose gel (Basica LE, Prona, Madrid, Spain) prepared with 0.5xTBE buffer with 4 V cm⁻¹ of the gel followed by staining (ethidium bromide, 1 µg ml⁻¹) and imaging in UV light in the ChemiDoc XRS+ System (BIO-RAD).

Multi-locus sequence typing (MLST) led to the determination of the sequence types (STs) for all tested bacilli. In order to this, seven housekeeping genes (*glp*, *gmk*, *ilv*, *pta*, *pur*, *pyc*, *tpi*) were amplified using primers and amplification conditions obtained from the PubMLST database (<http://pubmlst.org/bcereus>) in GeneAmp 9700 thermocycler. After the purification by the use of the Clean-up Kit (A&A Biotechnology) according to the manufacturer's protocol, all DNA samples in distilled nuclease-free water underwent sequence extension reaction. PCRs were performed by the use of GeneAmp 9700 in 25 cycles of subsequent denaturations (95°C, 15 sec.), annealings (50°C, 15 sec.) and elongations (60°C, 60 sec.). After the removal of the remaining dye terminators (ExTerminator Kit, A&A Biotechnology), purified fragments were sequenced with the ABI3500 DNA sequencer (Applied Biosystems). All sequences were visually examined using Chromas Lite 2.0.1., and aligned in BioEdit 7.0.1. (Hall 1999). The concatenated and aligned sequences of seven housekeeping genes were used for construction of a maximum likelihood dendrogram by the use of general time reversible model with gamma and invariable sites parameters, and with 1000 bootstrap iterations (GTR+G+I) in Mega 6. Clonal complexes of isolates possessing at least six identical alleles were discovered using PHYLOVIZ 1.1 and the geoBURST algorithm, as described by Feil et al. (2004).

2.4. PCR screening for the psychrotolerance and enterotoxins genes

All strains were PCR-screened for the presence of the *cspA* gene with motif ⁴ACAGTT⁹ (typical to psychrotolerant *B. weihenstephanensis* isolates), the *cspF* (positive control, present in both mesophiles and psychrotolerant strains), the *nhe* operon (genes *nheABC* encoding NHE enterotoxin), the *hbl* operon (genes *hblCDA* determining HBL toxin), the *cytK-1* and *cytK-2* genes, responsible for the cytotoxin K synthesis, and finally for the *ces* operon required for cereulide formation, using primers described in literature (as stated in Table 2S, Supporting information). For this purpose, the total DNA was prepared from overnight cultures in Luria-Bertani broth (Oxoid) using DNeasy Blood&Tissue Kit (Qiagen) by the use of automated system for nucleic acid isolation QIACube (Qiagen) according to a protocol for Gram-positive bacteria. DNA quantity and quality were estimated using NanoDrop 2000

spectrophotometer (Thermo Scientific). Only samples with O.D._{260/280} ratio >1.7, O.D._{260/230} >1.7 were used for subsequent amplification. All PCRs were carried out in GeneAmp 9700 thermal cycler and typical reaction mix was composed of: 12.5 µl 2x PCR Master Mix Plus (A&A Biotechnology, Gdynia, Poland), 1mM each oligonucleotide primer, 100 ng of template DNA and sterile double distilled water (ddH₂O) up to final volume of 25 µl. Typical amplification reaction included initial denaturation at 94°C for 3 minutes followed by 34 cycles of denaturation (95°C; 1 minute), annealing (temperatures listed in Table 2S, Supporting information; 1 minute) and elongation (72°C; 1 minute). Each reaction was finished by final elongation step at 72°C for 10 minutes. For the detection of the amplification products, QIAxcel capillary electrophoresis system (Qiagen) was used.

2.5. Immunochromatographic enterotoxin assays

On the basis of positive results in PCR screening for the *nhe* and *hbl* operons, 50 randomly selected strains representing proportionally all identified *B. cereus* group species underwent immunochromatographic Duopath Cereus Enterotoxins Test (Merck). For the procedure, isolates were grown in 10 ml of liquid CGY broth (Merck) for at least 18 h (to achieve identical turbidity) and then 200 µl of culture was transferred into the Falcon containing 20 ml of CGY broth with 1% glucose. This was cultivated for 6 h at 37°C with vigorous shaking and then cooled to room temperature prior to the test. Duopath Cereus Enterotoxins Test was used according to the manufacturer's protocol.

2.6. Statistical analyzes

Statistical significances of observed fluctuations in concentration of *B. cereus* *sl* in water were tested by the use of Statistica 6.0 software (one-way ANOVA). *Post-hoc* Tukey RIR test (with the credible interval 0.05) was applied to determine which samples differ significantly from the rest. The correctness of dendrograms showing *B. cereus* *sl* phylogeny was assessed by 1000 iterations of bootstrap test (resampling technique used for testing the reliability of an constructed phylogenetic tree)

in Mega 6 software. Next, the random distribution of genes encoding enterotoxins was examined by using a χ^2 (chi-square) test with 0.05 credible interval.

3. RESULTS

3.1. Concentration of *B. cereus* and *B. thuringiensis* spores and vegetative forms in water samples

In order to evaluate the population density of *B. cereus* *sl* in fresh water, 24 water samples coming out from two rivers and two distinct lakes were analyzed (i) for the total *B. cereus* *sl* count and (ii) for the presence of vegetative forms only. The dominating species was *B. cereus* with 98 isolates (66%). Next 36 isolates (24%) were identified as *B. thuringiensis*, and finally 14 isolates (10%) were assigned to *B. weihenstephanensis*. None of the isolates represented properties of *B. cytotoxicus*, *B. anthracis* or *B. mycoides*/*B. pseudomycoides*. *B. cereus* *sl* were isolated from 11 out of 12 samples collected during rainless weather and similarly, from 11 out of 12 samples obtained directly after rainfall. The total count of *B. cereus* *sl* in positive samples was variable and reached 200 CFU L⁻¹ at rainless weather, and 400 CFU L⁻¹ in samples collected after intensive rainfall, in both cases from Biebrza River in July. The concentration of vegetative forms also showed fluctuations dependent especially on sampling site (Figure 1). The concentration of *B. cereus* *sl* vegetative cells in lakes was ranging from 0 to 50 CFU L⁻¹, and in rivers from 0 to 100 CFU L⁻¹ without significant differences linked to rainfall. Finally, spore concentration in lakes were ranging from 0 to 25 CFU L⁻¹ (dry weather) and from 0 to 125 CFU L⁻¹ (after rainfall). In rivers we observed that spore concentration (0-100 CFU L⁻¹) at dry weather was lower from its concentration after intensive rainfall (25-325 CFU L⁻¹). It should be mentioned that concentration of spores in rivers after rainfall was significantly higher than in lakes (one-way ANOVA, F=13.41; p=0.002). Similar fluctuations at rainless weather were not statistically significant.

