Bestimmung des Endosporenanteils an der mikrobiellen Lebensgemeinschaft in Wattsedimenten

Determination of the abundance of endospores within the microbial community in tidal flat sediments

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für meine Familie

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Kurzfassung

Endosporen sind bakterielle Überdauerungsstadien, die keine nachweisbare Stoffwechselaktivität aufweisen und sehr lange Zeiträume überleben können. Es wird deshalb angenommen, dass der Anteil von Endosporen an der mikrobiellen Gemeinschaft in Sedimenten mit zunehmender Tiefe ansteigt. Bisher mangelte es jedoch an einem geeigneten Verfahren, um die Anzahl an Endosporen in Sedimenten zuverlässig bestimmen zu können.

In der vorliegenden Arbeit werden zwei Methoden zur Quantifizierung von Endosporen beschrieben, beide nutzen Dipicolinsäure (DPA) als Biomarker. Über diese Verfahren wurde der Endosporenanteil an der mikrobiellen Gemeinschaft in marinen Sedimenten abgeschätzt.

DPA lässt sich fluorimetrisch nach Komplexierung mit Terbium nachweisen. Diese Fluoreszenzmethode bietet eine sehr hohe Empfindlichkeit, in Sedimentextrakten wurde allerdings eine fast vollständige Auslöschung des Fluoreszenzsignals beobachtet. Außerdem wurde die Fluoreszenz der Terbiumdipicolinatkomplexe meist durch eine starke Untergrundfluoreszenz beeinflusst, welche höchstwahrscheinlich auf Huminstoffe zurückzuführen ist. Deshalb musste ein Verfahren zur Aufreinigung von Sedimentextrakten entwickelt werden.

Die erste Methode beruhte auf der Extraktion von DPA mit Essigsäureethylester und ermöglichte eine Bestimmung von DPA in Sedimentproben aus dem Rückseitenwatt der Insel Spiekeroog (deutsches Wattenmeer). Zur Umrechnung der Gehalte in Endosporenzahlen wurde ein mittlerer DPA-Gehalt von $2{,}24\times10^{-16}$ mol pro Spore angenommen. Dieser Mittelwert wurde für die Endosporen verschiedener Stämme ermittelt, die aus dem Rückseitenwatt in einer früheren Studie isoliert worden waren. Die Endosporen dieser Stämme zeigten keine großen Unterschiede in ihren DPA-Gehalten, auf der Basis des Mittelwerts sollte deshalb eine Bestimmung von Endosporenzahlen in den entsprechenden Wattsedimenten möglich sein. Über dieses analytische Verfahren wurde die quantitative Bedeutung von Endosporen in den untersuchten Wattsedimenten abgeschätzt. Allerdings wurde über die Extraktion mit Essigsäureethylester keine vollständige Abtrennung von störenden quenchenden und fluoreszierenden Substanzen erreicht. Die Quantifizierung von DPA in den aufgereinigten Sedimentextrakten erforderte deshalb ein sehr arbeitsintensives Standardadditionsverfahren, um Matrixeffekte zu berücksichtigen.

Eine höhere Empfindlichkeit bei gleichzeitig deutlich geringerer Analysenzeit wurde durch die Entwicklung eines HPLC-Systems zur fluorimetrischen Bestimmung von DPA über *Post-Column*-Komplexierung erreicht. Die Nachweisgrenze des entwickelten HPLC-Systems liegt bei 0,5 nmol DPA l^{-1} bzw. etwa 10^3 Endosporen pro ml. Auch für den schnellen kultivierungsunabhängigen Nachweis von Sporenkontaminationen in Nahrungsmitteln ist diese Methode geeignet. Dies wurde anhand der Analyse von Pfefferproben demonstriert. Pfeffer zeigt allgemein eine hohe Belastung mit Endosporen. Zur Aufreinigung der stark fluoreszierenden Pfefferextrakte wurde ein geeignetes Extraktionsverfahren entwickelt. Außerdem wurde gezeigt, dass das charakteristische Fluoreszenzspektrum der Terbiumdipicolinatkomplexe auch im Fall von Koelutionen eine Auswertung von Fluoreszenzchromatogrammen ermöglicht. Für die Quantifizierung von DPA in Sedimentproben aus dem Rückseitenwatt der Insel Spiekeroog war dieses erweiterte Detektionsverfahren jedoch nicht notwendig.

