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YEB DOUWE HOORNSTRA Tracking Cereulide Producing *Bacillus cereus* in Foods, Papermaking and Biowaste Management 3/2014

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DOUWE HOORNSTRA

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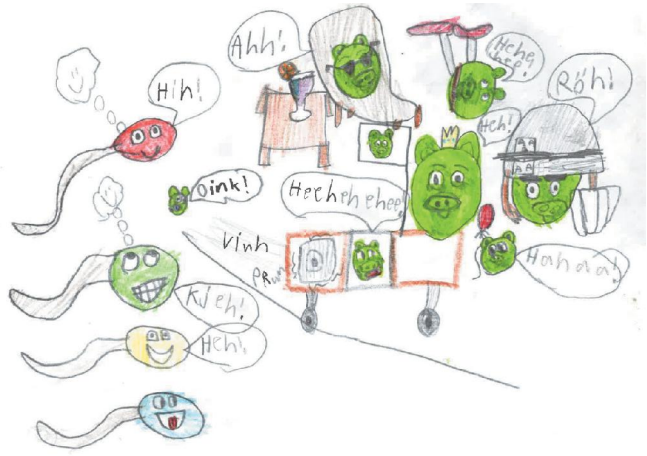

DEPARTMENT OF FOOD AND ENVIRONMENTAL SCIENCES
FACULTY OF AGRICULTURE AND FORESTRY
DOCTORAL PROGRAMME IN MICROBIOLOGY AND BIOTECHNOLOGY
UNIVERSITY OF HELSINKI

Tracking cereulide producing *Bacillus cereus* in foods, papermaking and biowaste management

Douwe Hoornstra

Department of Food and Environmental Sciences
Faculty of Agriculture and Forestry
University of Helsinki

Academic dissertation in Microbiology

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public criticism in auditorium B2 at the Viikki B Building, Latokartanonkaari 7, Helsinki, on October 31st, 2014 at 12 o'clock noon.

Supervisors: Prof. emeritus Mirja S. Salkinoja-Salonen
Department of Food and Environmental Sciences
Faculty of Agriculture and Forestry
University of Helsinki
Helsinki, Finland

Dr. Maria A. Andersson
Department of Food and Environmental Sciences
Faculty of Agriculture and Forestry
University of Helsinki
Helsinki, Finland

Reviewers: Prof Dr. Andreja Rajkovic
Department of Food Safety and Food Quality
Faculty of Bio-science engineering, Ghent University

Prof Dr. Heikki Ojamo
School of Chemical Technology
Department of Biotechnology and Chemical Technology

Opponent: Prof Dr. Tuula Heinonen
School of Medicine
University of Tampere

Front cover: Boar sperm cells, some will make it to become pigs and others will be used for toxicity analysis. Showing here the usefulness of boar spermatozoa. The drawing was made by Silvia Hoornstra, 8 years.

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I Hoornstra D, Andersson MA, Mikkola R, Salkinoja-Salonen MS. 2003. A new method for in vitro detection of microbially produced mitochondrial toxins. Toxicology in Vitro. 17: 745-51.

II Hoornstra D, Andersson MA, Johansson T, Pirhonen T, Hatakka M, Salkinoja-Salonen MS. 2004. Mitochondrial toxicity detected in a health product with a boar spermatozoan bioassay. ATLA-Alternatives to Laboratory Animals. 32: 407-16.

III Hoornstra D, Dahlman O, Jääskeläinen E, Andersson MA, Weber A, Aurela B, Lindell H, Salkinoja-Salonen MS. 2006. Retention of *Bacillus cereus* and its toxin, cereulide, in cellulosic fibres. Holzforschung. 60: 648–652

IV Govasmark E, Stäb J, Hoornstra D, Nesbakk T, Salkinoja-Salonen M. 2011. Chemical and microbiological hazards associated with recycling of anaerobic digested residue intended for agricultural use. Waste Management 31: 2577-2583.

V Hoornstra D, Andersson MA, Teplova VV, Mikkola R, Uotila LM, Andersson LC, Roivainen M, Gahmberg CG, Salkinoja-Salonen MS. 2013. Potato crop as a source of emetic *Bacillus cereus* and cereulide induced mammalian cell toxicity. Applied and Environmental Microbiology 79: 3534-3543.

The author's contribution

I. Douwe Hoornstra carried out the toxicological assays in cooperation with Maria Andersson, interpreted the results and wrote the main part in the paper.

II. Douwe Hoornstra carried out the toxicological assay using boar spermatozoa and the extractions in close cooperation with Maria Andersson, interpreted the results and wrote the paper.

III. Douwe Hoornstra did the *Bacillus cereus* work and prepared the hand sheets (paper) together with Birgit Aurela, prepared most of the leachates and analysed all leachates for toxicity and cereulide and wrote the main part of the paper.

IV. Douwe Hoornstra did the microbiological part in this project and wrote the microbiological part in the paper.

V. Douwe Hoornstra did the potato work, isolation, characterisation of the *B. cereus* endophytes, identification, toxicological analyses using the boar sperm cells, interpreted the results and wrote the paper together with the other authors.

Definitions of terms

Term	Definition
Antianginal drugs	Drugs that are used to relieve the symptoms of angina by restoring balance between oxygen supply and demand.
Depsipeptide	Peptide containing one or more ester bonds in addition to peptide bonds
Germination	The process in which a spore grows into a vegetative cell.
Sperm Combi Assay	Boar sperm motility inhibition assay with fluorescent dyes
Sporulation	The formation of spores
Thermal D value	The time (minutes) needed at a specific temperature to reduce the number of bacteria by one log
Toxin	Substance produced by a living organism toxic towards other living species

Abbreviations

Abbreviation	Full wording
Calcein AM	Calcein Acetoxy Methyl
CDC	Center for Disease Control and Prevention
$\Delta\psi_m$	Mitochondrial Membrane Potential
JC-1	5,5´,6,6´-tetrachloro-1,1´,3,3´-tetraethylbenzimidazolyl-carbocyanine iodide
LC/MS	Liquid Chromatography and Mass Spectrometry
NRPS	Non Ribosomal Peptide Synthetase
OD	Optical Density
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PKTE	Porcine Kidney Tubular Epithelial Cell
ROS	Reactive Oxygen Species

Abstract

Bacillus cereus is a ubiquitous food poisoning bacterium, but only producers of the emetic toxin, cereulide can be life threatening. Therefore a fast and reliable method is needed for identifying strains that produce the toxin. In this thesis the previously developed sperm bioassay was refined into a tool for the detection of microbially produced mitochondria toxic substances. This refined tool, Sperm Combi Assay effectively detected not only cereulide, but also other mitochondrial toxic substances and yielded data for classifying the substances into 3 different classes. The cereulide class (includes also valinomycin and enniatin) was characterised by a spotted mitochondrial sheath when stained with the membrane potential responsive dye JC-1. The gramicidin class (includes nigericin, calcimycin, monensin, narasin, salinomycin and antimycin A) was characterised by gradual fading of the dye during loss of the membrane potential. The ionomycin type (includes staurosporine, oligomycin) was characterised by loss of sperm motility with no changes of the mitochondrial membrane potential.

The novel Sperm Combi Assay was put to the test to clarify the cause of illness connected to consumption of food supplements (seaweed capsules). The Sperm Combi Assay revealed highly toxic contaminants in the capsules. The test revealed that the poisonous substance was in the seaweed inside the capsule, not involving the capsule shell. The currently available official methods had failed to disclose this information. The test further helped to identify bacteria responsible for producing the toxic substances in the seaweed. The effects shown by the Sperm Combi Assay were similar to the gramicidin type of response.

Next the Sperm Combi Assay was used to track potential sources of cereulide producers in food. We found cereulide producing *B. cereus* from potato tuber originating from the consumer market and had not undergone industrial treatment. We describe in this thesis how the initial plate count procedure can be improved to

recognise the cereulide producing *B. cereus* already on a plate. Cereulide producers have been reported in industrial foods and rice by many researchers. Our report appears to be the first one where potato crops were screened for cereulide producers by methods that were effective for detecting cereulide producing *B. cereus*. This development is important because the potato is a major food crop in the European diet.

Not only potatoes but many other plants such as trees in the forest contain cereulide producing *B. cereus* as an endophyte. The forest industry makes food packaging material from wood pulp. To trace the behaviour cereulide producing *B. cereus* contained in wood, we used the same protocol which was useful for finding the cereulide producing *B. cereus* in potato. We found that, of the spores of cereulide producing *B. cereus*, ca. 5 % may be retained in the paper product. The Sperm Combi Assay was used to track the distribution of cereulide, potentially released into the pulp slurry by cereulide producing *B. cereus*. We found that ca. 10 % present in the pulp slurry was recovered in the paper product. We measured if cereulide contained in paper could migrate from the paper product into packaged food and found almost all cereulide leached into simulated fatty foods, but less than the detection limit into warm or cold drinks.

Food waste contains valuable nutrients and should be recycled into agricultural production. The question arises about the fate of cereulide producing *B. cereus* contained in food. We studied the behaviour of cereulide producing *B. cereus* spores throughout four seasons in an industrial scale biowaste processing plant that involved a heating step of 24 min at 137 °C followed by anaerobic digestion. We traced cereulide producing *B. cereus* in the digested biowaste with the same culture methods and the Sperm Combi Assay which proved operative in potato and with non culture based methods (PCR). We showed that the liquid biofertilizer contained ca. 1000 total *B. cereus* per ml of which 10 % were *ces*B (indicator of

cereulide synthase) positive. This persistence of cereulide producing *B. cereus* under harsh conditions demonstrates that it is not possible to eradicate cereulide producers from the food cycle. Instead, we have to learn to design our food processing protocols so as to not give cereulide producers a chance to accumulate the cereulide toxin in foods.

In this study we used porcine sperm cells as a tool to detect cereulide (their sensitivity is high), with an EC_{100} (equal to lethal dose) 0.3 ng ml^{-1}. The question is if sperm sensitivity to cereulide reflects the sensitivity of other kinds of cells and tissues, for instance in the human body. We assessed this question by comparing the sensitivity of primary cells, cell lines and pancreatic islets towards cereulide. We found that the EC_{100} values for loss of mitochondrial membrane potential (24 h exposure) to human peripheral blood mononuclear cells and keratinocytes, porcine kidney epithelial cells and murine fibroblasts and pancreatic islets (containing the beta cells which produce insulin) ranged from 0.4 to 2 ng of cereulide per ml. The results show that different kinds of cells were similarly vulnerable to mitochondrial toxicity by cereulide. These results strongly indicate that the Sperm Combi Assay is useful not only for tracking cereulide in foods and environmental samples, but can also be useful in assessing human health risk by materials contaminated by cereulide. We also showed that the external concentration of potassium (300µM increased to 850-950 µM) influences the speed of the potassium efflux, measured in HaCaT and PBMC cells.

We introduced this Sperm Combi Assay to deal with a bacterium and/or toxin that can hardly be eliminated in foods. This assay is a fast and reliable method to detect mitochondria toxic substances as well as the reliable plating method for screening the producing organisms.

Because it is not possible to eliminate cereulide producing *B. cereus*, it should be kept under control by avoiding conditions permissive for cereulide production.

Tiivistelmä

Bacillus cereus on hyvin yleinen bakteeri ympäristössä, erityisesti kasveissa ja maaperässä. Elintarvikkeissa lisääntyessään sen toksineeja tuottavat kannat aiheuttavat ruokamyrkytyksiä. Sen monista toksiineista eli myrkkyaineista kereulidi, jota sanotaan myös oksetustoksiiniksi, on erityisen vaarallinen. Se tunnetaan jopa kuolemaan johtaneiden, vakavien myrkytysten aiheuttajana. Siksi tätä toksiinia tuottamaan kykenevien *B. cereus* -kantojen tunnistaminen on tärkeää. Tässä työssä kehitettiin kuumennusta kestävien toksiinien mittausmenetelmä, Sperm Combi Assay (SCA). Menetelmässä hyödynnetään sikataloudessa tuotettavaa siemennestettä. Eri toksiinit aiheuttavat sian siittiöissä erityyppisiä soluvammoja. Mikroskooppisesti todennettavien, kullekin toksiinityypille ominaisten soluvaurioiden avulla toksiinit voidaan tunnistaa suoraan elintarvikkeista ja mikrobinäytteistä. Siittiöreaktiivisia mikrobitoksiineja löytyi kolmenlaisia: 1. kereulidi ja sen kaltaisesti vaikuttavat, kuten kymmen- tai satakertaisesti vähemmän myrkylliset, valinomysiini ja enniatiini. Näille myrkyille on tunnusomaista siittiön hännän värjäytyminen pilkulliseksi JC-1 väriaineella, joka vaihtaa väriä solujen kalvopotentiaalin muuttuessa; 2. gramisidiinin kaltaiset myrkkyaineet nigerisiini, kalsimysiini, monensiini, narasiini, salinomysiini ja antimysiini A, joille tyypillistä oli JC-1 väriaineen ilmaiseman korkean kalvopotentiaalin vähittäinen sammuminen; 3. jonomysiinin kaltaiset myrkkyaineet, joille tyypillistä oli siittiöiden lakkaaminen uimasta ilman, että niiden kalvopotentiaalissa näkyy muutosta.

Uutta mittausmenelmää testattiin erään ravintolisäkapselien käyttöön liittyneen epidemian yhteydessä (merileväkapseleita). SCA-mittaus paljasti kapselien sisällön vahvan myrkyllisyyden, jota ei tavanomaisilla, viranomaiskäytössä olevilla menetelmillä voitu todeta. Myrkkyvaikutus liittyi kapselin sisältöön, ei kuoreen. Testillä selvitettiin, että myrkyn tuottajia olivat bakteerit, jotka olivat kapselin sisällössä epäpuhtauksina. Niiden tuottama myrkkyaine oli gramisidiini-tyyppinen.

SCA-testiä sovellettiin myös kereulidia tuottavien *B. cereus* -bakteerien kulkureittien paljastamiseen elintarviketuotantoketjussa. Havaitsimme, että perunassa on luontaisesti kereulidia tuottavia bakteereja jo ennen perunan elintarvikkeeksi jalostamista. Tässä väitöskirjassa kerrotaan, miten kereulidin tuottajat voidaan tunnistaa tätä tuottamattomien *B. cereus* -bakteerityyppien joukosta viljelymaljalla. Koska kereulidin tuottajia esiintyy monissa tuotantokasveissa, kuten riisissä, ja niiden itiöt kestävät poikkeuksellisen hyvin kuumuutta, niitä löytyy myös teollisesti tuotetuissa valmisteissa ja puolivalmisteissa. Tämä asia on tärkeä ymmärtää, jotta kereulidin tuottajien läsnäolo osataan ottaa huomioon ja pystytään minimoimaan niiden lisääntyminen tuotantoprosessin aikana. Kereulidia tuottavia *B. cereus* -bakteereja esiintyy luultavasti lähes kaikissa kasveissa, koska niitä löytyi kuusien (*Picea abies*) sisältäkin. Kuusihiokkeesta valmistetun sulpun sisältämistä kereulidin tuottajista n. 5 % kulkeutui valmiiseen paperiin saakka. Tutkimme simulointitestien avulla, siirtyykö kereulidi pahvista elintarvikkeeseen. Havaitsimme, että kulkeutumista tapahtui pahvista rasvaiseen elintarvikkeeseen mutta ei kuumaan tai kylmään juomaan.

Osa elintarvikkeista päätyy yhteiskunnassa biojätteeksi. Koska biojätteen ravinteet pitäisi saada kiertoon, peltojen lannoitteeksi, selvitimme kereulidia tuottavien *B. cereus* -bakteerien käyttäytymistä biojätteen kuumennus- (24 min, 137˚C) ja mädätysprosesseissa. Mittausmenetelminä oli SCA-, viljely- ja PCR-tekniikka (DNA tunnistus). Totesimme, että n. 10 % prosessin läpi selvinneistä *B. cereus* -bakteereista oli kereulidi-geenin suhteen positiivisia eli todennäköisiä tuottajia. Tulokset, jotka saatiin täysimittaisella biojätteen teollisen käsittelyn laitteistolla, eri työvaiheineen osoittivat, että kereulidia tuottavat *B. cereus* -bakteerit ovat osa luontoa, eivätkä ne ole tekniikan keinoin hävitettävissä elintarvikkeista.