Samples from rivers contained significantly more *B. cereus* *sl* (F=20.79, p=0.00004). However, the percentage of only *B. thuringiensis* isolates was significantly higher in the case of lake waters (43-53% of isolates from Wigry Lake and Garbas Lake were identified as *B. thuringiensis*, while in rivers this

value did not exceed 17%). Moreover, there was no difference in *B. cereus* *sl*/*B. thuringiensis* ratio depended on the rainfall just before sample collection.

The spore concentrations in rivers fluctuates significantly (one-way ANOVA, $F=4,973$; $p=0,035$ for rainless weather and $F=13,14$; $p=0,0021$ after intensive rainfall). Moreover, samples from July differ from those from remaining months (*post hoc* Tukey RIR test). In lakes similar situation took place. During rainless weather samples from July contained significantly more bacteria ($F=5,7$; $p=0,025$). In turns after rainfalls, lake water in August contained significantly less *B. cereus* *sl* cells than other samples ($F=4,36$; $p=0,47$). Next, statistical analyzes of vegetative cells concentration revealed no significance of their fluctuations with the exception of sample from lakes collected in August after intensive rainfall ($F=13,00$; $p=0,0022$).

3.2. Genetic diversity of *B. cereus* *sl* isolated from aqueous habitats

In order to assess the genetic diversity of 148 *B. cereus* *sl* from two lakes and two rivers, all isolates were investigated using REP-PCR. Altogether 76 different REP-PCR genetic profiles were found and among them 30 (39.5%) were unique. Representative profiles grouping at least two isolates is presented in Figure 2.

In spite of their diversity, identical *B. cereus* *sl* were widely distributed in the environments. Isolates of *B. weihenstephanensis* (profile A), and *B. cereus* (profiles B and C) were found in all four sampling sites. Next, identical *B. thuringiensis* strains (profiles D and E) were found in three different locations. Three emetic *B. cereus* isolates with the same REP-PCR profile, were obtained from Biebrza River (2 strains) and from Narew River (1 strain).

For the reconstruction of phylogeny, a multi-locus sequence typing method was applied. Among 148 isolates, altogether 38 distinct sequence types (STs) were identified. At the time of genotyping, four of them were not described in the PubMLST database, while the remaining profiles were already known. The level of the diversity at each sampling site was quite similar. For example, among isolates from

Biebrza River and Narew River, the ratios of isolates per STs were 2.54 and 2.56, respectively. In turn among bacilli from Wigry Lake and Garbas Lake, this ratio was lower (1.9-2.0). Calculated dN/dS ratio (non-synonymous/synonymous substitutions) was depending on a gene tested. It ranged from 0.0086 for the *pur* to 0.11 for the *glp* gene. Next, due to the clonal frame analysis, calculated r/m (recombination to mutation) ratio was higher in case of isolates obtained from lakes (1.2-2.25) than from rivers (0.65-1.4) indicating higher meaning of horizontal gene transfer in lakes than in rivers.

In order to find a model that describes the polymorphism the best, we performed a test of best-fit models of substitutions in Mega 6. On the basis of the lowest value of the Bayesian information criterion (BIC=26290.255), maximum likelihood model with the gamma distribution and invariable sites parameters was applied. The dendrogram of *B. cereus* *sl* phylogeny is presented in Figure 3. Isolates were assigned to *B. cereus* *sl* clades described by Guinebretiere et al. (2008). The most numerous clade III was represented by 42 *B. cereus* and *B. thuringiensis* isolates (8 STs). Next, clade V grouped 36 *B. cereus* and 4 *B. thuringiensis* isolates (with totally 8 distinct STs) and clade IV was composed of 36 isolates representing 12 STs. Psychrotolerant clade VI, besides reference strains, also includes 28 *B. weihenstephanensis*, and *B. thuringiensis* isolates with 9 different STs. Clade II was represented by only 2 identical *B. cereus* isolates. None bacilli (besides reference *B. pseudomycoides*) belonged to clade I. It should be noted that clades III, IV and VI were grouping *B. cereus* *sl* from all four sampling sites and clade V from three locations.

On the basis of single locus variants (SLVs), three distinct clonal complexes (CC-142, CC-665 and CC-8) and 29 wild-type singletons were distinguished (Figure 4). The CC-142 was the most heterogeneous. It comprised 4 distinct STs represented by 15 *B. cereus* wild-type isolates from all tested sampling sites and reference *B. cereus* ATCC 14579. CC-665 grouped 14 *B. cereus* isolates from Biebrza River and Wigry Lake. Finally CC-8 was composed of reference *B. thuringiensis* HD-73 strain and two distinct *B. thuringiensis* wild-type isolates from Biebrza River. It should be noted that 19 singletons grouped isolates from more than one location. Next, in relation to the temperature tolerance, CCs grouped only mesophilic bacilli, while all the cold-adapted bacteria and numerous mesophiles were assigned to several homogenous singletons.

Evaluation of panmictic characteristic of each population was performed by the calculation of the index of association (I_A , according to Maynard-Smith) independently for every sampling location. Obtained results ($I_A=1.9488$ for Biebrza River, $I_A=2.2899$ for Narew River and $I_A=2.9036$ for Wigry Lake) indicate significant ($P=0.000$) linkage disequilibrium, while the population from Garbas Lake appears to be panmictic ($I_A=0.0952$ and $P=0.565$).

3.3. The presence of psychrotolerant isolates belonging to the *B. cereus* group

All 148 *B. cereus* *sl* isolates were PCR-screened for the presence of the psychrotolerant signature ⁴ACAGTT⁹ (according to Francis et al. 1998) and tested for growth at 7°C. Totally 14 *B. weihenstephanensis* and 10 psychrotolerant *B. thuringiensis* isolates were found. Culture experiments (10 days at 7°C) confirmed psychrotolerance of all the *cspA* positive isolates. Bacteria devoid of the *cspA* gene usually grew well at 43°C and were unable to proliferate at 7°C. However two isolates were unable to multiply at either 43°C or 7°C.