In der vorliegenden Arbeit wurden bis zu 5,5 m lange Sedimentkerne von einem Sand- und einem Mischwattbereich auf ihren DPA-Gehalt hin untersucht. Bei den Analysen wurden DPA-Gehalte zwischen 0,02 und 4,4 nmol DPA g^{-1} Sediment bzw. Endosporengehalte zwischen 1×10^5 und 2×10^7 Endosporen g^{-1} Sediment (jeweils bezogen auf Trockengewicht) ermittelt. Interessanterweise spiegelten die Endosporen-Tiefenprofile die Lithologieänderungen in den Kernen wider. Die höchsten Sporenabundanzen wurden in dünnen schwarzen Schlickschichten bestimmt. Sandlagen waren durch deutlich geringere Endosporengehalte gekennzeichnet. In den oberen 50 cm der Sedimentkerne wurde ein Endosporenanteil von weniger als 1 % der Gesamtzellzahl bestimmt. In den tieferen Schichten stieg der Sporenanteil aber auf bis zu 10 % an.

Die relative Zunahme könnte auf die extreme Langlebigkeit von Endosporen zurückgeführt werden, während die Anzahl vegetativer Zellen mit zunehmender Sedimenttiefe in Folge von eintretendem Nährstoffmangel vermutlich deutlich abnimmt. Aus diesem Grund und im Hinblick auf die enorme vertikale Ausdehnung der marinen Sedimente wäre zu erwarten, dass Endosporen signifikant zur tiefen Biosphäre beitragen.

Summary

Bacterial endospores are resting stages without detectable metabolism. They can remain viable for a long time and hence might accumulate in sediments during burial. Because of methodological problems, the number of endospores in sediments has only rarely been quantified. Consequently, little is known about the quantitative contribution of endospores to the total number of procaryotic cells.

The present work reports on two protocols to determine the number of endospores. Furthermore, it provides quantitative cultivation-independent data concerning the contribution of endospores to total cell counts in marine sediments.

Both methods are based on the fluorimetric determination of dipicolinic acid (DPA), a spore specific compound, detected as complex after reaction with terbium chloride. This technique offers a high sensitivity, but suffers from interference of sediment constituents. Fluorescence of terbium dipicolinate complexes is almost completely quenched in sediment extracts, but also masked by background fluorescence due to humic substances. To overcome these interferences, a method for extraction of DPA with ethyl acetate was developed. This procedure was applied to determine the DPA content of sediment samples collected from tidal flats off the German North Sea coast (Wadden Sea). For conversion into endospore numbers an average DPA content of 2.24×10^{-16} mol per spore was used. This value was determined for the endospores of isolates obtained from tidal flats. The spores of these strains showed very similar DPA contents and should provide a suitable basis for an estimation of endospore numbers in the respective tidal flat sediments. This analytical approach permitted an insight into the quantitative importance of endospores in marine sediments. However, the quantification of DPA in purified sediment extracts required a standard addition procedure, since quenching was not completely inhibited and the signal was still affected by background fluorescence. In addition, purification and standard addition procedures were labour-intensive and time-consuming, emphasizing the need for a faster and more sensitive approach.

A higher sensitivity and shorter analysis time was achieved by development of an HPLC system for post-column complexation and fluorimetric detection of DPA. The HPLC method permitted the detection of 0.5 nmol DPA l^{-1}, corresponding to about 10^3 endospores per ml, and can also be used for the rapid assessment of endospore contamination in food products. This

was demonstrated by analysis of pepper, which generally is heavily loaded with endospores. For purification of highly fluorescent pepper extracts, a specific extraction protocol was developed. It was demonstrated that DPA can even be quantified in the case of coeluting substances by utilizing the characteristic emission spectrum of terbium dipicolinate complexes. However, this special quantification technique was not necessary for determination of DPA concentrations in marine sediments, the main objective of this study.

In the present work, up to 5.5 m long sediment cores were taken from a mixed and a sand flat in the backbarrier tidal flat of Spiekeroog Island and analyzed for their DPA depth profile. DPA contents ranged from 0.02 to 4.4 nmol DPA g^{-1} sediment dry weight, corresponding to 1×10^5 to 2×10^7 endospores g^{-1} sediment dry weight. The endospore depth profiles were irregular, but reflected the vertical changes in lithology. The highest endospore numbers were found in thin black mud layers, significantly lower numbers in sandy layers. In the uppermost 50 cm of the sediment section endospore numbers represented less than 1 % of the total cell counts. However, in the layers beneath their contribution to total cell counts apparently increased with depth reaching up to 10 %.