Koska siittiöt osoittautuivat tehokkaiksi ilmaisemaan kereulidin solumyrkyllisyyttä, halusimme selvittää, mikä on elimistön muiden solutyyppien

kereulidi-herkkyys. Käytimme testisoluina ihmisen, sian ja hiiren primaarisoluja ja solulinjoja. Mikroskoopilla ja ionielektrodeilla havaittiin, että solut vammautuivat kun ne altistuivat 24 tuntia kereulidi-pitoisuudelle 0.4–2 ng ml^{-1}. Tulokset osoittivat, että nisäkkään, kuten ihminen, monet eri kudos- ja solutyypit ovat herkkiä kereulidin myrkkyvaikutuksille. Tärkein soluvammojen syntyreitti oli ehkä kalium-ionien massiivinen vuoto ulos soluista, jonka kereulidi aiheutti ihmisen veren yksitumaisissa valkosoluissa ja ihon keratinosyytteissä jo muutaman minuutin altistuksen seurauksena.

Tässä työssä kehitetyllä SCA-menetelmällä voidaan tutkia vaikeasti tuhottavia bakteereja ja niiden toksiineja elintarvikkeissa. Menetelmällä havaitaan mitokondrioille myrkylliset aineet nopeasti ja luotettavasti. Lisäksi käytettiin maljausmenetelmää, jolla voidaan löytää kereulidin tuottajat.

Koska kereulidia tuottavien *B. cereus* -bakteerien tuhoaminen elintarvikkeesta on mahdotonta, on tärkeää pitää tuotteiden säilytysolosuhteet sellaisina, että kereulidin tuotto estyy.

1. Review of the literature

1.1 Introduction

Everything needs to go faster and be faster. In times of economic crisis the consumables also need to be cheaper. The world seems to be turning faster and everything needs to keep up, like food needs to be ready faster, we have no time to prepare it, we need the time to eat it. Many shops sell ready-to-eat foods which you warm up in a microwave. Since microwave warming up neither sterilizes nor pasteurizes the food, this fast way of food preparation and consumption comes hand in hand with a need to introduce new ways to ensure microbiological safety. Food needs to be edible and not harmful for the consumer. In days when more time than nowadays was invested in preparing the meals, potatoes (20 min) and vegetables (15 min) were boiled and meats were cooked or baked on the same day when these foods were consumed.

Heating of the food stuffs may be sufficient to inactivate the vegetative microbial cells; nevertheless the heat resistant microbial spores are likely to survive (Carlin *et al.* 2006). Currently meals and cooked but not sterilized food ingredients may be shelved for years (for instance: infant meals) and only need to be warmed up before consuming. In an industrial process, the load of microorganisms in a food will be effectively reduced when repeated steps of heating are applied, resulting in a shelf life of ~2-3 years at room temperature, when combined with vacuum, modified atmosphere packaging or reduced water activity (a_w). Nevertheless, problems may be caused by heat resistant endospore forming bacteria and/or by producers of heat stable toxin(s) (Table 1).

Table 1 Heat stable toxins and their producers linked to food borne illness (Summarized from Rajkovic 2014)

Toxins by producers	Heat stability of the toxin
Staphylococcal enterotoxin of *Staphylococcus aureus*, enterotoxins	2 min at 80 °C (staphylococcal enterotoxin A, as staphylococcal enterotoxin B is more heat stable than the staphylococcal enterotoxin A)
Cereulide of *Bacillus cereus*	80 min at 121 °C
Enterotoxin from *Clostridium perfringens* (CPE)	5 min at 60 °C
Botulin toxin of *Clostridium botulinum*	10 min at 80 °C

B. cereus is readily isolated from ready-to-eat foods (Rosenquist *et al.* 2005, Wijnands *et al*. 2006, Meldrum *et al*. 2009, Kotzekidou, 2013). An increase in consumption of these foods over the past decades did not yet result into an unacceptable increase of reported cases of food poisoning, possibly because of underreporting of the mild cases involving *B. cereus.* Or, maybe because the emetic type of food poisoning may be mistakenly reported as food poisoning caused by *Staphylococcus aureus* and the diarrheal type of food poisoning misinterpreted as *Clostridium perfringens* intoxication (Shinagawa, 1990).

In 2011, within the European Union 220 food-borne outbreaks were recorded as being caused by *Bacillus* toxins (3.9 % of the total 5648 reported food-borne outbreaks) (EFSA, 2013). Severe and even fatal cases of food borne illness by *B. cereus* have sporadically been reported in several countries (Naranjo *et al.* 2011, Shiota *et al.* 2010, Pósfay-Barbe *et al*. 2008, Dierick *et al*. 2005, Mahler *et al.* 1997, Dirnhofer *et al*. 1977, Takabe and Oya 1976, Temper 1963).

For reliable reporting, it is essential to be able to rapidly distinguish between the cases involving *Staph. aureus* heat stable protein toxin and those involving the peptide toxin of *B. cereus.* Currently this distinction is based on plating on specific agar media, selective chromogenic medium bacara or MYP for *B. cereus*,

incubating for 24 h (Talent *et al*. 2012) and Baird-Parker for *Staph. aureus*, incubating 45-48 h (Bennett and Lancette 2001). One should ask if 24 h is fast enough to detect *B. cereus*, when children have died within 13 h (Dierick *et al*. 2005) and 6½ h (Shiota *et al*. 2010) after consuming the food contaminated with emetic toxin producing *B. cereus*.

1.2 *Bacillus cereus*

1.2.1 Bacillus cereus group

The *Bacillus cereus sensu lato* is a species in the genus *Bacillus*. The genus *Bacillus* was established in 1872 by Ferdinand Cohn (Logan and de Vos 2009). *Bacillus* is a genus within the phylum Firmicutes (Logan and de Vos 2009). Ferdinand Cohn renamed the endospore forming aerobic Gram positive rod *Vibrio subtilis* as *Bacillus subtilis* (Harwood, 1989). The first described species, *Bacillus subtilis*, is the type species of this genus (Harwood, 1989). The genus *Bacillus* is one of the largest and most diverse among the Firmicutes genera, consisting presently (as of May 22nd, 2014) of 288 validly described species (Euzéby 2014). Of the *Bacillus species*, 7 belong to the *Bacillus cereus* group: *B. anthracis, B. thuringiensis, B. mycoides, B. pseudomycoides, B. cereus, B. cytotoxicus* and *B. weihenstephanensis* (Guinebretière *et al*. 2013). These members of the *B. cereus* group are distinguished from each other based on biochemical characters as shown in Table 2.

B. thuringiensis produces insecticidal δ-endotoxins and is used as an insecticide to protect farm plants (discussed by Vachon *et al*. 2012). The most dangerous member of the *B. cereus* group is the human pathogen *Bacillus anthracis*, causing anthrax in humans and herbivorous mammals (reviewed by Beyer and Turnbull 2009). The second pathogen is *B. cereus sensu stricto*, mostly linked to food

poisoning cases (Essen *et al*. 2000, Bennett *et al*. 2013, Agata *et al*. 2002, Kamga Wambo *et al*. 2011).

Toxins produced by members of the *B. cereus* group are compiled in Table 3. The strain *Bacillus cereus var. toyoi* is used as a probiotic in animal feed. It contributes to a better feed conversion (Williams *et al*. 2009).

Table 2 Differential characteristics of the species in *Bacillus cereus* group by biochemical traits (adopted from Logan and De Vos, 2009; Guinebretière *et al*. 2013; Goodwin *et al*. 1994)

Bacillus species	Motility	Rhizoid colonies	Parasporal crystals	Starch hydrolysis	Tyrosine degradation	Haemolysis	Glycogen	Salicin fermentation
B. anthracis	-	-	-	+	-	-	+	-
B. cereus sensu stricto	+	-	-	+[#]	+	+[#]	+	d[*]
B. weihenstephanensis	+	-	-	+	d	+	+	d
B. mycoides	-	+	-	+	+	+	+	d
B. pseudomycoides	-	+	-	nd	+	+	+	nd
B. thuringiensis	+	-	+	+	+	+	+	d
B. cytotoxicus	+	-	-	-	nd	nd	-	+

d different strains give different outcomes
nd no data
excepting most cereulide producers
* negative for cereulide producers

Table 3 Pathogenic substances in the *B. cereus* group (from Logan and de Vos 2009)

***Bacillus* species**	**Toxin(s)**	**Encoded by plasmid(s) or chromosomal**	**Reference**
B. anthracis	lethal factor, edema factor, protective antigen	plasmids pXO1, pXO2	Pilo & Frey 2011
B. cereus sensu stricto	hemolysin BL non-hemolytic enterotoxin enterotoxin FM cytotoxin K cereulide	 270 kb plasmid pCER270	 Hoton *et al.* 2009
B. weihenstephanensis	cereulide	large plasmid or chromosomal	Thorsen *et al*. 2006, Hoton *et al.* 2009
B. mycoides	none reported		
B. pseudomycoides	none reported		
B. thuringiensis	δ-endotoxins (Cry and Cyt toxins) 18 bacteriocins vegetative insecticidal proteins	plasmid plasmid and some chromosomal	Fagundes *et al*. 2011 de la Fuente-Salcido *et al*. 2013 Sattar *et al.* 2008
B. cytotoxicus	cytotoxin K	not known	Guinebretière *et al.* 2013

1.2.2 History of food-borne illness linked to B. cereus

Bacillus cereus is traditionally seen as a soil inhabitant. Although when the species *B. cereus* was first described in 1887, it was isolated from air in a cow shed (Frankland and Frankland 1887). Plazikowski was one of the first in 1906 to associate *B. cereus* with food poisoning in Europe (cited by Jay *et al*. 2005). It was not until the early 1950s that it was shown to cause food poisoning. The Norwegian scientist Steinar Hauge isolated *Bacillus cereus* from a dessert that was implicated in food borne illness of many people. Dr. Hauge turned to the Koch postulates, prepared vanilla sauce, inoculated it with the isolated *B. cereus* culture and after 24

h of incubation he drank the spiked vanilla sauce. He became ill 13 h later (Hauge 1955).

Reports on occurrence of the heat stable toxin of *B. cereus*, the active agent of the emetic syndrome started to emerge in the 1970s in Europe and in the US (cited by Jay *et al.* 2005, Anonymous 1975). In the USA it was related to mashed potatoes and in Europe to rice dishes, mostly take away food (Taylor and Gilbert 1975). Onset of the emetic illness occurred within 6 h and in most cases involved no diarrhoea (reviewed by Granum 2007). Therefore, it was a different type of illness compared to what Hauge described in 1955.

1.2.3 B. cereus in food

B. cereus is widespread in the environment. It has been isolated from plants, food crops and foods (Table 4). When ingested it rarely causes reportable illness. *B. cereus* does not spoil taste or consistency of the food, but it can cause serious illness, even death to consumers of the contaminated food (Takabe and Oya, 1976, Dirnhofer *et al*. 1977, Mahler *et al*. 1997, Dierick *et al*. 2005, Pirhonen *et al*. 2005, Kim J-B *et al*. 2010).

B. cereus is able to produce toxic metabolites and it produces heat stable spores (see persistence properties 1.4.1). One of the metabolites is cereulide, a heat stable peptide. The diarrheal toxins are proteins and assumed to be produced in the gut where they cause illness.

1.3 Toxic metabolites produced by *B. cereus* implicated in food borne illness

1.3.1 Heat stable toxin, cereulide

Certain strains of *B. cereus* can produce an emetic toxin, cereulide, which has been implicated in serious and fatal cases of food poisonings (Mahler *et al*. 1997, Dierick *et al*. 2005, Naranjo *et al.* 2011). These cereulide producing strains have a pXO1-like megaplasmid which encodes the cereulide synthetase (*ces*) NRPS (Ehling-Schulz *et al.* 2006). The heat stable toxin of *B. cereus* is a small cyclic depsipeptide, with a molecular formula of $C_{57}H_{96}O_{18}N_6$ with a m/z 1152 (Figure 1) and structure of (-OLeu-Ala-OVal-Val-)$_3$ (Agata *et al*. 1994), highly lipophilic (log K_{ow} of 5.96) and an ion carrier specific for potassium (Teplova *et al*. 2006). It is represented by two homologous molecules, cereulide and homocereulide (m/z 1166, Rasimus *et al*. 2012). Cereulide passes the mammalian cell membrane and dissipates the mitochondrial membrane potential, causing mitochondrial dysfunction (Mikkola *et al*. 1999). Cereulide was inactivated when 0.5 µg ml^{-1} cereulide was exposed to steam at 121 °C for > 20 min at pH > 9.5, whereas at pH 7 the cereulide retained its toxic activity for > 2 h when steam heated at 121 °C (Rajkovic *et al*. 2008).

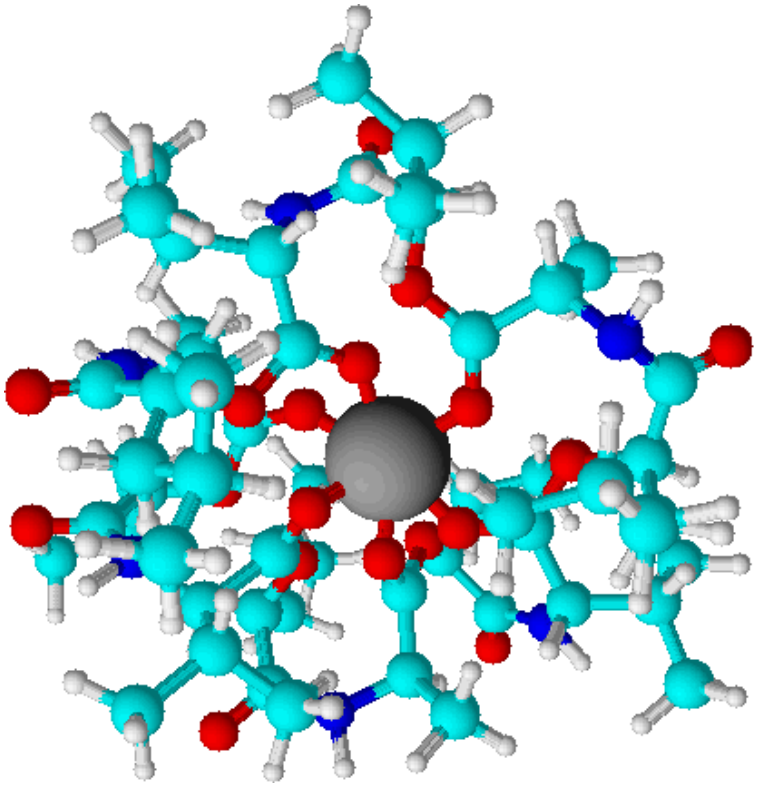

Figure 1. Cereulide. Cyan indicate carbon, red oxygen, blue nitrogen, white hydrogen and grey potassium (the picture was kindly provided by Dr R. Mikkola)

1.3.2 Diarrheal toxins

B. cereus produces several diarrheal toxins: hemolysin BL, enterotoxin FM, non-hemolytic enterotoxin and cytotoxin K. Producers of these diarrheal toxins have been isolated from foods (Kim *et al*. 2011, Batchoun *et al*. 2011, Granum 2007), but only three protein enterotoxins (non-haemolytic enterotoxin, haemolysin BL and cytotoxin K) have been implicated in food poisoning and were often connected to foods like pasta, desserts and milk (for a review see Granum 2007). Symptoms of the diarrheal illness start 10-24 h after ingestion (average 12 h).

The diarrheal toxins are proteins which can be inactivated by heating to 55 °C for 20 min (Turnbull *et al*. 1979).