3.4. The presence of toxin genes in *B. cereus* *sl* from fresh water

According to the PCR results, 95% of *B. cereus*, 63.9% of *B. thuringiensis* and 100% of *B. weihenstephanensis* isolates were positive for the *nhe* operon (*nheABC* genes) encoding Nhe enterotoxin. Next, the *hbl* operon (genes *hblCDA*) was present in 77.8% of *B. thuringiensis*, 64.3% of *B. weihenstephanensis* and 56.1% of *B. cereus* isolates. Finally, the *cytK* gene was detected in 46.9% of *B. cereus*, 78.6% of *B. weihenstephanensis* and 36.1% of *B. thuringiensis* strains (Figure 3). Moreover three *B. cereus* mesophilic isolates (7, 8, 150) were positive for the *ces* operon encoding cereulide synthetase. These emetic isolates were also positive for the *nhe* and negative for *hbl* and *cytK*. According to the χ^2 (chi-square) test, genes encoding enterotoxins were distributed randomly among *B. cereus* and *B. thuringiensis* isolates (*B. cereus*: $\chi^2=11.554$, $p=0.116$; *B. thuringiensis*: $\chi^2=3.108$, $p=0.875$). *B. weihenstephanensis* was not tested due to the insufficient number of isolates

for the χ^2 test. The isolates pertaining into the same ST were having the same enterotoxin genes, excluding ST-635 (*B. cereus*) and ST-1007 (*B. thuringiensis*), where differences in the *nhe* and *hbl* presence were observed.

The immunochromatographic Duopath Cereus Enterotoxins Test was applied to randomly select 25 different *B. cereus* *sl* isolates with *nhe* and/or *hbl* operons. Among them two *B. weihenstephanensis* isolates were negative for Hbl enterotoxin, while for the rest of the strains, the results of immunochromatographic tests were in agreement with PCR results.

4. DISCUSSION

Wide range of environments where isolates *B. cereus* *sl* were detected, includes also aquatic habitats (Iriarte et al. 2000; Ostensvik et al. 2004; Brillard et al. 2015). However, it has been believed that presence of these bacteria in soil or water is restricted rather to dormant spores and not vegetative cells that potentially might be metabolically active and carry their normal life cycle (Jensen et al. 2003). However, the results presented here show that vegetative cells were frequent in fresh water and their percentage requires taking them into account. All analyzes show that the level of *B. cereus* *sl* fluctuated with the significantly higher concentration in July, month with the highest average amount of atmospheric precipitation. This is in the agreement with observation that water cycle might be a potential source of *B. cereus* (Brillard et al. 2015). It also suggests that the rainfall could be important factor which influences on the microbiological quality of water, probably due to the surface runoff and contamination with wastewater. It may also lead to the presence of drug-resistant strains evidenced by Gao et al. (2012). However the soil still should be considered as the primary source of bacterial contamination of water. On the other hand, concentration of vegetative cells showed only mild changes between successive months. Thus their presence must be explained in different way. In the experiments performed by the authors (data not published), no proliferation of *B. cereus* *sl* was observed in fresh water. However vegetative cells persisted in water for a long time, probably due to their psychrotolerance or the ability of biofilm formation (Carlin et al. 2010; Cheng et al. 2014). We

cannot exclude that animal feces and invertebrates could be the source of vegetative cells. Next, this bacterial form may be associated with aquatic plants and abiotic factors, by the formation of the biofilms where they multiply. It has been shown that *B. cereus* and relatives can live in interactions with different animals (Jensen et al. 2003).

In samples collected after intensive rainfall there was even up to seven times more of *B. cereus* *sl* bacilli than during rainless weather. Moreover, flowing water contained significantly more cells and spores than standing waters by carrying more material washed from fields, forests and human activity areas.

The combination of membrane filtration method and subsequent incubation on the PTA and PTA+ media gave interesting data on the ecological properties of the *B. cereus* *sl* in natural environments. Bizzarri and Bishop (2007) demonstrated the presence of *B. thuringiensis* vegetative forms on the leaves of clover. However common occurrence of *B. cereus* *sl* vegetative cells in fresh water suggested that these bacteria can play important role in natural ecosystems. Moreover, besides being pathogens or symbionts of vertebrates and invertebrates (Jensen et al. 2003; Swiecicka 2008) they may occupy different ecological niches. It must be mentioned that presence of these species in natural habitats let them to penetrate into the industry, e.g. the dairy industry. It was suggested that primary source of *B. cereus* *sl* food contamination could be linked to the bacteria present in the dairy environment (Svensson et al. 2004; Bartoszewicz et al. 2008) and also the weather conditions might be important (McGuiggan et al. 2002). In general, cooling procedures usually preserve from germination and multiplication of bacilli and prevent from spoilage. However, occurrence of cold adapted bacilli in metabolically active stage in the environment should result in re-evaluation of procedures used in the dairy farms and industry in order to reduce the risk of contamination with *B. cereus* *sl* and their proliferation (Bartoszewicz et al. 2013).

The high level of genetic polymorphism of *B. cereus* and *B. thuringiensis* under study is in agreement with population genetic structure of these bacteria observed elsewhere (Lopez and Alippi 2007; Bartoszewicz et al. 2009; Swiecicka et al. 2013). Relying on this finding it can be suggested that *B.*