The relative increase can be explained by the extreme longevity of endospores, whereas numbers of vegetative cells are expected to decrease more rapidly due to starvation. Considering the vast vertical extension of the marine subsurface, it can be expected that endospores contribute substantially to the deep biosphere.

Abbildungsverzeichnis

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Abkürzungen

Abb.	Abbildung
ADP	Adenosindiphosphat
AMP	Adenosinmonophosphat
AO	Acridin Orange
ASE	*accelerated solvent extraction*
ATP	Adenosintriphosphat
CARD-FISH	*catalyzed reporter deposition-fluorescence in-situ hybridization*
CFU	*colony-forming units*
ChA	*chelidamic acid*
DAPI	4´-6-Diamino-2-phenylindol
DFG	Deutsche Forschungsgemeinschaft
DPA	*dipicolinic acid*
Fig.	*figure*
g	Erdbeschleunigung
GDP	Guanindiphosphat
GMP	Guaninmonophosphat
GP	Gröninger Plate
GTP	Guanintriphosphat
HPLC	*high-performance liquid chromatography*
ICBM	Institut für Chemie und Biologie des Meeres
JS	Janssand
MPN	*most probable number*
NAD	Nicotinsäureamid-Adenin-Dinucleotid
NSN	Neuharlingersieler Nacken
PCR	*polymerase chain reaction*
PDA	*photodiode array detector*
psi	*pounds per square inch*
RNA	*ribonucleic acid*
rRNA	*ribosomal ribonucleic acid*
RP-HPLC	*reversed-phase high-performance liquid chromatography*
SASPs	*small acid-soluble spore proteins*
TOC	*total organic carbon*
UV	Ultraviolett

1 Einleitung

1.1 Vorkommen und Aktivität von Prokaryonten in marinen Sedimenten

1.1.1 Die tiefe Biosphäre

In den letzten 20 Jahren wurde im Rahmen von internationalen Tiefseebohrprogrammen festgestellt, dass Mikroorganismen in marinen Sedimenten in hoher Anzahl vorhanden sind (Parkes et al., 1990, 1994). Mikroskopisch bestimmte Gesamtzellzahlen (z.B. Parkes et al., 2000) sowie der Nachweis von intakten Membranlipiden (Zink et al., 2003; Sturt et al., 2004) in Sedimentproben zeigten, dass selbst 800 Meter unter dem Meeresboden noch zahlreiche Prokaryonten vorkommen. Aktuellen Untersuchungen zufolge sind sogar in 1600 m Sedimenttiefe noch Prokaryonten nachzuweisen (R.J. Parkes, persönliche Mitteilung). Geht man von einer Maximaltemperatur für Leben von 121 °C aus (Kashefi & Lovley, 2003), so könnte die Erdkruste sogar bis in Tiefen von etwa viertausend Metern von Mikroorganismen besiedelt sein (Whitman et al., 1998).

Die Bedeutung dieser tiefen Biosphäre liegt in ihrem enormen Beitrag zur Biomasse der Erde. An der Sedimentoberfläche findet man typischerweise zwischen 10^8 und 10^9 Zellen pro cm^3 Sediment (Parkes et al., 1994). Höhere Zellzahlen können in Sedimentschichten auftreten, in denen den Mikroorganismen mehr Energie zum Beispiel durch das Aufeinandertreffen von Sulfat und Methan zur Verfügung steht (Parkes et al., 2005). Mit der Tiefe nehmen die Zellzahlen jedoch normalerweise kontinuierlich ab. Allerdings lassen sich knapp oberhalb der ozeanischen Kruste meist immer noch etwa 10^6 Zellen pro cm^3 Sediment über Epifluoreszenzmikroskopie nachweisen (z.B. Parkes et al., 1994). Auf der Grundlage dieser Zellzahlen wurde von Whitman et al. (1998) abgeschätzt, dass sich 3.6×10^{30} Prokaryonten in der marinen tiefen Biosphäre befinden. Diese enorme Anzahl an Mikroorganismen in marinen Sedimenten entspricht einer Biomasse von 3×10^{17} g Kohlenstoff bzw. etwa 30 % der gesamten Biomasse auf der Erde. Eine frühere Abschätzung von Parkes et al. (1994) belief sich aufgrund anderer Annahmen (unterschiedliche Tiefenextrapolation der Daten, Annahme eines geringeren Kohlenstoffgehalts der Zellen) auf immerhin 10 % der Gesamtbiomasse.