Table 4. Examples of sites where *B. cereus* has been reported

Habitat	Reference
Endophytes and epiphytes	
Norway spruce tree (*Picea abies*)	Hallaksela *et al*. 1991
broad-leaved dock (*Rumex obtusifolius*)	Collier *et al*. 2005
yellow-poplar (*Liriodendron tulipifera* L.)	Mikluscak and Dawson-Andoh 2004
apple leaf epiphyte, potato leaf, tomato leaf, quinoa leaf, cacao plants, lamb's quarters root (*Chenopodium album*), faba bean root and leaf	Melnick *et al*. 2012
figs	Hormazábal *et al*. 2004
peach	Mikami *et al*. 1994
Curly kale leaves	Thorsen *et al*. 2006
Food crops	
vegetable(s)	Wijnands *et al*. 2006
Farm environments, milk, milk products	
free stall bedding	Magnusson *et al*. 2007
milk (raw, UHT heated and pasteurised, milk powder)	Bartoszewicz *et al*. 2008, Ruusunen *et al*. 2013, Reis *et al*. 2013
dried milk products, milk with rice, flan, milk pudding, milk substitute, milk – cereal – rice, semolina with milk, mousse	
milk powder	Reyes *et al*. 2007
ice cream	Arslan *et al*. 2014, Messelhäusser *et al*. 2010
evaporated whey	Pirttijärvi *et al*. 1998
pasteurized milk filling machine	Eneroth *et al*. 2001
milk, milk products	Wijnands *et al*. 2006
Retail and restaurant foods	
retail foods marketed in Thailand	Ananchaipattana *et al*. 2012
food products marketed in Belgium	Samapundo *et al*. 2011
food samples collected in Morocco	Merzougui *et al*. 2014
US rice	Ankolekar *et al*. 2009
Chinese-style fried rice in Sri Lanka	Perera and Ranasinghe 2012
rice dishes in Belgium	Delbrassinne *et al*. 2012
ground roasted coffee	Chaves *et al*. 2012
rice and cereals in Korea	Park *et al*. 2009
vegetable puree (broccoli, carrot, courgette, split peas, potato)	Choma *et al*. 2000
poultry products sold in India	Sudershan *et al*. 2012
oil(s), fat(s), oil and fat products, fish, fish products, meat, meat products, ready-to-eat foods, vegetable products and pastry	Wijnands *et al*. 2006

rice and sunsik in Korea	Kim SK *et al*. 2009
Spices and additives	
flavorings	Wijnands *et al*. 2006
dried red pepper	Kim SK *et al*. 2009
Soil and soilless environment	
rice field soil	Ueda and Kuwabara, 1993.
surface soil	Hendriksen *et al*. 2006
pozzolana grains from a filtration unit in soilless culture tomato greenhouse	Déniel *et al*. 2004, Renault *et al*. 2007

1.4. Importance of spores

1.4.1. Persistence properties of B. cereus spores

B. cereus spores have a hydrophobic surface and as a result they adhere to steel and plastic surfaces (Faille *et al*. 2002, Granum 2002, 2007). Carlin *et al*. (2006) showed that *B. cereus* spores have a thermal D value at 90 ºC of ≥ 29 min and higher depending on the strain. Examples of harsh conditions where *B. cereus* spores were reported to persist or to survive are listed in Table 5. Préstamo *et al*. (2001) showed that a hydrostatic pressure of 1000 MPa for 15 min only reduced *B. cereus* viability to 40%. In the food and beverage processes 1-2 % w/v solutions of sodium hydroxide is often used in cleaning-in-place (CIP) protocols (Faille *et al*. 2013). Eighty to 99 % of the attached spores could effectively be detached from stainless steel coupons using a combination of mechanical and chemical action, pressure (4 Pa) combined with 0.5 % sodium hydroxide at 60 °C (Faille *et al*. 2013).

Table 5 Persistence properties of *Bacillus cereus* spores.

Showing conditions necessary to survive or not to survive

Condition	Reference
Survived	
steam boxes (80 °C), washing machine	Dohmae *et al*. 2008, Hosein *et al*. 2013
washing machine (71°C, 25 min is CDC guideline* and the disinfectant used was a quaternary ammonium compound or bleach)	Sifuentes *et al*. 2013
hydrostatic pressure (1000 MPa, 15 min at 20 °C)	Préstamo *et al*. 2001
washing machine (80 °C, > 10 min, requirement by Japanese law)	Sasahara *et al*. 2011
70% alcohol solution, 60 min	Turabelidze *et al*. 2013
pulsed light treatment (0.3 to 1.8 J cm^{-2} under an input voltage of 2.5 kV, 100 nm to 1100 nm)**	Planchon *et al*. 2011
antibacterial dishwashing liquid (0.05 %)	Kusumaningrum *et al*. 2002
Killed (> 90% reduction)	
skin disinfecting solutions povidone iodine solution (0.5 % wv iodine), savlon (1.5 % v/v chlorhexidine gluconate solution and 3.0 % cetrimide solution), "spirit" (70 % isopropyl alcohol)	Patel *et al*. 2013
heat resistance (D value at 90 °C ≥ 29 min)	Carlin *et al*. 2006
mechanical and chemical CIP (0.5 % aqueous sodium hydroxide and 4 Pa)	Faille *et al*. 2013

* Sehulster and Chinn (2003) ** tested with spores produced at 30 °C

1.4.2. Germination properties of Bacillus spores

The outgrowth of a spore to a vegetative cell is called germination. Most of the published papers on germination of *Bacillus* spores deal with the species *B. subtilis* (Setlow 2003, Moir 2006). Germination of the endospores is initiated when in the close environment germinants are detected, germinants such as amino acids, sugars or purine nucleosides, but the water activity (a_w), pH and temperature should be favouring bacterial growth. The germinants interact with the germinant receptors located behind the outer coat and cortex layers of the *Bacillus* spore (Setlow 2003). Subsequently the spore membranes increase in fluidity and monovalent cations (including H^+, K^+ and Na^+) are released from the spore core into the medium followed by release of Ca^{2+} and dipicolinic acid (Moir 2006). Subsequently the core becomes rehydrated, the peptidoglycan spore cortex is released and further uptake of water as well as expansion of the cell wall will take place. When protein mobility is returned, this allows enzyme action and the spore can germinate into a growing cell (Setlow 2003). Onset of the germination is measured by reading the optical density of the spore suspension at 600 nm. A drop of the optical density indicates germination.

De Vries (2006) showed that germinants (L-alanine or inosine or a combination of these two) affected *B. cereus* spores differently. Sixteen strains were analysed for the speed of germination with and without prior heat treatment and adding either one of the germinants or a combination of the two. The heat activated spores of 11 out of 16 strains responded to L-alanine (0.1 mM) and inosine (1 mM) by an OD_{600} decrease of > 45 % in 40 min, indicating a fast germination. When only one germinant was used (L-alanine or inosine) between one to four strains could germinate quickly. Only the *B. cereus* type strain (ATCC 14579) germinated rapidly under all tested conditions. Table 6 shows treatments to inhibit or promote germination.

Table 6 Germination of *Bacillus cereus* spores. Promoting and inhibiting germination conditions

Promoting germination	**Strains studied**	**Reference**
mild heat treatment (10 min at 70 ºC)	*Bacillus cereus* ATCC 14579^T	Den Besten *et al.* 2012
hypochlorite treatment	*Bacillus cereus*	Dohmae *et al*. 2008
L-alanine, inosine and heat activation	NIZO B436, NIZO B439, PAL2, PAL3, PAL5, PAL17, PAL18, PAL22, PAL25, PAL27, 61, 72, 43-92, 1230-88, F450183, ATCC 14579^T	Y de Vries 2006
Inhibiting germination		
temperature ≤ 10 °C	Emetic, diarrheal, clinical, food and environmental (n = 100)	Carlin *et al*. 2006
3 mM undissociated sorbic acid	*Bacillus cereus* ATCC 14579^T	van Melis *et al*. 2011
soya bean tempe extract	*B. cereus* ATCC 10987, NIZO B437, PAL20, PAL28, 55, *Bacillus weihenstephanensis* DSM11821^T and *B. subtilis* B20010	Roubos -van den Hil *et al*. 2010
supercritical CO_2 + ethanol	*Bacillus cereus* (KCTC 1012)	Park *et al.* 2013

1.5 Importance of mitochondria for the mammalian cell

Mitochondria (Figure 2) are called the power plants of the cell because through oxidative phosphorylation the mitochondria generate 80- 90 % of the ATP required for the mammalian cell to function (Wojtczak & Zablocki 2008). The first step of oxidative phosphorylation is the oxidation which takes place in the protein complexes I-IV (Figure 2B). Protein complexes I and II transfer electrons to ubisemiquinone (Q in figure 2B) which transports electrons to protein complex III. Cytochrome c transfers electrons to protein complex IV (Duchen, 2004). The redox reactions in these protein complexes create a proton motive force which is used to take the second step in the production of ATP. To synthesise one ATP it is estimated that three or four protons need to be translocated (Stock et al 1999). The processes (protein complexes I-V) are coupled as ATP generation requires the proton-motive force. Substances affecting this coupling are called uncouplers and they affect mitochondrial function.

The mitochondria also play an essential role in apoptosis (Wojtczak & Zablocki 2008).

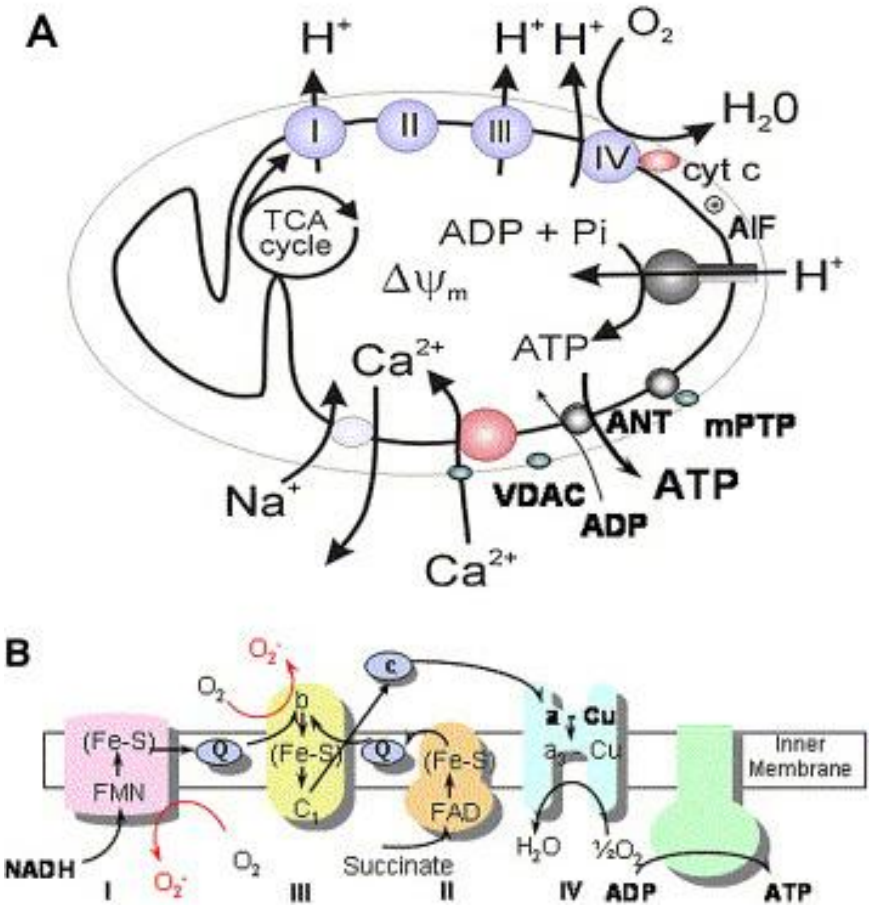

Figure 2 Oxidative phosphorylation

Panel A shows the functional components of the mitochondrion. Panel B shows the complexes in a more detailed scheme (Duchen 2004). ANT, Adenine Nucleotide Translocase; AIF, Apoptosis Inducing Factor; VDAC, Voltage Dependent Anion Channel; mPTP, Mitochondrial Permeability Transition Pore; TCA cycle, Tricarboxylic acid cycle.

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1.5.1 Mitochondria as a vulnerable cellular organelle

1.5.1.1 Mitochondria as target for pharmaceuticals

Exposure to substances adversely affecting mitochondria may be harmful for humans (Table 7). Iatrogenic mitochondrial toxic substances have been implicated in diseases such as ischaemia, diabetes, several neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and cancer (compiled by Scatena 2012, Duchen 2004). Currently, therapeutic pharmaceuticals are being reinvestigated to assess the risk for mitochondrial dysfunction. Dysfunction can be the primary effect of the drug or side effect, examples shown in Table 7 (from Szewczyk and Wojtczak 2002).

Table 7. Examples of drugs identified as harmful to mitochondria

Class of the drug	Therapeutic use	Adverse effect	Reference
nonsteroidal anti-inflammatory drugs (e.g. pirprofen, ibuprofen)	arthritis	inhibition of fatty acid β-oxidation	Mehta *et al*. 2008
antianginal drugs, treatment for myocardial ischemia (e.g. amiodarone)	controlling cardiac arrhytmias	affects thyroid, lung pulmonary, liver	Mehta *et al*. 2008
antidepressant drugs (nefazodone, trazodone, buspirone)	blocking the serotonine uptake	hepatotoxicity	Dykens *et al*. 2008
antidiabetic drugs (glibenclamide, glibonuride, glisoxepide)	to increase the insulin level in blood	mito K_{ATP}channel was blocked	Skalska *et al*. 2005

1.5.1.2 Mitochondria as target for microbially produced toxins

Several microbially produced mitochondrial toxic substances and their producers are found in the environment, food, food crops or indoor (air) environments (Andersson 1999).

In the pharmaceutical industry the tools used for assaying mitochondrial toxicity of synthetic chemical substances include *in vitro* assays, measurement of the reactive oxygen species (ROS), the membrane potential and intracellular ATP (Table 8).

Table 8. *In vitro* mitochondrial toxicity assays used in the pharmaceutical industry

Bioindicator	What and how	Reference
HepG2 cells (human liver carcinoma cell line)	cellular ATP content using CellTiter-GloCell Viability Assay	Dykens *et al*. 2008
isolated rat liver mitochondria	respiration, using oxygen sensitive probe	Dykens *et al*. 2008
human hepatocyte imaging assay	1. mitochondrial membrane potential using tetramethyl rhodamine methyl ester 2. nuclei and lipids DNA using 1,5-bis[[2-(dimethylamino)ethyl]amino]-4,8-dihydroxyanthracene-9,10-dione 3. reactive oxygen species (ROS) using 5-(and-6)-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate acetyl ester	Dykens *et al*. 2008
PBMC	thymidine kinase 2 (TK2) and R2 subunit of ribonucleotide reductase (P53R2). qRT-PCR, Western blotting	Liu *et al*. 2013
HepG2 cell	mitochondrial membrane potential using tetramethyl rhodamine methyl ester, plasma membrane permeability (TO-PRO-3), nuclear morphology using Hoechst33342	Tsiper *et al*. 2012

1.6 Methods for the detection of heat stable mitochondrial toxin(s) produced by *B. cereus*

1.6.1 Bioassays for the emetic B. cereus toxin

In 1976 the first toxin detection method was published for detecting the emetic toxin of *B. cereus*. This involved a test using whole animals, executed using rhesus

monkey (Melling et al. 1976). Table 9 compiles the currently described *in vitro* and *in vivo* methods for the detection of *B. cereus* emetic toxin. The emetic *B. cereus* toxin *in vivo* testing was long delayed because rodents proved to be insensitive in feeding tests towards this toxin (Yokoyama *et al*. 1999).

Table 9 Methods for the detection of the emetic toxin of *B. cereus*

***in vitro* methods**	**Reference**
HEp2 cell assay	Hughes *et al*. 1988, Mikami *et al*. 1994
Boar sperm motility inhibition assay	Andersson *et al*. 1997, Andersson *et al*. 2004
***in vivo* methods**	
Rhesus monkeys (*Macaca mulatta*)	Melling *et al*. 1976
Asian house shrew (*Suncus murinus*)	Agata *et al*. 1995
Chemical assay	
HPLC	Agata *et al*. 1994, Mikkola *et al*. 1999
LC/MS	Häggblom *et al*. 2002

The HEp-2 cell (human carcinoma of the larynx) assay was introduced in 1988 to replace the animal (monkey) feeding test (Hughes *et al*. 1988). The Asian house shrew was introduced in 1995 as a reliable test animal for detection of the emetic toxin (Agata *et al*. 1995). The boar sperm motility assay was the second *in vitro* test, introduced in 1997, to detect heat stable toxins in bacterial methanol extracts (Andersson *et al*. 1997). A year later it was the tool used for quantifying the mitochondrial toxic substance cereulide (Figure 3, Andersson *et al*. 1998).