cereus *sl* present in water samples have a variety of origins with the soil as the primary source. In spite of the lack of strains significantly dominating in aquatic habitats, isolates representing profiles 3, 4, 6, 7, and 10 in the REP-PCR typing were spread in almost all sampling sites. Thus they might have specific features (e.g. lower nutritional requirements or biofilm formation abilities) that increases their fitness. MSLT revealed a slightly lower differentiation of strains obtained from rivers. Water in lakes contained bacteria from its internal populations as well as bacilli carried by the tributary rivers, including microflora washed from soil environments. Moreover, conjugation may play important role here (Thomas et al. 2001). Only strains adapted to poor environment probably could germinate or maintain vegetative cell stage. Next, in standing water, recombination played more important role, what suggests that this could be the niche where bacteria complete their life cycle, not only occasionally occur (Furlaneto et al. 2000). Next, examined populations appeared not to be panmictic (excluding strains from Garbas Lake), what should lead to their clonal structure. Purifying selection usually eliminates forms with insufficient fitness and reduces the diversity. However, we observed high polymorphism what is in good agreement with other findings (Jung et al. 2011; Liu et al. 2014; Miller et al. 2016; Yang et al. 2017). Both observations were not mutually exclusive. Water is not completely homogenous environment, and its trophy, temperature or biotic elements may show huge impact on bacterial populations, as shown in case of soil strains (Drewnowska and Swiecicka 2013). Thus, natural selection favors genotypes adapted to numerous distinct niches. In turn, horizontal gene transfer (clearly shown especially for lakes on the basis of *r/m* ratio), including plasmids (Modrie et al. 2010) and chromosomal elements (Bohm et al. 2015), probably was in opposition to the purifying selection and complicated the population genetic structure. Concluding these facts, we are likely to observe two opposing forces. One contributes to increasing diversity through continuous supply of bacteria, conjugation, and heterogeneity of the environment, while the second force reduces diversity by purifying selection.

Unclear and polyphyletic structure of the entire *B. cereus* *sl* causes confusion in taxonomy. Phenotypic properties often do not correlate with genetic clustering. Only selected features, like psychrotolerance (typical for phylogenetic clade VI), strong virulence (clade IV) or thermotolerance and cytotoxicity

(clade VII) are in compliance with phylogeny (Guinebretiere et al. 2008). Other properties, mainly enterotoxicity and synthesis of parasporal crystal inclusions are distributed in distinct phylogenetic groups and widespread in fresh water environments (Drewnowska and Swiecicka 2013; Soborg et al. 2013). All these facts suggest that we deal with a complex species with several ecotypes (Swiecicka et al. 2013) and the classification should be revised on the basis of phylogenetic approach, not phenotypic properties (Okinaka and Kaim 2016). Nevertheless *B. cereus* *sl* remains interesting object for evolutionary studies (Raymond and Bonsall 2013).

Common enterotoxicity of *B. cereus* and related bacilli, in opposition to rare emetic characteristics, cannot be surprising. Popularity of genes encoding enterotoxins might have an adaptive value, probably in case of low availability of oxygen, e.g. in aquatic habitats. Depending on the water trophic, concentration of oxygen changes and reaches the lowest value in eutrophic water with high content of organic matter. However, all collected samples were similar in physiochemical properties (Table 1S). The lower, than in other studies (e.g. Drewnowska and Swiecicka 2013) frequency of the *nhe* operon could be linked to aerobic conditions optimal for *B. cereus* *sl*. Next, there was a good correlation between the presence of particular operons encoding non-hemolytic and hemolytic enterotoxins with their expression. Only a few *B. weihenstephanensis* isolates did not produce toxins in spite of their operons. This may be the explanation why usually *B. cereus*, not *B. weihenstephanensis* is linked to human food poisonings, even if both bacteria are positive in PCR for genes responsible for their toxicity.

Concluding all these observations, it is worth noting that *B. cereus* *sl* is common in aquatic habitats and omitting vegetative cells may lead to significant underestimation of the total content of these bacilli. Next, intensive rainfalls could decrease microbiological quality of water that could be, in some circumstances, used in the industry and in production of organic food products. Spraying of *B. thuringiensis*-based insecticides can result in changes of soil and water microflora in the same manner as it influenced the intestinal microflora of people working in greenhouses (Jensen et al. 2002). Finally, taxonomic and phylogenetic approach for the investigation of the diversity bring distinct results and criteria for the identification of group members (especially for new *B. cereus* *sl* members)

are difficult to use in practice. The recognition of *B. cereus* *sl* as one polymorphic and polyphyletic species with several ecotypes, appears to be optimal.

ACKNOWLEDGMENTS

This study was supported by National Science Centre grant (DEC-2011/03/B/NZ8/02835) to Marek Bartoszewicz. Authors are also thankful to Monika Zambrzycka and Grzegorz Zambrowski for the excellent technical assistance. MLST was accomplished in Laboratory of Applied Microbiology, University of Bialystok, Poland.

REFERENCES

- Bartoszewicz, M., Hansen, B.M., and Swiecicka, I. 2008. The members of the *Bacillus cereus sensu lato* are commonly present contaminants of fresh and heat-treated milk. *Food Microbiol.* 25: 588-596.
- Bartoszewicz, M., Bideshi, D.K., Kraszevska, A., Modzelewska, E., and Swiecicka, I. 2009. Natural isolates of *Bacillus thuringiensis* display genetic and psychrotrophic properties of *Bacillus weihenstephanensis*. *J. Appl. Microbiol.* 106: 1967-1975.
- Bartoszewicz, M., Kroten, M.A., and Swiecicka, I. 2013. Germination and proliferation of emetic *Bacillus cereus sensu lato* strains in milk. *Folia Microbiol.* 58: 529-535.
- Bartoszewicz, M., and Marjanska, P.S. 2017. Milk-originated *Bacillus cereus sensu lato* strains harbouring *Bacillus anthracis*-like plasmids are genetically and phenotypically diverse. *Food Microbiol.* 67: 23-30.
- Bizzarri, M.F., and Bishop, A.H. 2007. Recovery of *Bacillus thuringiensis* in vegetative form from the phylloplane of clover (*Trifolium hybridum*) during a growing season. *J. Invertebr. Pathol.* 94: 38-47.