1.1.2 Abschätzung der Aktivität von Zellen in der tiefen Biosphäre

Die über Epifluoreszenzmikroskopie bestimmten Gesamtzellzahlen geben keine Informationen über die Stoffwechselaktivität der detektierten Zellen. Zur Bestimmung von Gesamtzellzahlen werden Fluoreszenzfarbstoffe wie zum Beispiel Acridin Orange (AO) oder DAPI (4´-6-Diamino-2-phenylindol) verwendet. Diese Farbstoffe binden unabhängig vom metabolischen Zustand an die DNA von Zellen. Die über AO oder DAPI bestimmten Gesamtzellzahlen umfassen daher sowohl metabolisch aktive als auch inaktive sowie tote Zellen (Kepner & Pratt, 1994). Aufgrund dieser Tatsache ist davon auszugehen, dass die Biomasse in der tiefen Biosphäre auf der Grundlage der mit AO bestimmten Gesamtzellzahlen überschätzt wird (Smith & D'Hondt, 2006).

Eine Unterscheidung von aktiven und inaktiven Zellen ist für die Bestimmung der Aktivität der Prokaryonten notwendig. Über die Modellierung des Flusses von Elektronenakzeptoren kann die mittlere metabolische Aktivität von Populationen in der tiefen Biosphäre abgeschätzt werden (z.B. D'Hondt et al., 2002). Die berechneten Aktivitäten sind jedoch um Größenordnungen geringer als die in Kultivierungsexperimenten und in oberflächennahen Sedimenten bestimmten Umsatzraten (D'Hondt et al., 2002). Nach bisherigen Kenntnissen sind diese Aktivitäten zu gering, um den Energiebedarf für den Erhaltungsstoffwechsel von Mikroorganismen zu decken (Price & Sowers, 2004). Auf der Grundlage dieser Daten ergibt sich eine mittlere Verdoppelungszeit von über 1000 Jahren für die Mikroorganismen in der tiefen Biosphäre (Jørgensen & D'Hondt, 2006).

Eine mögliche Erklärung für die geringen mittleren Aktivitäten wäre ein signifikanter Anteil von inaktiven Überdauerungsstadien oder toten Zellen an der Gesamtzellzahl (Jørgensen & D'Hondt, 2006). Luna et al. (2002) haben über spezielle Färbetechniken abgeschätzt, dass tote und inaktive Zellen selbst in wenigen Zentimetern Sedimenttiefe einen Anteil von über 90 % an der Gesamtzellzahl haben könnten. Berücksichtigt man nur den Anteil aktiver Bakterien an der Gesamtzellzahl, so würden in diesem Fall die zellspezifischen Aktivitäten um eine Größenordnung höher liegen. Die Quantifizierung von aktiven Mikroorganismen und die Unterscheidung von inaktiven und toten Zellen ist deshalb Gegenstand aktueller Forschung. Eine empfindliche Möglichkeit zum Nachweis aktiver Mikroorganismen bietet die *catalyzed reporter deposition-fluorescence in-situ hybridization* (CARD-FISH). Bei dieser Methode nutzt man die Tatsache, dass der rRNA-Gehalt von Zellen in etwa proportional zu ihrer metabolischen Aktivität ist (Molin & Givskov,

1999). Zum Anfärben aktiver Zellen verwendet man Oligonukleotid-Sonden, die kovalent an ein spezielles Enzym gebunden sind. Nach erfolgreicher Anlagerung der Sonden an die rRNA der Zellen katalysiert dieses Enzym die Produktion eines fluoreszierenden Signalstoffes, der in der Zelle akkumuliert und somit zu einem deutlichen Fluoreszenzsignal führt (Pernthaler et al., 2002). Schippers et al. (2005) haben diese Methode verwendet, um den Anteil aktiver Zellen an Populationen in der tiefen Biosphäre abzuschätzen. Dabei stellten sie fest, dass sich über CARD-FISH nur maximal 30 % der über AO angefärbten Zellen detektieren lassen. Dies weist darauf hin, dass nur ein Bruchteil der über AO erfassten Zellen einen aktiven Metabolismus aufweist. Ein Großteil der Zellen könnte folglich tot oder inaktiv sein. Es wäre auch denkbar, dass ein signifikanter Anteil der Zellen in Form von inaktiven Überdauerungsstadien – wie zum Beispiel Endosporen – vorliegt.