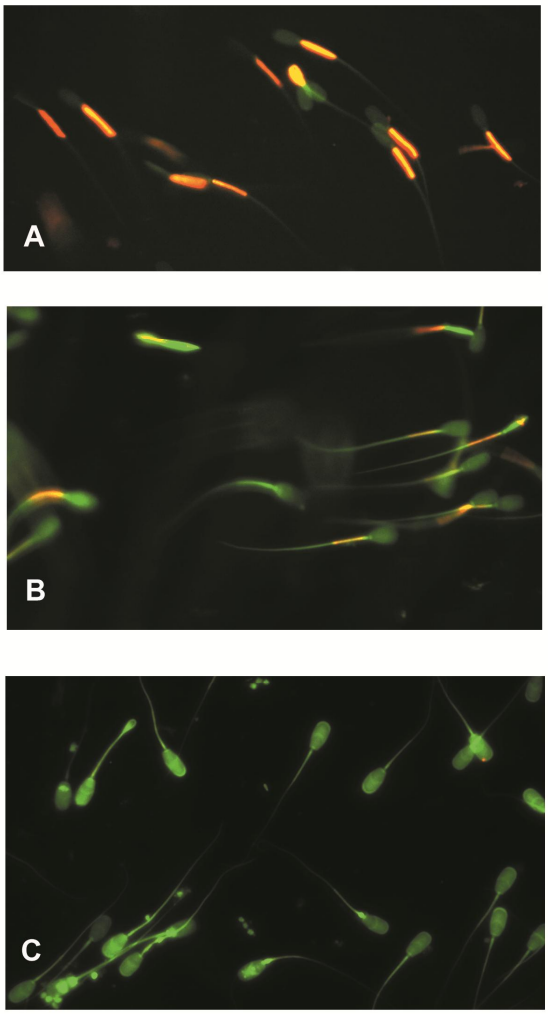

Figure 3. Fluorescence micrographs of boar sperm cells exposed to cereulide for 24 h and then stained with the fluorophoric dye JC-1, a dye that changes fluorescence emission in response to decreasing $\Delta\psi$, by a shift from orange to green. Panel A, spermatozoa exposed to 0 ng cereulide per ml. Panel B exposed to 5 ng cereulide per ml. Panel C exposed to 50 ng cereulide per ml. The sperm head is ca. 8 µm by 4 µm

1.6.2 Chemical assays for cereulide

Chemical methods for detecting the toxin cereulide using HPLC were described by Agata *et al*. (1994), Mikkola *et al*. (1999) and a quantitative LC/MS assay by Häggblom *et al*. (2002).

1.6.3 Detection of cereulide synthase genes

Cereulide is not a protein, but rather a molecule consisting of amino acids and hydroxyl acids linked by alternating ester bonds and peptide bonds.

The genes responsible for the biosynthesis of cereulide were first identified in 2004 (Toh *et al*. 2004, Horwood *et al.* 2004, Ehling-Schulz *et al*. 2005). Since then, specific PCR primers have been developed for the genes responsible for the biosynthesis of cereulide, as listed in Table 10.

Table 10. PCR methods for detection of the cereulide genes

Target gene	**Primer identification**	**Primer sequence**	**Reference**
*Ces*A	CER1 EMT1	ATCATAAAGGTGCGAACAAGA AAGATCAACCGAATGCAACTG	Horwood *et al*. 2004
*Ces*B	EM1-f EM1-r	GACAAGAGAAATTTCTACGAGCAAG TACAAT GCAGCCTTCCAATTACTCCTTCTGCC ACAGT	Ehling-Schulz *et al*. 2004
NRPS	BE-f BE-r	ACTTAGATGATGCAAGACTG TTCATAGGATTGACGAATTTT	Toh *et al*. 2004
Ces	CesF1 CesR1 CesR2	GGTGACACATTATCATATAAGGTG GTTTTCTGGTAACAGCGTTTCTAC GTAAGCGAACCTGTCTGTAACAACA	Ehling-Schulz *et al*. 2005

2. Aims of the study

1. Upgrade the boar spermatozoa motility inhibition *in vitro* bioassay to detect toxic substances targeted to mitochondria, simple to perform and widely applicable.
2. Determine the applicability of the *in vitro* assay to food stuffs as well as bacterial cultures.
3. Use the assay to track the environmental reservoirs of emetic toxin producing *Bacillus cereus* and their potential ports of entry into food processes and food packaging.4. Assess the potential for the presence of cereulide producing *B. cereus* in liquid fertilizer processed from biowaste and its possible concerns for organic farming.
5. Evaluate the adverse effects of cereulide on mammalian primary cells and cell lines.

3. Materials and methods

The methods used by the author of this study are described in the publications, as shown in Table 11.

Table 11. Methods used in this thesis

Method	**Described in Paper**
Toxicity assay	
Sperm Combi Assay	I
Rapid sperm motility inhibition assay	V
Methods for extraction/leaching of the toxin	
Methanol extraction	II
Mechanical extraction	III

Ethanol leaching	III
Chemical assay for quantification of cereulide	
LC/MS analysis	V, III
Bioassay for toxic effects of cereulide	
Fluorogenic dyes for visualizing the membrane potential ($\Delta\psi$) and intactness of the cell membrane: JC-1, PI Viability staining with calcein-AM	I I
Methods for identification of *B. cereus*	
Automated ribotyping	V
PCR with primers specific for *B. cereus*	IV
PCR method for verifying the presence of the cereulide synthethase gene(s)	
PCR primers specific for *ces*B	IV

4. Results and Discussion

4.1 Detection of mitochondrial toxicity using eukaryotic cells as indicators

We extended the boar sperm motility inhibition assay initially described by Andersson *et al*. (1997) to make it applicable for assessing the presence of mitochondrial toxic substances, known and unknown and microbial metabolites in composite matrices. Boar spermatozoa were shown to be useful for this purpose because the rapid sperm motility mainly depends on intact mitochondrial function (Andersson 1999). Mitochondria are located in the proximal part of the sperm tail

(Figure 4). The porcine sperm cell contains around 70 mitochondria (Andersson 1999) and damage to the mitochondria results to cessation of sperm motility.
A big difference between human, bovine sperm cells and boar spermatozoa is that boar spermatozoa are essentially unable to support rapid motility by anaerobic glycolysis and depend to a much greater degree on oxidative respiration than e.g. human sperm cells (Pasupuleti, 2007).

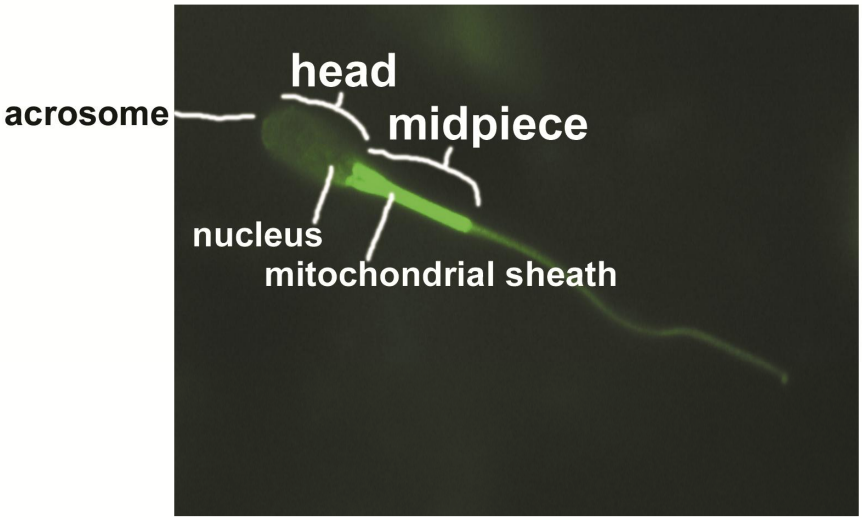

Figure 4. Microscopic view of a boar sperm cell showing the membranes, mitochondria and sperm head. The sperm head is ca. 8 µm by 4 µm.
(the picture was kindly provided by Dr C. Constantin-Apetroaie).

To distinguish between cellular damages affecting or not affecting the mitochondria we used the membrane potential responsive fluorescent stain 5,5´,6,6´-tetrachloro-1,1´,3,3´-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1). A down shift in the mitochondrial membrane potential is visible as the change of the fluorescent emission of JC-1 from bright orange to green (Fig 1A and 1D in

Paper I). The electric potential across the mitochondrial inner membrane is 180 to 200 mV (reviewed by Wojtczak and Zablocki, 2008), created by a proton gradient.

Staining with propidium iodide combined with calcein AM was used to reveal the permeability barrier status of the cell membrane. Uptake of propidium iodide (PI) by the cells indicates damage to the cell membrane permeability barrier. Calcein AM was used to show intactness of the cell membrane, as the cytoplasm fluoresces green when calcein is liberated from its acetoxymethyl ester by intracellular esterases. Green fluorescence in the cytoplasm indicates that the cell membrane is intact, preventing leak out of the calcein. When PI enters the cell, DNA (nucleus) will fluoresce red revealing that the plasma membrane permeability barrier had been lost.

In Paper I twenty substances were analysed for mitochondrial effects. Of these 18 were microbially produced and 12 were previously known to affect mitochondria (Table 12). Exposure of the sperm cells to each of the 20 substances affected the sperm motility; 14 substances also caused loss of the mitochondrial membrane potential visible in JC-1 stained cells. Six substances (cereulide, gramicidin, ionomycin, nigericin, oligomycin, valinomycin) affected the spermatozoan mitochondria, inside of an intact plasma membrane of cells, when exposed in extended boar semen to concentrations of ≤ 5 ng ml^{-1} within 4 d of exposure. This was shown by staining the cells with the live dead stain calcein AM and propidium iodide (Table 2 in Paper I). Gramicidin and cereulide depolarized the mitochondrial sheath inside the sperm cells at exposure concentrations of 0.5-1 ng ml^{-1} of extended boar semen (Table 2 in Paper I). Gramicidin and cereulide displayed different toxicokinetics: the time required to reach the maximal depolarizing effect was for cereulide 24 h and for gramicidin several days (4 d).

Combining the parameters obtained for motility inhibition, loss of $\Delta\psi_m$ and loss of the cell (plasma) membrane permeability barrier function, revealed three distinct modes of mitochondrial toxicity. First type: cereulide, valinomycin, a mixture of enniatin A, A_1, B, B_1 caused spotwise depolarisation of the mitochondrial sheath (Figure 3 B and Fig 1 C in Paper I). Valinomycin and cereulide reached their maximal toxic effect on the mitochondria (motility inhibited and $\Delta\psi_m$ lost) within 24 h, whereas with the enniatin mixture it took a much higher concentration and longer time, up to 4 d. Valinomycin and cereulide were $\geq$ 200 times more toxic towards the sperm cells than was the enniatin mixture (Table 2 in Paper I).

The second mode of mitochondrial toxicity was displayed by gramicidin (a mixture of A, B, C, D), antimycin A, calcimycin A23187, monensin, narasin, salinomycin and nigericin. These substances caused a gradual quenching of the JC-1 fluorescence in the mitochondrial sheath (Fig. 1B in Paper I). Antimycin A, calcimycin A23187, gramicidin and nigericin reached their full toxic potential within 24 h, whereas narasin, salinomycin and monensin, were after 4 d of exposure 1000 times more toxic than after 24 h (Table 2 in Paper I).

A third interesting mode of toxic action towards the spermatozoan mitochondria was observed with staurosporine, oligomycin (a mixture of A, B, C) and ionomycin. These substances terminated sperm motility at an exposure concentration 200-1000 fold below that where the mitochondrial membrane potential was dissipated. When exposed for 4 d to motility inhibiting concentrations of these substances, the mitochondrial $\Delta\psi$ was similar to that of none exposed sperm cells (Fig 1A in Paper I).

Table 12. Microbially produced mitochondrial toxic substances (compiled from Wong *et al*. 1977, Mikkola *et al*. 1999, Gräfe 1992)

Substance and the known biochemical activity	**Producing organism**	**Target in mitochondria (Fig 2)**	**Ionophoric affinity for**
Potassium ionophore			
cereulide	*Bacillus cereus*	complex V	$K^+ > Na^+ > H^+ > Ca^{2+}$ (Mikkola 1999)
enniatin	*Fusarium sp.*	complex V	$NH_4^+ > K^+ > Rb^+ > Na^+$
valinomycin	*Streptomyces tsusimaensis* *S. fulvissimus* *S. griseus*	complex V	$Rb^+ > K^+ >> NH_4^+$
salinomycin	*S. albus*	complex V	$K^+ > Na^+ > Cs^+ > Ca^{2+}$
Protonophore			
nigericin	*S. hygroscopicus*	complex V	$K^+ > Rb^+ > Na^+ > Cs^+ > Li^+$
gramicidin	*Brevibacillus brevis*		$H^+ > Rb^+ > NH_4^+ > K^+ > Na^+ > Li^+$
Calcium ionophore			
calcimycin	*S. chautreusensis*	complex V	$Mn^{2+} > La^{3+} > Ca^{2+}$
ionomycin	*S. conglobatus*		$Ca^{2+} > Mg^{2+} >> Sr^{2+}$
Sodium ionophore			
monensin	*S. cinnamonensis*	complex V	$Na^+ >> K^+ > Rb^+ > Li^+ > Ca^{2+}$
Ionophoric antibiotic			
narasin	*S. aureofaciens*	Complex V	$Na^+ > K^+ = Rb^+ > Cs^+ > Li^+$
Other			**Mechanism of action**
antimycin A	*Streptomyces sp.*	complex III	Inhibits electron transfer from cytochrome b to cytochrome c
staurosporine	*S. staurosporeus*	complex V	Inhibitor of protein kinase C
oligomycin	*Streptomyces sp.*	complex V	Inhibitor of the mitochondrial ATPase

4.2 Introducing the Sperm Combi Assay to test extracts prepared from cultured bacteria or from food

We tested a food supplement marketed as 'health promoting'. A sample of capsules containing seaweed had been sent to the Finnish Food Safety Authority EVIRA after consumers of the capsules had reported illness, repeated vomiting 30 min after consumption. By the time when the seaweed capsules were delivered to us, EVIRA had already investigated the capsules, applying standard food analytical methods by International Organization for Standardization (ISO) and Nordisk Metodik Kommittè for Livsmedel (NMKL) (Table 2 in Paper II). These tests involve culturing for 24 h. Slow growers, if present, would have been unlikely detected. We cultivated the capsule content on tryptic soy agar plates at room temperature for up to 20 d to include also the slow growing bacteria. Colonies showing antagonism towards co-growing bacteria or molds on the same plate were selected for analysis.

The capsule coat and the content (seaweed powder) were separately extracted with methanol and analysed using Sperm Combi Assay. We found that methanol soluble substance(s) from the seaweed powder, but not those from the capsule coat, inhibited motility and also depolarised the mitochondrial membrane potential of the sperm cells (Table 13). These events took place at exposure concentrations as low as 11 µg of seaweed extract d.wt. ml^{-1} in extended boar semen within 4 days (Table 1 in Paper II).

Extracts were also prepared from individual bacterial colonies subcultured from the extended culture plates of the seaweed content. These extracts were analysed for mitochondrial toxic substances using the spermatozoan motility inhibition assay. It was found that spore forming actinobacterial and endospore forming *Bacillus* isolates from the seaweed yielded extracts that inhibited sperm motility and

depolarised the mitochondrial membrane potential of the sperm cells (Table 4 in Paper II).

Extracts prepared from the capsule content indicated a distinct mode of action towards sperm cells (motility inhibition and depolarisation of the mitochondrial $\Delta\psi$, Table 13). We then saw a similar mode of action with extracts prepared from bacteria isolated from the capsule content (Table 13). We compared the mitochondrial effects to the known effects observed in Table 2 in Paper 1. An actinobacterial isolate "A" showed similarity to the cereulide type of toxicity. Other actinobacterial isolates, "B" and "C", showed similarity in the toxic response to gramicidin. Further actinobacterial isolates, D, E, F, revealed responses similar to ionomycin. The *Bacillus* isolate, "G", showed a response not seen in any of the 18 substances tested in paper I: it depolarised the $\Delta\psi$ of the plasma membrane as well as that of the mitochondria.