- Bohm, M.-E., Huptas, C., Krey, V.M., and Scherer, S. 2015. Massive horizontal gene transfer, strictly vertical inheritance and ancient duplications differentially shape the evolution of *Bacillus cereus* enterotoxins operons *hbl*, *cytK* and *nhe*. BMC Evolution Biol. 15: 246.
- Brillard, J., Dupont, C.M.S., Berge, O., Dargaignaratz, C., Oriol-Gagnier, S., Doussan, C., Broussolle, V., Gillon, M., Clavel, T., and Berard, A. 2015. The water cycle, a potential source of the bacterial pathogen *Bacillus cereus*. Biomed Res. Int. 2015: 356928.
- Caamano-Antelo, S., Fernandez-No, I.C., Bohme, K., Ezzat-Alnakip, M., Quintela-Baluja, M., Barros-Velazquez, J., and Calo-Mata, P. 2015. Genetic discrimination of foodborne pathogens and spoilage *Bacillus* spp. based on three housekeeping genes. Food Microbiol. 46: 288-298.
- Carlin, F., Brillard, J., Broussolle, V., Clavel, T., Dupont, C., Jobin, M., Guinebretiere, M.-H., Auger, S., Sorokin, A., and Nguyen-The, C. 2010. Adaptation of *Bacillus cereus*, an ubiquitous worldwide-distributed foodborne pathogen, to a changing environment. Food Res. Int. 43: 1885-1894.
- Cheng, Z., Meng, X., Wang, H., Chen, M., and Li, M. 2014. Isolation and characterization of broad spectrum coaggregating bacteria from different water systems for potential use in bioaugmentation. PloS One 15:e94220.
- Dierick, K., van Coillie, C., Swiecicka, I., Meyfroidt, G., Devlieger, H., Meulemans, A., Hoedemaekers, G., Fourie, L., Heyndrickx, M., and Mahillon, J. 2005. A fatal family outbreak of *Bacillus cereus* food poisoning. J. Clin. Microbiol. 43: 4277-4279.
- Douville, M., Gagne, F., Blaisse, C., and Andre, C. 2007. Occurrence and persistence of *Bacillus thuringiensis* (Bt) and transgenic Bt corn *cryIAb* gene from an aquatic environment. Ecotoxicol. Environ. Saf. 66: 195-203.
- Drewnowska, J.M. and Swiecicka, I. 2013. Eco-genetic structure of *Bacillus cereus sensu lato* populations from different environments in Northeastern Poland. PloS One 8: e80175.

- Ehling-Schulz, M., Fricker, M., and Scherer, S. 2004. Identification of emetic toxin producing *Bacillus cereus* strains by a novel molecular assay. FEMS Microbiol. Lett. 232: 189-195.
- Feil, E.J., Li, B.C., Aanensen, D.M., Hanage, W.P., and Spratt, B.G. 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J. Bacteriol. 186: 1518-1530.
- Francis, K.P., Mayr, R., von Stetten, F., Stewart, G.S.A.B., and Scherer, S. 1998. Discrimination of psychrotrophic and mesophilic strains of the *Bacillus cereus* group by PCR targeting of major cold shock protein genes. Appl. Environ. Microbiol. 64: 3515-3529.
- Furlaneto, L., Ostrensky Saridakis, H., and Nagy Arantes, O.M. 2000. Survival and conjugal transfer between *Bacillus thuringiensis* strains in aquatic environment. Braz. J. Microbiol. 31: 233-238.
- Gao, P., Mao, D., Luo, Y., Wang, L., Xu, B., and Xu, L. 2012. Occurrence of sulfonamide and tetracycline-resistant bacteria and resistance genes in aquaculture environment. Water Res. 46: 2355-2364.
- Guinebretiere M.-H., Fagerlund A., Granum P.E., and Nguyen-The C. 2006. Rapid discrimination of *cytK*-1 and *cytK*-2 genes in *Bacillus cereus* strains by a novel duplex PCR system. FEMS Microbiol. Lett. 259: 74-80.
- Guinebretiere, M.-H., Thompson, F.L., Sorokin, A., Normand, P., Dawyndt, P., Ehling-Schulz, M., Svensson, B., Sanchis, V., Nguyen-The, C., Heyndrickx, M., and De Vos, P. 2008. Ecological diversification in the *Bacillus cereus* group. Environ. Microbiol. 10: 851-865.
- Guinebretiere, M.-H., Auger, S., Galleron, N., Contzen, M., De Sarrau, B., De Buyser, M.-L., Lamberet, G., Fagerlund, A., Granum, P.E., Lereclus, D., De Vos, P., Nguyen-The, C., and Sorokin, A. 2013. *Bacillus cytotoxicus* sp. nov. is a novel thermotolerant species of the *Bacillus cereus* group occasionally associated with food poisoning. Int. J. Syst. Evol. Microbiol. 63: 31-40.

- Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41: 95-98.
- Hansen, B.M., and Hendriksen, N.B. 2001. Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. Appl. Environ. Microbiol. 67: 185-189.
- Hendriksen, N.B., and Hansen, B.M. 2006. Detection of *Bacillus thuringiensis kurstaki* HD1 on cabbage for human consumption. FEMS Microbiol. Lett. 257: 196-111.
- Ichimatsu, T., Mizuki, E., Nishimura, K., Akao, T., Saitoh, H., Higuchi, K., and Ohba, M. 2000. Occurrence of *B. thuringiensis* in fresh water of Japan. Curr. Microbiol. 40: 217-220.
- Iriarte, J., Porcar, M., Lecadet, M.-M., and Caballero, P. 2000. Isolation and characterization of *Bacillus thuringiensis* strains from aquatic environments in Spain. Curr. Microbiol. 40: 402-408.
- Jensen, G.B., Larsen, P., Jacobsen, B.L., Madsen, B., Smidt, L., and Andrup, L. 2002. *Bacillus thuringiensis* in fecal samples from greenhouse workers after exposure to *B. thuringiensis*-based pesticides. Appl. Environ. Microbiol. 48: 4900-4905.
- Jensen, G.B., Hansen, B.M., Eilenberg, J., and Mahillon, J. 2003. The hidden lifestyles of *Bacillus cereus* and relatives. Environ. Microbiol. 5: 631-640.
- Jimenez, G., Urdiain, M., Cifuentes, A., Lopez-Lopez, A., Blach, A.R., Tamames, J., Kampfer, P., Kolsto, A-B., Ramon, D., Martinez, J.F., Codoner, F.M., and Rosello-Mora, R. 2013. Description of *Bacillus toyonensis* sp. nov., a novel species of the *Bacillus cereus* group, and pairwise genome comparison of the species of the group by means of ANI calculations. Syst. Appl. Microbiol. 36: 323-391.
- Jung, M.Y., Kim, J.S., Peak, W.K., Lim, J., Lee, H., Kim, P.I., Ma, J.Y., Kim, W., and Chang, Y.H. 2011. *Bacillus manliponensis* sp. nov., a new member of the *Bacillus cereus* group isolated from foreshore tidal flat sediment. J. Microbiol. 49: 1027-1032.

- Lechner, S., Mayr, R., Francis, K.P., Pruß, B.M., Kaplan, T., Wießner_Gunkel, E., Stewart, G.S.A.B., and Scherer, S. 1998. *Bacillus weihenstephanensis* sp. nov. is a new psychrotolerant species of the *Bacillus cereus* group. Int. J. Syst. Evol. Microbiol. 48: 1373-1382.
- Liu, B., Liu, G.H., Hu, G.P., Sengonca, C., Cetin, S., Lin, N.Q., Tang, J.Y., Tang, W.Q., and Lin, Y.Z. 2014. *Bacillus bingmayongensis* sp. nov., isolated from the pit soil of Emperor Qin's Terra-cotta warriors in China. Antonie van Leeuwenhoek 105: 501-510.
- Lopez, A.C., and Alippi, A.M. 2007. Phenotypic and genotypic diversity of *Bacillus cereus* isolates recovered from honey. Int. J. Food Microbiol. 117: 175-184.
- McGuiggan, J.T.M., McCleery, D.R., Hannan, A., and Gilmour, A. 2002. Aerobic spore-forming bacteria in bulk raw milk: factors influencing the numbers of psychrotrophic, mesophilic and thermophilic *Bacillus* spores. Int. J. Dairy Technol. 55: 100-107.
- Melnick, R.L., Testen, A.L., Poleatewich, A.M., Backman, P.A., and Bailey, B.A. 2012. Detection and expression of enterotoxin genes in endophytic strains of *Bacillus cereus*. Lett. Appl. Microbiol. 54: 468-474.
- Miller, R.A., Beno, S.M., Kent, D.J., Carroll, L.M., Martin, N.H., Boor, K.J., and Kovac, J. 2016. *Bacillus wiedmannii* sp. nov., a psychrotolerant and cytotoxic *Bacillus cereus* group species isolated from dairy food and dairy environments. Int. J. Syst. Evol. Microbiol. 66: 4744-4753.
- Mizuki, E., Maeda, M., Tanaka, R., Lee, D-W., Hara, M., Akao, T., Yamashita, S., Kim, H.-S., Ichimatsu, T., and Ohba, M. 2001. *Bacillus thuringiensis*: a common member of microflora in activated sludges of a sewage treatment plant. Curr. Microbiol. 42: 422-425.
- Modrie, P., Beuls, E., and Mahillon, J. 2010. Differential plasmid transfer dynamics of pAW63 plasmid among members of the *Bacillus cereus* group in food microcosms. J. Appl. Microbiol. 108: 888-897.

- Okinaka, R.T., and Keim, P. 2016. The phylogeny of *Bacillus cereus sensu lato*. Microbiol. Spectr. 4(1): TBS-0012-2012.
- Ostensvik, O., From, C., Heidenreich, B., O'Sullivan, K., and Granum, P.E. 2004. Cytotoxic *Bacillus* spp. belonging to the *B. cereus* and *B. subtilis* groups in Norwegian surface waters. J. Appl. Microbiol. 96: 987-993.
- Raymond, B., and Bonsall, M.B. 2013. Cooperation and the evolutionary ecology of bacterial virulence: the *Bacillus cereus* group as a novel study system. Bioessays 35: 706-716.
- Reyes-Ramirez, A., and Ibarra, J.E. 2005. Fingerprinting of *Bacillus thuringiensis* type strains and isolates by using *Bacillus cereus* group-specific repetitive extragenic palindromic sequence-based PCR analysis. Appl. Environ. Microbiol. 71: 1346-1355.
- Schleifer, K., and Whitman, W.B. 2009. Bergey's manual of systematic bacteriology: The Firmicutes. 2nd ed. New York, Springer.
- Soborg, D.A., Hendriksen, N.B., Kilian, M., and Kroer, N. 2013. Widespread occurrence of bacterial human virulence determinants in soil and freshwater environments. Appl. Environ. Microbiol. 79: 5488-5497.
- Stenfors Arnesen, L.P., Fagerlund, A., and Granum, P.E. 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol. Rev. 32: 579-606.
- Svensson, B., Ekelund, K., Ogura, H., and Christiansson, A. 2004. Characterisation of *Bacillus cereus* isolated from milk silo tanks at eight different dairy plants. Int. Dairy. J. 14: 17-27.
- Swiecicka, I. 2008. Natural occurrence of *Bacillus thuringiensis* and *Bacillus cereus* in eukaryotic organisms: a case for symbiosis. Biocontrol Sci. Technol. 18: 221-239.
- Swiecicka, I., Bartoszewicz, M., Kasulyte-Creasey, D., Drewnowska, J., Murawska, E., Yernazarova, A., Łukaszuk, E., and Mahillon, J. 2013. Diversity of thermal ecotypes and potential pathotypes of *Bacillus thuringiensis* soil isolates. FEMS Microbiol. Ecol. 85: 262-272.

Thomas, D.J.I., Morgan, A.W., Whipps, J.M., and Saunders, J.R. 2001. Plasmid transfer between *Bacillus thuringiensis* subsp. *israelensis* strains in laboratory culture, river water, and dipteran larvae. *Appl. Environ. Microbiol.* 67: 330-338.

Vilas-Boas, L.A., Peruca, A.P.S., and Arantes, O.M.N. 2007. Biology and taxonomy of *Bacillus cereus*, *Bacillus anthracis*, and *Bacillus thuringiensis*. *Can. J. Microbiol.* 53: 673-687.

Yang, Y., Yu, X., Zhan, L., Chen, J., Zhang, Y., Zhang, J., Chen, H., Zhang, Z., Zhang, Y., Lu, Y., and Mei, L. 2017. Multilocus sequence type profiles of *Bacillus cereus* isolates from infant formula in China. *Food Microbiol.* 62: 46-50.

Zwick, M.E., Joseph, S.I., Didelot, X., Chen, P.E., and Bishop-Lilly, K.A. 2012. Genomic characterization of the *Bacillus cereus sensu lato* species: backdrop to the evolution of *Bacillus anthracis*. *Genome Res.* 22: 1512-1524.

FIGURE CAPTIONS

Figure 1S (Supporting information). Location of sampling sites in Northeastern Poland.

Figure 1. Changes in the concentration of *B. cereus* *sl* spores and vegetative forms concentration in fresh water of NE Poland during (a) dry weather, and (b) after intensive rainfalls. The detection limit for *B. cereus* *sl* was 25 CFU L⁻¹.

Figure 2. The genetic diversity of *B. cereus* *sl* from fresh water assessed by the use of the REP-PCR fingerprinting.