The Sperm Combi Assay thus enables a conclusion that several differently acting toxins and their producers were involved in the adverse health effects of the capsules. The results cited above also demonstrate the value of the sperm cell assay for distinguishing between the constituents of a commercial product from metabolites emitted by bacteria contaminating the product.

Table 13. Compilation of the toxic effects of the bacterial isolates and capsule content from paper II

	Tested toxicity outcomes		
Tested sample	**Motility inhibition**	**Δψ of plasma membrane**	**Δψ of mitochondria**
Capsule content of batches	+	no effect	depolarisation
E1 2242	+	no effect	depolarisation
E1 3037	+		depolarisation
E1 3038			
Spore forming actinobacteria	+	hyperpolarisation	depolarisation
"A"	+	no effect	depolarisation
"B" and "C"	+	no effect	no effect
"D", "E" and "F"			
Endospore-former "G" and "H"	+	depolarisation	depolarisation

4.3 Scientific and practical impact of the Sperm Combi Assay.

Since the year 2003 when Paper I, introducing the Sperm Combi Assay, was published the assay has repeatedly been used by other research groups to detect cereulide (Stenfors Arnesen *et al*. 2008, Rajkovic *et al*. 2007, From *et al*. 2007, Bauer *et al*. 2010). Our method has been compared to more recently introduced assays by Yamaguchi *et al*. (2013) and Stark *et al*. (2012). It has been applied to reference substances other than cereulide, enniatin (Uhlig *et al*. 2006, Ivanova *et al*. 2006, Jestoi 2008, Logrieco *et al*. 2008, Lee *et al*. 2008) and salinomycin (Boehmerle and Endres 2011). Paper I has also been cited showing the known effects of cereulide on sperm cell motility and the ionophoric properties (Rajkovic *et al*. 2006, 2007).

Since then, improvements for extraction of cereulide have been introduced by Yamaguchi *et al*. (2013) for extracting cereulide from foods and quantifying it with the LC/MS method. Synthetic cereulide has been used as an external standard by

Ueda *et al*. (2012) and Biesta-Peters *et al*. (2010) instead of valinomycin used by Häggblom *et al*. (2002). $^{13}C_6$ cereulide was used as an internal standard by Stark *et al*. (2012). The Sperm Combi Assay is semi quantitative, with a detection limit of 0.3 ng cereulide ml^{-1}. Our test additionally distinguishes at least 3 different types of mitochondrial toxicity which have not been reported as detectable by the other assays (as of 24.07.2014). Boar spermatozoa are commercially available in most countries and do not need advanced cell cultivation facilities to be maintained.

If a drug is found toxic for mitochondria there is a reason for re-evaluation of its safety. Mitochondrial toxicity is the main reason for withdrawal of drugs from the market (Dykens *et al*. 2008). The Sperm Combi Assay is a useful addition to the tests available for this purpose as it is fast and metabolically significant because it results in motility inhibition.

In further work the Sperm Combi Assay was used for screening, tracking and quantifying mitochondrial toxic substances produced by microorganisms. One of my colleagues could identify the toxin (Antimycin A) of a *Streptomyces* isolated from potato (Kotiaho *et al*. 2007). Other colleagues showed that also other toxic heatstable mitochondria toxic substances, besides cereulide, could be detected in extracts prepared from foods after an exposure of 1 h (Rasimus *et al*. 2012, Apetroaie-Constantin *et al*. 2009).

4.4 Where are the environmental reservoirs of cereulide producing *B. cereus*?

Bacillus cereus is widely distributed in the environment and has been isolated from almost everywhere (Table 4). However cereulide producing strains have so far been found in only a few environments: as endophytes in spruce, potatoes (This thesis Papers III, V), from peach fruit (*Prunus persica*, 5 out of 20 isolates, Mikami *et al*.

1994), from dried figs (Hormazábal *et al*. 2004) and from unhulled rice, chaff and rice bran (Ueda and Kuwabara, 1993).

Endophytic *B. cereus* is reported in plant materials: leaves, branches and tree roots (Mikluscak and Dawson-Andoh 2004, Melnick *et al*. 2012). Nevertheless, cereulide producers were so far reported from two species only: spruce trees (Hallaksela *et al*. 1991) and potato (in the present work). Being an endospore former, this species is likely to survive most kinds of industrial processing, for example processing timber into pulp and paper, or fermenting and drying cacao beans to manufacture chocolate products.

4.5 Migration of cereulide and the producing organism in industrial non-food processing

4.5.1 Migration during paper making

Paper III describes problems potentially arising from the presence of cereulide producing endophytic *B. cereus* when spruce wood is processed into pulp for paper making. We tracked the route of migration of viable *B. cereus* during the processing of paper pulp into packaging paper.

Migration of *B. cereus* and cereulide containing pulp slurry was simulated by spiking the wood pulp (Fig 1 in paper III, point A) with a known amount of an endophytic *B. cereus* strain originating from live Norway spruce (*Picea abies*). In parallel, similar pulp was spiked with an inactivated (autoclaved) spruce endophyte *B. cereus* NS58 (contained 90 µg of cereulide per 600 ml of pulp slurry). From this pulp we produced handsheets using a laboratory sheet former (Figure 1 in paper III). Five percent of the spiked cfu *B. cereus* were recovered from the handsheets,

two percent of the cfu as spores. Ten percent of the 90 µg cereulide spiked in the slurry was recovered from the handsheets.

If cereulide producing *B. cereus* are present in materials used to package food, the question arises whether the contained cereulide could leach from the package into the packaged food. We simulated the leaching of cereulide from the handsheets (2.9 µg cereulide per g of hand sheet) by the method described in the European Standards, EN 645:1993, EN 647:1993, EN 1186-15:2002. Ethanol was the surrogate for fatty foods.
The results showed that almost all cereulide present in the handsheets leached into ethanol but none into cold or hot drink. Therefore, if cereulide is present in the packaging material it may be expected to readily leach into fatty food. The high leachability into fatty foods is explicable by the high octanol/water partition coefficient, Log K_{ow} 5.96, of cereulide (see 1.3.1). Other chemicals with a similarly high Log K_{ow} (PCDD/PCDF, Urbaniak 2013) have been reported to readily migrate from the body of the paper board into the hydrophobic coating (polyethylene) of the paperboard (Kitunen and Salkinoja-Salonen 1991).

4.5.2 Fate of B. cereus and cereulide during industrial biowaste processing

Cereulide producing *B. cereus* are frequently found in many foods e.g. rice, pasta, ice cream and infant foods (Shaheen 2009, Messelhäusser *et al*. 2010). Therefore, cereulide producers will be present in food waste. We asked the question whether cereulide or cereulide producers would pass in an active state through thermophilic anaerobic bioprocessing of food waste at an industrial scale.

The flow sheet in Figure 5 explains the processing of municipal biowaste at an industrial size biowaste processing facility in Norway. The biowaste is processed into biogas and digestates (liquid and solid). The solid digestate fraction is mixed

with wood chips and further processed by composting and offered for sale. The liquid digestate is recycled as biofertilizer for organic farming.

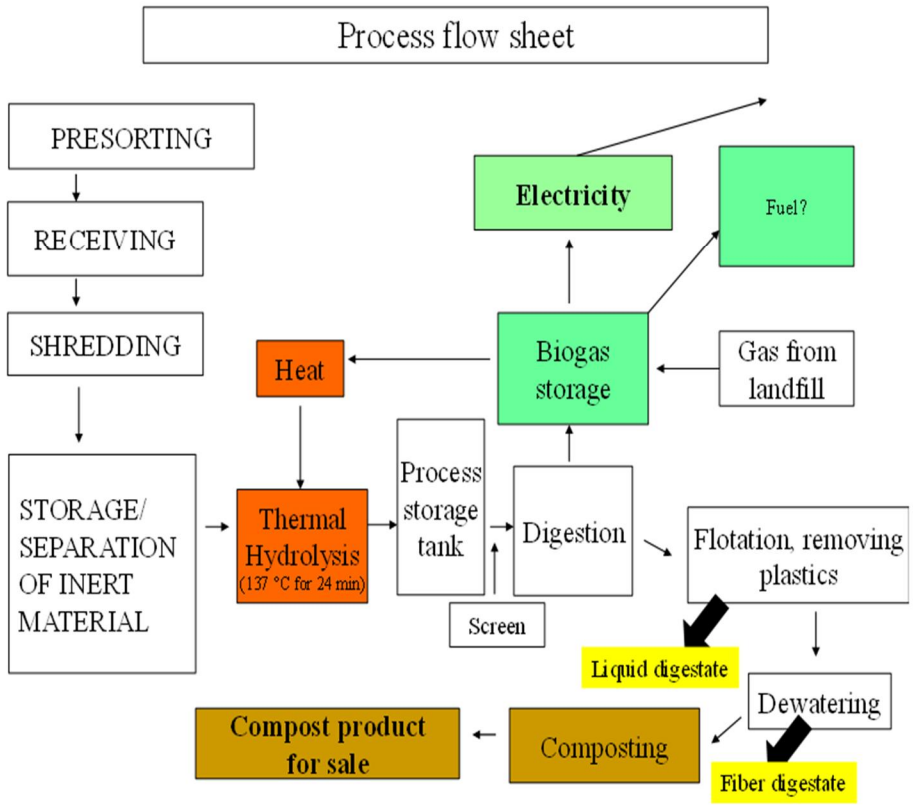

Figure 5 Flow sheet of the biowaste gasification plant of Mjøsanlegget AS, located at Lillehammer, Norway. The biowaste entered the process at the presorting. Samples were taken at the indicated with arrows.

Liquid digestate was sampled from the biowaste process throughout different seasons of the year and analysed for *B. cereus*, by viable counting and non-culture based methods. *B. cereus* was isolated from the digestates by plating on tryptic soy agar, mannitol egg yolk polymyxin B agar (MYP) and chromogenic *B. cereus* agar. The *B. cereus*-like colonies were pure cultured and analysed for toxicity using the

sperm motility inhibition assay (Paper I). DNA was extracted from the liquid digestate, quantified and inspected for the presence of *B. cereus* group members using primers as described by Hansen *et al*. (2001) specific for *B. cereus* group members. Cereulide synthethase genes were searched for and quantified using primers specific for the *ces*B gene (listed in Table 10). For quantitation the cereulide producing standard strain *B. cereus* F4810/72 was used as reference material (Mikkola *et al*. 1999, Ehling-Schulz *et al*. 2004, Dierick *et al*. 2005, Ankolekar *et al*. 2009).

We found an average of 10^4 *B. cereus* genomes per ml of the liquid digestate and of this 10 % contained the *ces*B gene. Heat stable spore content in the liquid fraction ranged from 10^3 to 10^6 CFU ml^{-1}. Of these 10^2 to 10^3 per ml, i.e. 0.1 to 10 %, were identified by PCR as viable *B. cereus*. Eleven pure cultured *B. cereus* isolates were checked with the sperm assay, two produced substance(s) that inhibited sperm motility and one isolate possessed the *ces*B gene. The isolates negative for the *ces*B gene produced a substance similarly toxic as cereulide. This substance could be the substance paenilide previously described by Rasimus *et al*. (2012) produced by *Paenibacillus tundrae,* as *Paenibacilli* are ubiquitous in plant (vegetable) material (Suominen 2005).

4.6 Environmental impact of *B. cereus* in food waste

When liquid digestate from biowaste biogasification is used to fertilize agricultural soil, the *B. cereus* present will meet indigenous soil *B. cereus.* Does this have an impact on the agro crops? To answer that question we should know the *B. cereus* content in food crops and in soil. Scottish agricultural soil was reported to contain 10^3 CFU of *B. cereus* per gram (Altayar and Sutherland, 2006).

Rice, pasta and potatoes are foods daily consumed all over the world. Rice and pasta have been regularly investigated for *B. cereus* and been implicated in food borne illness (Bennett *et al*. 2013, Pirhonen *et al*. 2005, Mahler *et al*. 1997, reviewed by Logan 2011). In Northern countries like Norway and Finland, potato is the likely bulk crop, not rice. Potato as a source for *B. cereus* has only sparsely been investigated (Altayar and Sutherland 2006, Doan and Davidson 2000, Fangio *et al*. 2010). Ready-to-eat potato products, potato purée, dehydrated and rehydrated potato products have been analysed for *B. cereus* content (Carlin *et al*. 2000, Rajkovic *et al*. 2006, Turner *et al*. 2006, King *et al*. 2007). Turner *et al*. and Rajkovic *et al*. investigated the samples for emetic toxin producers, but found none.

We investigated the occurrence and toxigenicity of cereulide producing *B. cereus* of locally purchased potatoes (Figure 6). Cereulide producers represented around 10% of the colonies on non-selective culture plates, tryptic soy agar, after incubation for 7-10 days. Based on these findings cereulide producing *B. cereus* is in my opinion approximately as common in raw potato as it is in raw rice (Ueda and Kuwabara, 1993). In Korea Kim SK *et al*. (2009) investigated raw rice, but did not find any cereulide producing isolates. This may have been because they investigated the samples according to the ISO standard protocol i.e. with MYP agar and 24 h incubation at 30 °C, continued by looking for "typical" *B. cereus* colonies (β-haemolytic). We showed in our Paper V that cereulide producers are atypical *B. cereus* colonies, being mostly starch and salicin negative and weakly haemolytic (Apetroaie *et al*. 2005). Ekman *et al*. (2012) showed that in potassium low media cereulide producers out competed non-producers. This ability will give competition advantage for cereulide positive endophytes, because potassium levels inside the extracellular space of plant tissue are low, due to uptake of potassium by the plant from the soil water for its own growth.

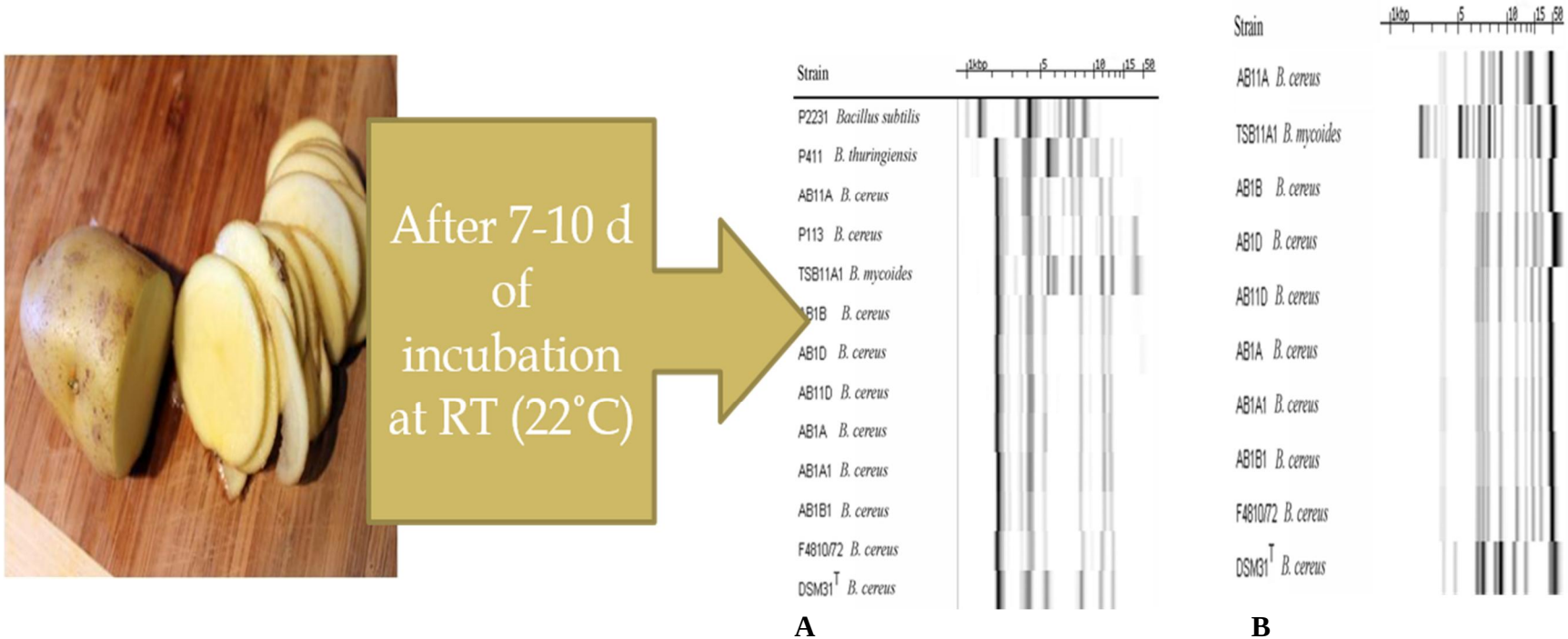

Figure 6. Identification of the presumptive *Bacillus cereus* isolates from the tuber interior of potatoes. The ribopatterns in panel A are obtained after cleavage of the DNA obtained from the presumptive *B. cereus* isolates with the restriction enzyme *Eco*RI and panel B is cleaved with *Pvu*II.
Cereulide producing strains were AB1A, AB1B, AB1D, AB1A1, AB1B1 and AB11D.