Profiling performed according to Reyes-Ramirez and Ibarra (2005) was applied to investigate the total genetic diversity of *B. cereus* *sl* isolates from fresh water. Here only representative profiles, which include at least three isolates were shown. M-marker line (GeneRuler 100 bp Plus, Fermentas), Species: *Bc-B. cereus*, *Bt-B. thuringiensis*, *Bw-B. weihenstephanensis*. Thermotolerance: M-

mesophilic and P-psychrotolerant characteristics. Finally, *nhe*, *hbl* and *cytK* indicate presence or absence of the corresponding genes for enterotoxin synthesis.

Figure 3. Reconstruction of phylogenetic diversity of *B. cereus* *sl* from fresh water by maximum likelihood method.

The evolutionary history was inferred by the use of the maximum likelihood method based on the general time reversible model with gamma distribution and invariable sites parameters. The bar length indicates the number of substitutions per site (0.01). For each branch, a proper sequence type number (ST) is given in white boxes for wild-type strains and gray boxes for reference strains. Bootstrap values (for 1000 iterations) are above the branches. Species: *Bc-B. cereus*, *Bt-B. thuringiensis*, *Bw-B. weihenstephanensis*, *Ba-B. anthracis*, *Bm-B. mycoides*, *Bp-B. pseudomycoides*, Nd-not determined. Number of isolates corresponds to the number of isolates of each particular ST. Toxicity shows the presence of genes responsible for synthesis of enterotoxins. Psychrotolerance depicts psychrotolerant (P) or mesophilic (M) characteristics and location depicts adequate sampling sites. All isolates were assigned to phylogenetic clades described previously (Guinebretiere et al., 2008).

Figure 4. Population structure of *B. cereus* *sl* from fresh water assessed by geoBURST analysis (Feil et al., 2004) in relation to their sampling site (A), cold adaptation (B) and taxonomic data (C).

Isolates were grouped into clonal complexes (CCs) by the use of Phyloviz 1.0. Clonal complexes were defined on the basis of single locus variants (SLV) as groups of isolates with six or seven identical alleles in MLST analysis. The diameter of a circle indicates the number of isolates representing each particular Sequence Type. *Bc-B. cereus*, *Bt-B. thuringiensis*, *Bw-B. weihenstephanensis*, *Ba-B. anthracis*, *Bm-B. mycoides*, *Bp-B. pseudomycoides*.

Figure 1. Changes in the concentration of *B. cereus* s/l spores and vegetative forms concentration in fresh water of NE Poland during (a) dry weather, and (b) after intensive rainfalls. The detection limit for *B. cereus* s/l was 25 CFU L⁻¹.

Line	Species		Psychrotolerance	<i>nhe</i>	<i>hbl</i>	<i>cytK</i>
M	-					
1	Bc		M	+	-	-
2	Bt		M	-	+	+
3	Bc		M	+	+	-
4	Bw		P	+	-	-
5	Bc		M	+	+	+
6	Bc		M	+	+	-
7	Bt		M	+	+	+
8	Bw		P	+	+	+
9	Bc		M	+	+	+
10	Bt		P	+	+	-
11	Bc		M	+	+	-
12	Bc		M	-	+	+
13	Bw		P	+	+	-

Figure 2. The genetic diversity of *B. cereus* *sl* from fresh water assessed by the use of the REP-PCR fingerprinting. Profiling performed according to Reyes-Ramirez and Ibarra (2005) was applied to investigate the total genetic diversity of *B. cereus* *sl* isolates from fresh water. Here only representative profiles, which include at least three isolates were shown. M-marker line (GeneRuler 100 bp Plus, Fermentas), Species: *Bc*-*B. cereus*, *Bt*-*B. thuringiensis*, *Bw*-*B. weihenstephanensis*. Thermotolerance: M-mesophilic and P-psychrotolerant characteristics. Finally, *nhe*, *hbl* and *cytK* indicate presence or absence of the corresponding genes for enterotoxin synthesis.

Figure 3. Reconstruction of phylogenetic diversity of *B. cereus* s.l. from fresh water by maximum likelihood method. The evolutionary history was inferred by the use of the maximum likelihood method based on the general time reversible model with gamma distribution and invariable sites parameters. The bar length indicates the number of substitutions per site (0.01). For each branch, a proper sequence type number (ST) is given in white boxes for wild-type strains and gray boxes for reference strains. Bootstrap values (for 1000 iterations) are above the branches. Species: *Bc*-*B. cereus*, *Bt*-*B. thuringiensis*, *Bw*-*B. weihenstephanensis*, *Ba*-*B. anthracis*, *Bm*-*B. mycoides*, *Bp*-*B. pseudomycoides*, Nd-not determined. Number of isolates corresponds to the number of isolates of each particular ST. Toxicity shows the presence of genes responsible for synthesis of enterotoxins. Psychrotolerance depicts psychrotolerant (P) or mesophilic (M) characteristics and location depicts adequate sampling sites. All isolates were assigned to phylogenetic clades described previously (Guinebretiere et al., 2008).

Figure 4. Population structure of *B. cereus* s/l from fresh water assessed by geoBURST analysis (Feil et al., 2004) in relation to their sampling site (A), cold adaptation (B) and taxonomic data (C). Isolates were grouped into clonal complexes (CCs) by the use of Phyloviz 1.0. Clonal complexes were defined on the basis of single locus variants (SLV) as groups of isolates with six or seven identical alleles in MLST analysis. The diameter of a circle indicates the number of isolates representing each particular Sequence Type. *Bc*-*B. cereus*, *Bt*-*B. thuringiensis*, *Bw*-*B. weihenstephanensis*, *Ba*-*B. anthracis*, *Bm*-*B. mycoides*, *Bp*-*B. pseudomycoides*.

190x120mm (96 x 96 DPI)

Table 1S. Characteristics of rainfall and physiochemical properties of the water collected from Narew River, Biebrza River, Wigry Lake and Garbas Lake in three successive months.

Property	June				July				August			
	Narew	Biebrza	Wigry	Garbas	Narew	Biebrza	Wigry	Garbas	Narew	Biebrza	Wigry	Garbas
Rainfall duration [h]	7	7	8	8	11	10	8	7	12	12	11	12
Rainfall intensity [mm]	21	17	28	35	49	46	41	39	31	36	30	29
pH ^{a)}	7.9/7.9	8.0/7.9	7.6/7.6	7.5/7.6	7.8/7.8	8.1-8.1	7.6/7.6	7.6/7.6	7.8/7.9	8.0/8.0	7.5/7.6	7.6/7.6
Electric conductivity [$\mu\text{S cm}^{-1}$] ^{a)}	280/350	310/380	290/300	330/360	300/320	330/380	330/350	320/330	290-310	330/360	340/370	350/370
Dissolved organic carbon [mg l^{-1}] ^{a)}	8.3/8.6	8.6/8.8	5.6/5.7	5.1/5.3	8.6/8.6	8.1/8.3	4.9/5.2	5.7/5.8	7.9/8.0	8.0/8.1	5.3/5.4	5.6/5.6
Total nitrogen [mg l^{-1}] ^{a)}	0.8/0.8	1.2/1.3	0.7/0.6	1.1/1.3	1.3/1.2	1.3/1.3	0.8/1.0	1.0/1.2	0.8/0.8	0.9/1.1	0.6/0.7	1.1/1.0
Organic phosphorus [$\mu\text{g l}^{-1}$] ^{a)}	180/230	160/250	140/160	110/120	210/200	180/200	150/180	120/160	230/280	230/220	130/170	140/130

a) properties of water samples collected during rainless weather/properties of water samples collected after intensive rainfall

1 **Table 2S. Oligonucleotide primers and amplification conditions used in the study.**

2 Each amplification reaction included 34 cycles. F, forward primer; R, reverse primer.

3 Symbols in degenerated primers are in accordance to the IUPAC code. References are given

4 below.

5

Target	Sequence of primers (5'→3')	Annealing temperature [°C]	References
<i>nheA</i>	F: TACGCTAAGGAGGGGCA R: GTTTTATTGCTTCATCGGCT	60	Hansen and Hendriksen 2001
<i>nheB</i>	F: CTATCAGCACTTATGGCAG R: ACTCCTAGCGGTGTTCC	55	Hansen and Hendriksen 2001
<i>nheC</i>	F: CGGTAGTGATTGCTGGG R: CAGCATTGCTACTTGCCAA	58	Hansen and Hendriksen 2001
<i>hblA</i>	F: CAAGGTGCAGATGTTGATGC R: GAACGCCCGAATATTGAG	55	Melnick et al. 2012
<i>hblC</i>	F: AATGGTCATCGGAACTCTAT R: CTCGCTGTTCTGCTGTTAAT	56	Hansen and Hendriksen 2001
<i>hblD</i>	F: AATCAAGAGCTGTCACGAAT R: CACCAATTGACCATGCTAAT	57	Hansen and Hendriksen 2001
<i>cytK-1</i>	F: GATAATATGACAATGTCTTTAAA R: GGAGAGAAACCGCTATTTGT	60	Guinebretiere et al. 2006
<i>cytK-2</i>	F: CAATCCCTGGCGCTAGTGCA R: GTGIAGCCTGGACGAAGTTGG	60	Guinebretiere et al. 2006
<i>ces</i>	F: GACAAGAGAAATTCTACGAGCAAGTACAAT R: GCAGCCTTCCAATTACTCCTTCTGCCACAGT	60	Ehling-Schulz et al. 2004
<i>cspA</i>	F: GAGGAAATAATTATGACAGTT R: CTTYTTGGCCTTCTTCTAA	50	Francis et al. 1998
<i>cspF</i>	F: CTTYTTGGCCTTCTTCTAA R: GAGATTAAATGAGCTGTAA	50	Francis et al. 1998
<i>REP</i>	F: ATTAAAGTTTCACTTTAT R: TTTAATCAGTGGG	42	Reyes-Ramirez and Ibarra 2005
<i>glpF</i>	F: GCGTTTGTGCTGGTGTAAGT R: CTGCAATCGGAAGGAAGAAG	59	pubmlst.org

<i>gmk</i>	F: ATTTAAGTGAGGAAGGGTAGG R: GCAATGTTCACCAACCACAA	56	pubmlst.org
<i>ilvD</i>	F: CGGGGCAAACATTAAGAGAA R: GGTTCGTGTCGTTCCATTC	58	pubmlst.org
<i>pta</i>	F: GCAGAGCGTTTAGCAAAAGAA R: TGCAATGCGAGTTGCTTCTA	56	pubmlst.org
<i>pur</i>	F: CTGCTGCGAAAAATCACAAA R: CTCACGATTCGCTGCAATAA	56	pubmlst.org
<i>pyc</i>	F: GCGTTAGGTGGAACGAAAG R: CGCGTCCAAGTTATGGAAT	57	pubmlst.org
<i>tpi</i>	F: GCCCAGTAGCACTTAGCGAC R: CCGAAACCGTCAAGAATGAT	58	pubmlst.org

6

1. Ehling-Schulz M., Fricker M., Scherer S. 2004. Identification of emetic toxin producing *Bacillus cereus* strains by a novel molecular assay. FEMS Microbiol. Lett. 232: 189-195.
2. Francis K.P., Mayr R., von Stetten F., Stewart G.S.A.B., and Scherer S. 1998. Discrimination of psychrotrophic and mesophilic strains of the *Bacillus cereus* group by PCR targeting of major cold shock protein genes. Appl. Environ. Microbiol. 64: 3515-3529
3. Guinebretiere M.-H., Fagerlund A., Granum P.E., and Nguyen-The C. 2006. Rapid discrimination of *cytK*-1 and *cytK*-2 genes in *Bacillus cereus* strains by a novel duplex PCR system. FEMS Microbiol. Lett. 259: 74-80.
4. Hansen B.M., and Hendriksen N.B. 2001. Detection of enterotoxigenic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. Appl. Environ. Microbiol. 67: 185-189.
5. Melnick R.L., Testen A.L., Poleatewich A.M., Backman P.A., and Bailey B.A. 2012. Detection and expression of enterotoxin genes in endophytic strains of *Bacillus cereus*. Lett. Appl. Microbiol. 54: 468-474.
6. Reyes-Ramirez A., and Ibarra J.E. 2005. Fingerprinting of *Bacillus thuringiensis* type strains and isolates by using *Bacillus cereus* group-specific repetitive extragenic palindromic sequence-based PCR analysis. Appl. Environ. Microbiol. 71: 1346-1355.

27

28

29