Would the views on the prevalence of cereulide producing *B. cereus* change, if like we described in Paper V, the researchers would look for atypical colonies which are weakly haemolytic, salicin and starch negative? As the famous Dutch scientist, Professor David Mossel wrote in 1967: "it might be wise to study also a reasonable proportion of egg yolk-negative, mannitol-negative *Bacillus* colonies cultured from foods on MYP-agar. When these are found morphologically typical, they should be submitted to the tests which were found invariable positive for *B. cereus*: anaerobic dissimilation of glucose, gelatin liquefaction, nitrate reduction, and profuse growth on chloral hydrate agar" (Mossel *et al*. 1967).

4.7 Patterns of cereulide toxicity on mammalian cells and cell lines

Cereulide producing *B. cereus* have been isolated from foods and from health troubled indoor environments (Apetroaie *et al*. 2005). Cereulide, the mitochondrial toxic substance, has been shown to cause emetic illness in humans, apes and shrews. Cereulide is toxic to boar sperm cells (1.6.1) but is this similar toxicity towards cells of other body organs?

To answer this question we exposed human (peripheral blood mononuclear cells, keratinocytes), porcine (sperm, kidney tubular epithelial cell line) and murine (pancreatic islets and fibroblasts) cells to cereulide. We found that the cytotoxic effects on four of the cells tested (except the MIN-6 cells) were similar as for the porcine sperm cells. The murine pancreatic islets lost their cell membrane integrity, indicating necrotic cell death, by exposure to cereulide at concentrations 2 log units lower (< 0.007µg ml^{-1}) than did the other five cell types. In the six tested cell types, exposure to cereulide caused dissipation of the mitochondrial $\Delta\Psi$ within 24 h at exposure concentrations below 10 ng ml^{-1} in absence of disruption of the plasma membrane barrier towards propidium iodide. Earlier was shown that low

concentrations of cereulide (1 ng cereulide ml^{-1}) caused necrosis of porcine pancreatic Langerhans islets (Virtanen *et al*. 2008). The question remains, why the pancreatic islet (MIN-6) cells lost the plasma membrane permeability barrier in response to extremely low exposure concentration of cereulide. The pancreatic islet has an architecture structure and pattern of ATP generation different from that of the other cells. This may explain the extreme sensitivity of the beta cells to cereulide. Other cells shift to anaerobic glycolysis when the rate of ATP generation by oxidative phosphorylation is too low for the cell maintenance and proliferation.For anaerobic glycolysis one of the proteins required is a monocarboxylate transporter that helps lactate and pyruvate diffuse through the membrane, but this protein MCT1 needs to be very low in bèta cells to prevent inappropriate insulin release (Schuit *et al*. 2012). Also the generation of pyruvate in anaerobic glycolysis would cause problems in pancreatic islets as it is essential in the secretion of insulin (Patterson *et al*. 2014).

4.8 Practical use of this thesis outcome

We showed that cereulide producing *B. cereus* are everywhere. The formation of highly heat resistant spores as well as the heat stable toxin makes it essential to have a way to detect and identify the toxin and the producer in food. In the preparation of food it is essential to make sure the heat stable spores possibly present in the food do not get the chance to germinate. Dangerous practices are preparing the food and leaving it for hours or days at room temperature before consumption. Or storing heated foods or leftovers so that the food is not fast enough cooled down to a temperature below 6 °C or is not heated to above 55 °C i.e. conditions allowing cereulide biosynthesis to proceed. When cooking for example potatoes, the vegetative *B. cereus* cells will not survive. In heated food spores will germinate due to nutrients available from the broken potato cells.

It is not possible to eliminate cereulide producing *B. cereus* from foods. To prevent intoxication of the consumer it is essential to avoid conditions of food storage that are permissive for cereulide production.

4.9 Final comments

In vitro and *in vivo* assays for identifying adverse effects on mitochondria in substances intended as drugs are needed in the pharmaceutical industry to improve drug safety (reviewed by Dykens and Will 2007). The pharmaceutical industry focuses on identifying drugs with undesired effects towards mitochondria, but as shown here, not only pharmaceuticals can cause negative effects towards mitochondria. We showed that microbially produced substances should be taken into account when the toxic load on mitochondria is assessed.

We introduced in this thesis a fast assay which unlike the PCR methods can detect more than just one toxin in a target oriented way. We showed the method to be useful also when searching for toxins affecting different mitochondrial targets in the oxidative phosphorylation chain, not just those caused by cereulide. The principal difference between PCR based methods and the sperm motility inhibition assay is the detection of the toxin by its toxicity. The presence of the gene (*ces*) does not reveal whether and how much toxin is actually being produced (Shaheen 2009). There are factors that can influence the amount of cereulide produced, like free amino acids. Glycine (Apetroaie-Constantin *et al*. 2008) L-leucine and L-valine (Jääskeläinen *et al*. 2004) have been implicated to increase cereulide production, when supplied as free amino acids. Nevertheless, these amino acids are still used as food additives without requirement for EU-registration (E-code).

5. Conclusions

1. The bioassay utilizing porcine spermatozoa as test cells was shown suitable for distinguishing mitochondrial toxic effects from other cytotoxic effects of known and unknown microbially produced toxins.
2. We showed that combining the boar sperm motility inhibition assay with the membrane potential responsive dye (JC-1), and the live-dead dyes propidium iodide and calcein-AM, yielded a test set that was uniquely effective in detecting mitochondrial toxic substances in the metabolome of bacteria isolated from potato crop, in food supplements or in industrial waste processing residues.
3. Our results indicate that when *B. cereus* is present in the wood pulp used for manufacturing food packaging paperboard, *B. cereus* spores may end up in the packaging material. Results of experimental leachings indicated that cereulide, if present in paper, may leach from the paper into a packaged fatty food.
4. The distribution of cereulide during paper making was: ~ 10 % of the cereulide present in pulp was recovered in the paper.
5. Cereulide was > 100 fold more cytotoxic towards murine insulin producing pancreatic islet cells, than to other mammalian somatic cells or porcine spermatozoa. Necrotic death of pancreatic islet cells to following exposure to cereulide arouses concern when assumed that cereulide is toxic towards human pancreatic islet cells (beta cells) similarly as to the murine.

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7. References

Agata N, More M, Ohta M, Suwan S, Ohtani I, Isobe M. 1994. A novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation in HEp-2 cells. FEMS Microbiology Letters 121: 31-34.

Agata N, Ohta M, Mori M, Isobe M. 1995. A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. FEMS Microbiology Letters 129: 17-20.

Agata N, Ohta M, Yokoyama K. 2002. Production of *Bacillus cereus* emetic toxin (cereulide) in various foods. International Journal of Food Microbiology 73: 23-27.

Altayar M, Sutherland AD. 2006. *Bacillus cereus* is common in the environment but emetic toxin producing isolates are rare. Journal of Applied Microbiology 100: 7-14.

Ananchaipattana C, Hosotani Y, Kawasaki S, Pongsawat S, Latiful BM, Isobe S, Inatsu Y. 2012. Prevalence of foodborne pathogens in retailed foods in Thailand. Foodborne Pathogens and Disease 9: 835-840.

Andersson MA, Nikulin M, Köljalg U, Andersson MC, Rainey F, Reijula K, Hintikka EL, Salkinoja-Salonen M. 1997. Bacteria, molds, and toxins in water-damaged building material. Applied and Environmental Microbiology 63: 387-393.

Andersson MA, Mikkola R, Helin J, Andersson MC, Salkinoja-Salonen M. 1998. A novel sensitive bioassay for detection of *Bacillus cereus* emetic toxin and related depsipeptide ionophores. Applied and Environmental Microbiology 64: 1338-1343.

Andersson M. 1999. Bacterial diversity and toxicity in air, indoor environment and foods. Ph.D thesis. University of Helsinki, Department of Applied Chemistry and Microbiology, Helsinki, Finland. p. 13

Andersson MA, Jääskeläinen EL, Shaheen R, Pirhonen T, Wijnands LM, Salkinoja-Salonen MS. 2004. Sperm bioassay for rapid detection of cereulide-producing *Bacillus cereus* in food and related environments. International Journal of Food Microbiology 94: 175-183.

Ankolekar C, Rahmati T, Labbé RG. 2009. Detection of toxigenic *Bacillus cereus* and *Bacillus thuringiensis* spores in U.S. rice. International Journal of Food Microbiology 128: 460-466.

Anonymous. 1975. **'***Bacillus cereus* food poisoning' in CDC weekly report Morbidity and Mortality Vol 24. No 36. For the week ending September 6, 1975.

Apetroaie C, Andersson MA, Spröer C, Tsitko I, Shaheen R, Jääskeläinen EL, Wijnands LM, Heikkilä R, Salkinoja-Salonen MS. 2005. Cereulide-producing strains of *Bacillus cereus* show diversity. Archives of Microbiology 184: 141-151.

Apetroaie-Constantin C, Shaheen R, Andrup L, Smidt L, Rita H, Salkinoja-Salonen M. 2008. Environment driven cereulide production by emetic strains of *Bacillus cereus*. International Journal of Food Microbiology 127: 60-67.

Apetroaie-Constantin C, Mikkola R, Andersson MA, Teplova V, Suominen I, Johansson T, Salkinoja-Salonen M. 2009. *Bacillus subtilis* and *B. mojavensis* strains connected to food poisoning produce heat stable toxin amylosin. Journal of Applied Microbiology 103: 1976-1985.

Arslan S, Eyi A, Küçüksarı R. 2014. Toxigenic genes, spoilage potential, and antimicrobial resistance of *Bacillus cereus* group strains from ice cream. Anaerobe 25: 42-46.

Batchoun R, Al-Sha'er AI, Khabour OF. 2011. Molecular characterization of *Bacillus cereus* toxigenic strains isolated from different food matrices in Jordan. Foodborne Pathogens and Disease 8: 1153-1158.

Bartoszewicz M, Hansen BM, Swiecicka I. 2008. The members of the *Bacillus cereus* group are commonly present contaminants of fresh and heat-treated milk. Food Microbiology 25: 588-596.

Bauer T, Stark T, Hofmann T, Ehling-Schulz M. 2010. Development of a stable isotope dilution analysis for the quantification of the *Bacillus cereus* toxin cereulide in foods. Journal of Agricultural and Food Chemistry 58: 1420-1428.

Bennett SD, Walsh KA, Gould LH. 2013. Foodborne disease outbreaks caused by *Bacillus cereus, Clostridium perfringens,* and *Staphylococcus aureus* — United States, 1998-2008. Clinical Infectious Diseases 57: 425-433

Bennett RW, Lancette GA. 2001. Chapter 12 *Staphylococcus aureus.* in Bacteriological Analytical Methods (BAM) of the US Food and Drug Administration, 8th Edition, Revision A . Online. Accessed 02.06.2014 http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm071429.htm

Beyer W, Turnbull PCB. 2009. Anthrax in animals. Molecular Aspects of Medicine 30: 481-489

Biesta-Peters EG, Reij MW, Blaauw RH, in 't Veld PH, Rajkovic A, Ehling Schulz M, Abee T. 2010. Quantification of the emetic toxin cereulide in food products by liquid chromatography-mass spectrometry using synthetic cereulide as a standard. Applied and Environmental Microbiology 76: 7466-7472.

Boehmerle W, Endres M. 2011. Salinomycin induces calpain and cytochrome c-mediated neuronal cell death. Cell Death and Disease 2, e168; doi:10.1038/cddis.2011.46

Carlin F, Guinebretiere MH, Choma C, Pasqualini R, Braconnier A, Nguyen-The C. 2000. Spore-forming bacteria in commercial cooked, pasteurized and chilled vegetable purées. Food Microbiology 17:153–165.

Carlin F, Fricker M, Pielaat A, Heisterkamp S, Shaheen S, Salkinoja-Salonen M, Svensson B, Nguyen-the C, Ehling-Schulz M. 2006. Emetic toxin-producing strains of *Bacillus cereus* show distinct characteristics within the *Bacillus cereus* group. International Journal of Food Microbiology 109: 132-138.

Chaves JQ, Cavados C de F, Vivoni AM. 2012. Molecular and toxigenic characterization of *Bacillus cereus* and *Bacillus thuringiensis* strains isolated from commercial ground roasted coffee. Journal of Food Protection 75: 518-522.

Choma C, Guinebretière MH, Carlin F, Schmitt P, Velge P, Granum PE, Nguyen-The C. 2000. Prevalence, characterization and growth of *Bacillus cereus* in commercial cooked chilled foods containing vegetables. Journal of Applied Microbiology 88: 617-625.

Collier FA, Elliot SL, Ellis RJ. 2005. Spatial variation in *Bacillus thuringiensis/cereus* population within the phyllosphere of broad-leaved dock (*Rumex obtusifolius*) and surrounding habitats. FEMS Microbiology Ecology 54: 417-425.

de la Fuente-Salcido NM, Casados-Vásquez LE, Barboza-Corona JE. 2013. Bacteriocins of *Bacillus thuringiensis* can expand the potential of this bacterium to other areas rather than limit its use only as microbial insecticide. Canadian Journal of Microbiology 59: 515-522.

Delbrassinne L, Andjelkovic M, Dierick K, Denayer S, Mahillon J, van Loco J. 2012. Prevalence and levels of *Bacillus cereus* emetic toxin in rice dishes randomly collected from restaurants and comparison with the levels measured in a recent foodborne outbreak. Foodborne Pathogens and Disease 9: 809-814.

den Besten HMW, van Melis CCJ, Sanders JW, Nierop Groot MN, Abee T. 2012. Impact of sorbic acid on germination and outgrowth heterogeneity of *Bacillus cereus* ATCC 14579 spores. Applied and Environmental Microbiology 78: 8477-8480.

Déniel F, Rey P, Chérif M, Guillou A, Tirilly Y. 2004. Indigenous bacteria with antagonistic and plant-growth-promoting activities improve slow-filtration efficiency in soilless cultivation. Canadian Journal of Microbiology 50: 499-508.

de Vries, YP. 2006. Chapter 7: Germination Capacity and Heat Resistance of Spores from Naturally occurring *Bacillus cereus* Strains, in thesis: *Bacillus cereus* spore formation, structure, and germination. Thesis Wageningen University, Wageningen, the Netherlands. p 93-102.
http://library.wur.nl/WebQuery/edepot/121760 visited 07.05.2012

Dierick K, van Coillie E, Swiecicka I, Meyfroidt G, Devlieger H, Meulemans A, Hoedemaekers G, Fourie L, Heyndrickx M, Mahillon J. 2005. Fatal family outbreak of *Bacillus cereus*-associated food poisoning. Journal of Clinical Microbiology 43: 4277-4279.

Dirnhofer D, Sonnabend O, Sonnabend W. 1977. Eine tödlich verlaufene Lebensmittelvergiftung durch Bacillus cereus. (A fatal food poisoning by *Bacillus cereus*), in german. Rechtsmedizin 80: 139-151.

Doan CH, Davidson PM. 2000. Microbiology of potatoes and potato products: a review. Journal of Food Protection 63: 668-683.

Dohmae S, Okubo T, Higuchi W, Takano T, Isobe H, Baranovich T, Kobayashi S, Uchiyama M, Tanabe Y, Itoh M, Yamamoto T. 2008. *Bacillus cereus* nosocomial infection from reused towels in Japan. Journal of Hospital Infection 69: 361-367. doi:10.1016/j.jhin.2008.04.014

Duchen MR. 2004. Mitochondria in health and disease: perspective on a new mitochondrial biology. Molecular Aspects of Medicine 25: 365-451.

Dykens JA, Jamieson JD, Marroquin LD, Nadanaciva S, Xu JJ, Dunn MC, Smith AR, Will Y. 2008. In vitro assessment of mitochondrial dysfuntion and cytotoxicity of nefazodone, trazodone, and buspirone. Toxicological Sciences 103: 335-345.
Dykens JA, Will Y. 2007. The significance of mitochondrial toxicity testing in drug development. Drug Discovery Today 12: 777-785.
EFSA. 2013. The European Union summary report on trends and sources of zoonoses,
zoonotic agents and foodborne outbreaks in 2011. EFSA Journal 11(4): p. 193-239.
Ehling-Schulz M, Fricker M, Scherer S. 2004 Identification of emetic toxin producing *Bacillus cereus* strains by a novel molecular assay. FEMS Micrbiology Letters 232: 189-195.
Ehling-Schulz M, Vukov N, Schulz A, Shaheen R, Andersson M, Märtlbauer E, Scherer S. 2005. Identification and partial characterization of the nonribosomal peptide synthetase gene responsible for cereulide production in emetic *Bacillus cereus*. Applied and Environmental Microbiology 71: 105-113.
Ehling-Schulz M, Fricker M, Grallert H, Rieck P, Wagner M, Scherer S. 2006. Cereulide synthetase gene cluster from the emetic *Bacillus cereus:* Structure and location on a mega virulence plasmid related to *Bacillus anthracis* toxin plasmid pXOI. BMC Microbiology 6: 20.
Ekman JV, Kruglov A, Andersson MA, Mikkola R, Raulio M, Salkinoja-Salonen M. 2012. Cereulide produced by *Bacillus cereus* increases the fitness of the producer organism in low-potassium environments. Microbiology 158: 1106-1116.
Eneroth Å, Svensson B, Molin G, Christiansson A. 2001. Contamination of pasteurized milk by *Bacillus cereus* in the filling machine. Journal of Dairy Research 68: 189-196.
Essen R, de Ruyter C, de Wit M. 2000. Massale voedselvergiftiging in het Kotterbos te
Almere (Massive food poisoning in Kotterbos at Almere) In Dutch. Infectieziekten Bulletin **11**:205–207.
European standard EN 645. 1993. Paper and board intended to come into contact with foodstuffs – Preparation of a cold water extract.
European standard EN 647. 1993. Paper and board intended to come into contact with foodstuffs – Preparation of a hot water extract.
European standard EN 1186-15. 2002. Materials and articles in contact with foodstuffs – Plastics – Part 15: Alternative test methods to migration into fatty food simulants by rapid
extraction into iso-octane and/or 95% ethanol.
Euzéby JP. 2014: List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. (List of Prokaryotic names with Standing in Nomenclature. http://www.bacterio.net). Online visited 02.06.2014. http://www.bacterio.net/bacillus.html

Fagundes RBS, Picoli EAT, Lana UGP, Valicente FH. 2011. Plasmid patterns of efficient and inefficient strains of *Bacillus thuringiensis* against *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae). Neotropical Entomology 40: 600-606.

Faille C, Jullien C, Fotaine F, Bellon-Fontaine M-N, Slomianny C, Benezech T. 2002. Adhesion of *Bacillus* spores and *Escherichia coli* cells to inert surfaces: role of surface hydrophobicity. Can J. Microbiol 48: 728-738.

Faille C, Bénézech T, Blel W, Ronse A, Ronse G, Clarisse M, Slomianny C. 2013. Role of mechanical vs. chemical action in the removal of adherent *Bacillus* spores during CIP procedures. Food Microbiology 33: 149-157.

Fangio MF. Roura SI, Fritz R. 2010. Isolation and identification of *Bacillus* spp. and related genera from different starchy foods. Journal of Food Science 75: M218-M221.

Frankland GC, Frankland PF. 1887**.** Studies on some new micro-organisms obtained from air. Philosophical Transactions of the Royal Society London Biological Sciences 178:257–287.

From C, Hormazabal V, Granum PE. 2007. Food poisoning associated with pumilacidin-producing *Bacillus pumilus* in rice. International Journal of Food Microbiology 115: 319-324.

Goodwin AE, Spencer Roy Jr J, Grizzle JM, Terrell Goldsby Jr, M. 1994. *Bacillus mycoides*: a bacterial pathogen of channel catfish. Diseases of Aquatic Organisms 18: 173-179.

Gräfe, U. 1992. Biochemie der Antibiotika: Struktur-Biosynthese-Wirkmechanismus. Spektrum Akademischer Verlag GmbH, Heidelberg. pp. 282.

Granum PE. 2002. *B. cereus* and food poisoning. In: Application and Systematics of *Bacillus* and relatives, edited by Berkeley R, Heyndrickx M, Logan N, and de Vos P. Blackwell Publishing Company. MA. USA. p 37-46.

Granum PE. 2007. *B. cereus*. In: Food Microbiology. Fundaments and Frontiers 3rd edition. Edited by Doyle M. and Beuchat L. ASM Press Washington, DC., p 445-456.

Guinebretière M-H, Auger S, Galleron N, Contzen M, De Sarrau B, De Buyser M-L, Lamberet G, Fagerlund A, Granum PE, Lereclus D, De Vos P, Nguyen-The C, Sorokin A. 2013. *Bacillus cytotoxicus* sp. nov. is a novel thermotolerant species of the *Bacillus cereus* group occasionally associated with food poisoning. International Journal of Systematic and Evolutionary Microbiology 63: 31-40.

Häggblom MM, Apetroaie C, Andersson MA, Salkinoja-Salonen MS. 2002. Quantitative analysis of cereulide, the emetic toxin of *Bacillus cereus* produced under various conditions. Applied and Environmental Microbiology 68: 2479-2483.

Hallaksela AM, Väisänen O, Salkinoja-Salonen M. 1991. Identification of *Bacillus* species isolated from *Picea abies* by physiological tests, phage typing and fatty acid analysis. Scandinavian Journal of Forest Research 6: 365-377.

Hansen BM, Leser TD, Hendriksen NB. 2001. Polymerase chain reaction assay for the detection of *Bacillus cereus* group cells. FEMS Microbiology Letters 202: 209-213.

Harwood CR. 1989. Introduction to the biotechnology of *Bacillus*. In: Biotechnology Handbooks – *Bacillus*. Series editors. T. Atkinson and R.F. Sherwood, Plenum Press New York and London, Vol 2, p 1-2.
Hauge S. 1955. Food poisoning caused by aerobic spore-forming bacilli. Journal of Applied Bacteriology 18: 591-595.
Hendriksen NB, Hansen BM, Johansen JE. 2006. Occurrence and pathogenic potential of *Bacillus cereus* group bacteria in a sandy loam. Antonie van Leeuwenhoek 89: 239-249.
Hormazábal V, Østensvik Ø, O'Sullivan K, Granum PE. 2004. Quantification of *Bacillus cereus* emetic toxin (cereulide) in figs using LC/MS. Journal of Liquid Chromatography & Related Technologies 27: 2531-2538.
Horwood PF, Burgess GW, Oakey HJ. 2004. Evidence for non-ribosomal peptide synthetase production of cereulide (the emetic toxin) in *Bacillus cereus*. FEMS Microbiology Letters 23: 319-324.
Hosein IK, Hoffman PN, Ellam S, Asseez T-M, Fakokunde A, Silles J, Devereux E, Kaur D, Bosanquet J. 2013. Summertime *Bacillus cereus* colonization of hospital newborns traced to contaminated laundered linen. Journal of Hospital Infection 85: 149-154.
Hoton FM, Fornelos N, N'Guessan E, Hu X, Swiecicka I, Dierick K, Jääskeläinen E, Salkinoja-Salonen M, Mahillon J. 2009. Family portrait of *Bacillus cereus* and *Bacillus weihenstephanensis* cereulide-producing strains. Environmental Microbiology Reports 1: 177-183.
Hughes S, Bartholomew B, Hardy JC, Kramer JM. 1988. Potential application of a HEp-2 cell assay in the investigation of *Bacillus cereus* emetic-syndrome food poisoning. FEMS Microbiology Letters 52: 7-12.
Ivanova L, Skjerve E, Eriksen GS, Uhlig S. 2006. Cytotoxicity of enniatins A, A1, B, B1, B2 and B3 from *Fusarium avenaceum*. Toxicon 47: 868-876.
Jääskeläinen EL, Häggblom MM, Andersson MA, Vanne L, Salkinoja-Salonen MS. 2003. Potential of *Bacillus cereus* for producing an emetic toxin, cereulide, in bakery products: quantitative analysis by chemical and biological methods. Journal of Food Protection 66: 1047-1054.
Jääskeläinen EL, Häggblom MM, Andersson MA, Salkinoja-Salonen MS. 2004. Atmospheric oxygen and other conditions affecting the production of cereulide by *Bacillus cereus* in food. International Journal of Food Microbiology 96: 75-83.
Jay JM, Loessner MJ, Golden DA. 2005. Chapter 24: Food poisoning caused by Gram-positive sporeforming bacteria in: Modern food microbiology. 7th edition. Springer Science and Business Media, Inc. New York. pp. 567-590.
Jestoi M. 2008. Emerging *Fusarium*-mycotoxins fusaproliferin, beauvericin, enniatins, and moniformin—a review. Critical Reviews in Food Science and Nutrition 48: 21-49.
Kamga Wambo GO, Burckhardt F, Frank C, Hiller P, Wichmann-Schauer H, Zuschneid I, Hentschke J, Hitzbleck T, Contzen M, Suckau M, Stark K. 2011. The proof of the pudding is in the eating: an outbreak of emetic syndrome after a

kindergarten excursion, Berlin, Germany, December 2007. Eurosurveillance 16: pii=19839.
Kim J-B, Jeong H-R, Park Y-B, Kim J-M, Oh D-H. 2010. Food poisoning associated with emetic-type of *Bacillus cereus* in Korea. Foodborne Pathogens and Disease 7: 555-563.
Kim J-B, Kim J-M, Cho S-H, Oh H-S, Choi NJ, Oh D-H. 2011. Toxin gene profiles and toxin production ability of *Bacillus cereus* isolated from clinical and food samples. Journal of Food Science 76: T25-T29.
Kim SK, Kim K-P, Jang SS, Shin EM, Kim M-J, Oh S, Ryu S. 2009. Prevalence and toxigenic profiles of *Bacillus cereus* isolated from dried red peppers, rice and sunsik in Korea. Journal of Food Protection 72: 578-582.
King NJ, Whyte R, Hudson JA. 2007. Presence and significance of *Bacillus cereus* in dehydrated potato products. Journal of Food Protection 70: 514-520.
Kitunen VH, Salkinoja-Salonen MS. 1991. Distribution of PCDDS/PCDFS in bleached paperboard and explanation for the migration of PCDDS/PCDFS from paper and board products. Chemosphere 23: 1561-1568.
Kotiaho M, Aittamaa M, Andersson MA, Mikkola R, Valkonen JPT, Salkinoja-Salonen M. 2007. Antimycin A-producing nonphytopathogenic *Streptomyces turgidiscabies* from potato. Journal of Applied Microbiology 104: 1332-1340.
Kotzekidou P. 2013. Microbiological examination of ready-to-eat foods and ready-to-bake frozen pastries from university canteens. Food Microbiology 34: 337-343.
Kusumaningrum HD, van Putten MM, Rombouts FM, Beumer RR. 2002. Effects of antibacterial dishwashing liquid on foodborne pathogens and competitive microorganisms in kitchen sponges. Journal of Food Protection 65: 61-65.
Lee H-S, Song H-H, Jeong J-H, Shin C-G, Choi S-U, Lee C. 2008. Cytotoxicities of enniatins H, I, and MK1688 from *Fusarium oxysporum* KFCC 11363P. Toxicon 51: 1178-1185.
Logan NA. & De Vos P. 2009. Genus I Bacillus Cohn 1872. In: Bergey's Manual of Systematic Bacteriology 2nd Edition. Volume 3 The firmicutes. Editors Paul De Vos, George M. Garrity, Dorothy Jones, Noel R. Krieg, Wolfgang Ludwig, Fred A. Rainey, Karl-Heinz Schleifer and William B. Whitman. Springer Dordrecht Heidelberg London New York. p 21-128.
Logan NA. 2011. *Bacillus* and relatives in foodborne illness. Journal of Applied Microbiology 112: 417-429.
Liu K, Sun Y, Liu D, Yin J, Qiao L, Shi Y, Dong Y, Li N, Zhang F, Chen D. 2013. Mitochondrial toxicity studied with the PBMC of children from the Chinese national pediatric highly active antiretroviral therapy cohort. PLoS ONE 8: e57223.
Logrieco A, Moretti A, Mule G, Paciollla C, Ritieni A. 2008. Advances on the toxicity of the cereulide contaminant *Fusarium* esadepsipeptides. Cereal Research Communications Supplement B 36: 303-313.
Magnusson M, Svensson B, Kolstrup C, Christiansson A. 2007. *Bacillus cereus* in free-stall bedding. Journal of Dairy Science 90: 5473-5482.

Mahler H, Pasi A, Kramer JM, Schulte P, Scoging AC, Bär W, Krähenbühl S. 1997. Fulminant liver failure in association with the emetic toxin of *Bacillus cereus*. The New England Journal of Medicine 336:1142-1148.

Mehta R, Chan K, Lee O, Tafazoli S, O'Brien PJ. 2008. Chapter 3: "Drug-associated mitochondrial toxicity." In: Drug-Induced mitochondrial dysfunction, edited by James A. Dykens and Yvonne Will. John Wiley & Sons, Inc. New Jersey. USA. p 71-126.

Meldrum RJ, Mannion PT, Garside J. on behalf of the Welsh food microbiology forum. 2009. Microbiological quality of ready-to-eat food served in school in Wales, United Kingdom. Journal of Food Protection 72: 197-201.

Melling J, Capel BJ, Turnbull PCB, Gilbert RJ. 1976. Identification of a novel enterotoxigenic activitiy associated with *Bacillus cereus*. Journal of Clinical Pathology 29: 938-940.

Melnick RL, Testen AL, Poleatewich AM, Backman PA, Bailey BA. 2012. Detection and expression of enterotoxin genes in endophytic strains of *Bacillus cereus*. Letters in Applied Microbiology 54: 468-474.

Merzougui S, Lkhider M, Grosset N, Gautier M, Cohen N. 2014. Prevalence, PFGE typing, and antibiotic resistance of *Bacillus cereus* group isolated from food in Morocco. Foodborne Pathogens and Disease 11: 145-149.

Messelhäusser U, Kämpf P, Fricker M, Ehling-Schulz M, Zucker R, Wagner B, Busch U, Höller C. 2010. Prevalence of emetic *Bacillus cereus* in different ice creams in Bavaria. Journal of Food Protection 73: 395-399.

Mikami T, Horikawa T, Murakami T, Matsumoto T, Yamakawa A, Murayama S, Katagiri S, Shinigawa K, Suzuki M. 1994. An improved method for detecting cytostatic toxin (emetic toxin) of *Bacillus cereus* and its application to food samples. FEMS Microbiology Letters 119: 53-58.

Mikkola R, Saris NEL, Grigoriev PA, Andersson MA, Salkinoja-Salonen MS. 1999. Ionophoric properties and mitochondrial effects of cereulide the emetic toxin of *B. cereus*. European Journal of Biochemistry 263: 112-117.

Mikluscak M, Dawson-Andoh BE. 2004. Microbial colonizers of freshly sawn yellow-poplar (*Liriodendron tulipifera* L.) lumber in two seasons: Part 2. Bacteria. Holzforschung 58:182–188.

Moir A. 2006. How do spores germinate? Journal of Applied Microbiology 101: 526-530.

Mossel DAA, Koopman MJ, Jongerius E. 1967. Enumeration of *Bacillus cereus* in foods. Applied Microbiology 15: 650-653.

Naranjo M, Denayer S, Botteldoorn N, Delbrassinne L, Veys J, Waegenaere J, Sirtaine N, Driesen RB, Sipi KR, Mahillon J, Dierick K. 2011. Sudden death of a young adult associated with *Bacillus cereus* food poisoning. Journal of Clinical Microbiology 49: 4379-4381.

Park HS, Choi HJ, Kim M-D, Kim KH. 2013. Addition of ethanol to supercritical carbon dioxide enhances the inactivation of bacterial spores in the biofilm of *Bacillus cereus*. International Journal of Food Microbiology 116: 207-212.

Park Y-B, Kim J-B, Shin S-W, Kim J-C, Cho S-H, Lee B-K, Ahn J, Kim J-M, Oh D-H. 2009. Prevalence, genetic diversity, and antibiotic susceptibility of *Bacillus cereus* strains isolated from rice and cereals collected in Korea. Journal of Food Protection 72: 612-617.

Pasupuleti V. 2007. Role of glycolysis and respiration in sperm metabolism and motility.
A thesis submitted to Kent State University in partial fulfillment of the requirements for the degree of Master of Science. p. 8.

Patel TG, Shukla RV, Gupte SC. 2013. Impact of donor arm cleaning with different aseptic solutions for prevention of contamination in blood bags. Indian Journal of Hematology and Blood Transfusion 29: 17-20.

Patterson JN, Cousteils K, Lou JW, Manning Fox JE, MacDonald PE, Joseph JW. 2014. Mitochondrial metabolism of pyruvate is essential for regulating glucose-stimulated insulin secretion. The Journal of Biological Chemistry 289: 13335-13346.

Perera ML, Ranasinghe GR. 2012. Prevalence of *Bacillus cereus* and associated risk factors in Chinese-style fried rice available in the city of Colombo, Sri Lanka. Foodborne Pathogens and Disease 9: 125-131.

Pilo P, Frey J. 2011. *Bacillus anthracis:* Molecular taxonomy, population genetics phylogeny and patho-evolution. Infection, Genetics and Evolution 11: 1218-1224.

Pirhonen TI, Andersson MA, Jääskeläinen EL, Salkinoja-Salonen MS, Honkanen-Buzalski T, Johansson TM-L. 2005. Biochemical and toxic diversity of *Bacillus cereus* in a pasta and meat dish associated with a food-poisoning case. Food Microbiology 22: 87-91.

Pirttijärvi TSM, Ahonen LM, Maunuksela LM, Salkinoja-Salonen MS. 1998. *Bacillus cereus* in a whey process. International Journal of Food Microbiology 44: 31-41.

Planchon S, Dargaignaratz C, Levy C, Ginies C, Broussolle V, Carlin F. 2011. Spores of *Bacillus cereus* strain KBAB4 produced at 10 °C and 30 °C display variations in their properties. Food Microbiology 28: 291-297.

Pósfay-Barbe KM, Schrenzel J, Frey J, Studer R, Korff C, Belli DC, Parvex P, Rimensberger PC, Schäppi MG. 2008. Food poisoning as a cause of acute liver failure. The Pediatric Infectious Disease Journal 27: 846-847.

Préstamo G, Arabas J, Fonberg-Broczek M, Arroyo G. 2001. Reaction of *B. cereus* and peroxidase enzymes under pressures > 400 MPa. Journal of Agricultural and Food Chemistry 49: 2830-2834.

Rajkovic A, Uyttendaele M, Courtens T, Heyndrickx M, Debevere J. 2006. Prevalence and characterisation of *Bacillus cereus* in vacuum packed potato puree. International Journal of Food Science and Technology 41: 878-884.

Rajkovic A, Uyttendaele M, Debevere J. 2007. Computer aided boar semen motility analysis for cereulide detection in different food matrices. International Journal of Food Microbiology 114: 92-99.

Rajkovic A, Uyttendaele M, Vermeulen A, Andjelkovic M, Fitz-James I, in't Veld P, Denon Q, Vérhe R, Debevere J. 2008. Heat resistance of *Bacillus cereus* emetic toxin, cereulide. Letters in Applied Microbiology 46: 536-541.
Rajkovic A. 2014. Microbial toxins and low level of foodborne exposure. Trends in Food Science & Technology DOI: 10.1016/j.tifs.2014.04.006
Rasimus S, Mikkola R, Andersson MA, Teplova VV, Venediktova N, Ek-Kommonen C, Salkinoja-Salonen M. 2012. Psychrotolerant *Paenibacillus tundrae* isolates from barley grains produce new cereulide-like depsipeptides (paenilide and homopaenilide) that are highly toxic to mammalian cells. Applied and Environmental Microbiology 78: 3732-3743.
Reis AS, Montanhini MTM, Bittencourt JVM, Destro MT, Bersot LS. 2013. Gene detection and toxin production evaluation of hemolysin BL of *Bacillus cereus* isolated from milk and dairy products marketed in Brazil. Brazilian Journal of Microbiology 44: 1195-1198.
Renault D, Déniel F, Benizri E, Shier D, Barbier G, Rey P. 2007. Characterization of *Bacillus Pseudomonas* strains with suppressive traits isolated from tomato hydroponic-slow filtration unit. Canadian Journal of Microbiology 53: 784-797. doi:10.1139/W07-046.
Reyes JE, Bastías JM, Gutiérrez MR, Rodríguez M de la O. 2007. Prevalence of *Bacillus cereus* in dried milk products used by Chilean school feeding program. Food Microbiology 24: 1-6.
Rosenquist H, Smidt L, Andersen SR, Jensen GB, Wilcks A. 2005. Occurrence and significance of *Bacillus cereus* and *Bacillus thuringiensis* in ready-to-eat food. FEMS Microbiology Letters 250: 129-136.
Roubos-van den Hil PJ, Dalmas E, Nout MJR, Abee T. 2010. Soya bean tempe extracts show antibacterial activityt against *Bacillus cereus* cells and spores. Journal of Applied Microbiology 109: 137-145.
Ruusunen M, Salonen M, Pulkkunen H, Huuskonen M, Hellström S, Revez J, Hänninen ML, Fredriksson-Ahomaa M, Lindström M. 2013. Pathogenic bacteria in Finnish bulk tank milk. Foodborne Pathogens and Disease 10: 99-106.
Sasahara T, Hayashi S, Morisawa Y, Sakihama T, Yoshimura A, Hirai Y. 2011. *Bacillus cereus* bacteremia outbreak due to contaminated hospital linens. European Journal of Clinical Microbiology and Infectious Diseases. 30(2):219-26.
Samapundo S, Heyndrickx M, Xhaferi R, Devlieghere F. 2011. Incidence, diversity and toxin gene characteristics of *Bacillus cereus* group strains isolated from food products marketed in Belgium. International Journal of Food Microbiology 150: 34-41.
Sattar S, Biswas PK, Hossain MA, Maiti MK, Sen SK, Basu A. 2008. Search for vegetative insecticidal proteins (VIPs) from local isolates of *Bacillus thuringiensis* effective against lepidopteran and homopteran insect pests. Journal of Biopesticides 1: 216-222.
Scatena R. 2012. Chaper 15: Mitochondria and Drugs. In Advances in Mitochondrial Medicine, Advances in Experimental Medicine and Biology, editors

Roberto Scatena, Patrizia Bottoni and -Bruno Giardina. Springer Science+Business Media B.V. 942: 329-346.
doi: 10.1007/978-94-007-2869-1_15.
Schuit F, van Lommel L, Granvik M, Goyvaerts L, de Faudeur G, Schraenen A, Lemaire K. 2012. β-cell-specific gene repression: a mechanism to protect against inappropriate or maladjusted insulin secretion? Diabetes 61: 969-975.
Sehulster L, Chinn RY. 2003. Guidelines for environmental infection control in health-care facilities. Recommendations of CDC and the Healthcare Infection Control Practices Advisory Committee (HICPAC). MMWR Recommendations and Reports 52:1–42
Setlow P. 2003. Spore germination. Current Opinion in Microbiology 6: 550-556.
Shaheen R. 2009. *Bacillus cereus* spores and cereulide in food-borne illness. Ph.D thesis. University of Helsinki, Department of Applied Chemistry and Microbiology, Helsinki, Finland. https://helda.helsinki.fi/bitstream/handle/10138/20853/bacillus.pdf?sequence=2 visited 07.05.2014.
Shinagawa K. 1990. Analytical methods for *B. cereus* and other *Bacillus* species. International Journal of Food Microbiology 10: 125-142.
Shiota M, Saitou K, Mizumoto H, Matsusaka M, Agata N, Nakayama M, Kage M, Tatsumi S, Okamoto A, Yamaguchi S, Ohta M, Hata D. 2010. Rapid detoxification of cereulide in *Bacillus cereus* food poisoning. Pediatrics 125: e951-e955.
Sifuentes LY, Gerba CP, Wearts I, Engelbrecht K, Koenig DW. 2013. Microbial contamination of hospital reusable cleaning towels. American Journal of Infection Control 41: 912-915.
Skalska J, Debska G, Kunz WS, Szewczyk A. 2005. Antidiabetic sulphonylureas activate mitochondrial permeability transition in rat skeletal muscle. British Journal of Pharmacology 145:785-791.
Stark T, Marxen S, Rütschle A, Lücking G, Scherer S, Ehling-Schulz M, Hofmann T. 2012. Mass spectrometric profiling of *Bacillus cereus* strains and quantitation of the emetic toxin cereulide by means of stable isotope dilution analysis and HEp-2 bioassay. Analytical and Bioanalytical Chemistry 405: 191-201.
Stenfors Arnesen LP, Fagerlund A, Granum PE. 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. FEMS Microbiology Reviews 32: 579-606.
Stock D, Leslie AGW, Walker JE. 1999. Molecular architecture of the rotary motor in ATP synthase. Science 286: 1700-1705.
Sudershan RV, Kumar RN, Kashinath L, Bhaskar V, Polasa K. 2012. Microbiological hazard identification and exposure assessment of poultry products sold in various localities of Hyderabad, India. The Scientific World Journal doi: 10.1100/2012/736040
Suominen I. 2005. *Paenibacillus* and *Bacillus* related to paper and spruce tree. Ph.D thesis. University of Helsinki, Department of Applied Chemistry and Microbiology, Helsinki, Finland.Table 4, p 16-19.

http://ethesis.helsinki.fi/julkaisut/maa/skemi/vk/suominen2/paenibac.pdf visited 11.07.2014.
Szewczyk A. and Wojtczak L. 2002. Mitochondria as a pharmacological target. Pharmacological Reviews 54: 101-127.
Takabe F, Oya M. 1976. An autopsy case of food poisoning associated with *Bacillus cereus*. Forensic Science 7: 97-101.
Talent SM, Rhodehamel EJ, Harmon SM, Bennett RW. 2012. Chapter 14 *Bacillus cereus.* in Bacteriological Analytical Methods (BAM) of the US Food and Drug Administration, 8th Edition, Revision A . Online. Accessed 02.06.2014 http://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm070875.htm
Taylor AJ, Gilbert RJ. 1975. *Bacillus cereus* food poisoning: a provisional serotyping scheme. Journal of Medical Microbiology 8: 543-550.
Temper K. 1963. Exitus letalis nach Lebensmittelvergiftung durch Bazillus cereus. (Lethal outcome following food intoxication by *Bacillus* cereus) in German. Zeitschrift fuer die Gesamte Hygiene und ihre Grenzgebiete. **9**: 481-490.
Teplova VV, Mikkola R, Tonshin AA, Saris NE, Salkinoja-Salonen MS. 2006. The higher toxicity of cereulide relative to valinomycin is due to its higher affinity for potassium at physiological plasma concentration. Toxicology and Applied Pharmacology 210:39–46.
Thorsen L, Hansen BM, Nielsen KF, Hendriksen NB, Phipps RK, Bjørn Budde B. 2006. Characterization of emetic *Bacillus weihenstephanensis*, a new cereulide-producing bacterium. Applied and Environmental Microbiology 72: 5118-5121.
Toh M, Moffitt MC, Henrichsen L, Raftery M, Barrow K, Cox JM, Marquis CP, Neilan BA. 2004. Cereulide, the emetic toxin of *Bacillus cereus*, putatively a product of nonribosomal peptide synthesis. Journal of Applied Microbiology 97: 992-1000.
Tsiper MV, Sturgis J, Avramova LV, Parakh S, Fatig R, Juan-Garcia, A, Li N, Rajwa B, Narayanan P, Qualls Jr CW, Robinson JP, Davisson VJ. 2012. Differential mitochondrial toxicity screening and multi-parametric data analysis. PLoS ONE 7: e45226.
Turabelidze G, Gee JE, Hoffmaster AR, Manian F, Butler C, Byrd D, Schildknecht S, Chavez Hauser L, Duncan M, Ferrett R, Evans D, Talley C. 2013. Contaminated ventilator air flow sensor linked to *Bacillus cereus* colonization of newborns. Emerging Infectious Diseases 19: 781-783.
Turnbull PCB, Kramer JM, Jørgensen K, Gilbert RJ, Melling J. 1979. Properties and production characteristics of vomiting, diarrheal, and necrotizing toxins of *Bacillus cereus*. The American Journal of Clinical Nutrition 32: 219-228.
Turner NJ, Whyte R, Hudson JA, Kaltovei SL. 2006. Presence and growth of *Bacillus cereus* in dehydrated potato flakes and hot-held, ready-to-eat potato products purchased in New Zealand. Journal of Food Protection 69: 1173-1177.
Ueda S, Kuwabara Y. 1993. An ecological study of *Bacillus cereus* in rice crop processing – putative emetic toxin production by *B. cereus* strains isolated at

various stages of the process. Journal of Antibacterial and Antifungal Agents 21: 499-502.
Ueda S, Nakajima H, Iwase M, Shinagawa K, Kuwabara Y. 2012. LC-MS analysis of the emetic toxin, cereulide, produced by *Bacillus cereus*. Biocontrol Science 17: 191-195.
Uhlig S, Torp M, Heier BT. 2006. Beauvericin and enniatins A, A1, B and B1 in Norwegian grain: a survey. Food Chemistry 2: 193-201.
Urbaniak M. 2013. Chapter 4: Biodegradation of PCDDs/PCDSs and PCBs. In Biodegradation – Engineering and Technology. Edited by Rolando Chamy and Francisca Rosenkranz. ISBN 978-953-51-1153-5
Vachon V, Laprade R, Schwartz J-L. 2012. Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: A critical review. Journal of Invertebrate Pathology 111: 1-12.
van Melis CCJ, Nierop Groot MN, Abee T. 2011. Impact of sorbic acid on germinant receptor-dependent and -independent germination pathways in *Bacillus cereus.* Applied and Environmental Microbiology, 77: 2552–2554.
Virtanen SM, Roivainen M, Andersson MA, Ylipaasto P, Hoornstra D, Mikkola R, Salkinoja-Salonen MS. 2008. In vitro toxicity of cereulide on porcine pancreatic Langerhans islets. Toxicon 51: 1029-1037.
Wijnands LM, Dufrenne JB, Rombouts FM, in 't Veld PH, van Leusden FM. 2006. Prevalence of potentially pathogenic *Bacillus cereus* in food commodities in the Netherlands. Journal of Food Protection 69: 2587-2594.
Williams LD, Burdock GA, Jiménez G, Castillo M. 2009. Literature review on safety of Toyocerin ®, a non-toxigenic and non-pathogenic *Bacillus cereus* var. *toyoi* preparation. Regulatory Toxicology and Pharmacology 55: 236-246.
Wojtczak L, Zablocki K. 2008. Chapter 1: Basic mitochondrial physiology in cell viability and death" in Drug-Induced mitochondrial dysfunction. edited by James A. Dykens and Yvonne Will. John Wiley & Sons, Inc. New Jersey. USA. p 3-35.
Wong DT, Berg DH, Hamill RH, Wilkinson JR. 1977. Ionophorous properties of narasin a new poly ether mono carboxylic acid antibiotic in rat liver mitochondria. Biochemical Pharmacology 26: 1373-1376.
Yamaguchi M, Kawai T, Kitagawa M, Kumeda Y. 2013. A new method for rapid and quantitative detection of the *Bacillus cereus* emetic toxin cereulide in food products by liquid chromatography-tandem mass spectrometry analysis. Food Microbiology 34: 29-37.
Yokoyama K, Ito M, Agata N, Isobe M, Shibayama K, Horii T, Ohta M. 1999. Pathological effects of synthetic cereulide, an emetic toxin of *Bacillus cereus*, is reversible in mice. FEMS Immunology and Medical Microbiology 24: 115-120.