1.2 Endosporen als Überdauerungsstadien

1.2.1 Klassifizierung der endosporenbildenden Bakterien

Überdauerungsstadien werden von einer Reihe von Bakterien bei eintretendem Nährstoffmangel oder zum Überleben extremer Umweltbedingungen gebildet. Im Allgemeinen werden diese differenzierten Zellen als Sporen bezeichnet, in einigen Fällen spricht man auch von Cysten. Eine besonders widerstandsfähige Art von Sporen findet man unter den Bakterien des Phylums *Firmicutes*. Bei diesen werden die Sporen im Inneren der Bakterienzellen gebildet, sie werden deshalb als Endosporen bezeichnet (z.B. Marahiel & Zuber, 1999).

Endosporenbildende Bakterien sind vorwiegend in Böden und Sedimenten zu finden (Slepecky, 1972; Slepecky & Leadbetter, 1983). Aufgrund ihrer hohen Resistenz und Langlebigkeit können sich Endosporen aber auch äolisch, zum Beispiel als Anhaftung an Saharastaub, über weite Strecken verbreiten (Gorbushina et al., 2007).

Endosporenbildner sind meist stäbchenförmige, Gram-positive Bakterien. Als die bekanntesten Vertreter sind die Gattung *Bacillus*, die strikt aerobe, aber auch fakultativ anaerobe Arten umfasst, sowie die strikt anaerobe Gattung *Clostridium* zu nennen. Des Weiteren findet man die Möglichkeit zur Sporenbildung bei Vertretern der Gattung *Desulfotomaculum* sowie unter den Thermophilen der Gattung *Thermoactinomyces*. Kokkenförmige En-

dosporenbildner sind die strikt aeroben Bakterien der Gattung *Sporosarcina* (Hobbs & Cross, 1983; Perry & Staley, 1997). Es gibt aber auch Gram-negative Endosporenbildner. Dazu gehören Vertreter der Gattungen *Desulfotomaculum* und *Desulfosporosinus* (Campbell & Postgate, 1965; Stackebrandt et al., 1997) sowie die Gattung *Sporomusa* (Möller et al., 1984).

Endosporenbildende Bakterien zeigen eine hohe metabolische Diversität. Das Potential zur Sporenbildung findet man beispielsweise bei Milchsäure-Gärern (z.B. *Sporolactobacillus*), Sulfatreduzierern (z.B. *Desulfotomaculum*), homoacetogenen (z.B. *Sporomusa*), syntrophen (z.B. *Syntrophospora*) sowie anoxygen phototrophen Bakterien (z.B. *Heliobacterium*) und N_2-Fixierern (z.B. *Clostridium pasteurianum*). Eine Unterscheidung der Arten ist unter anderem mikrokopisch anhand der Form der Sporen und deren Lage in der Mutterzelle möglich (Madigan et al., 2000).

Die meisten Sporenbildner produzieren eine Endospore, *Anaerobacter polyendosporus* kann allerdings bis zu fünf Endosporen pro Zelle bilden (Duda et al., 1987). Gewöhnlich dient die Bildung von Endosporen jedoch nicht der Reproduktion.

1.2.2 Sporulationszyklus

Initiierung der Sporulation Endosporen stellen kein obligat zu durchlaufendes Stadium im Lebenszyklus eines Endosporenbildners dar. Zur Bildung von Sporen kommt es normalerweise nur am Ende der exponentiellen Wachstumsphase, wenn das Substrat verbraucht ist oder der Mangel eines anderen Faktors wie N- oder P-Quelle limitierend wirkt (Vinter, 1969). Eine Sporulation von Zellen lässt sich zum Beispiel durch den kontrollierten Entzug von Nährstoffen in chemisch definierten Medien oder durch Resuspension wachsender Zellen in ein Nährstoffmangelmedium induzieren (Sterlini & Mandelstam, 1969).

Die Nährstofflimitierung führt zu einer Verringerung der intrazellulären Konzentrationen an Guanindiphosphat (GDP) und Guanintriphosphat (GTP), wodurch vermutlich die Sporulation induziert wird (Lopez et al., 1979; Freese, 1981). Mitani et al. (1977) haben gezeigt, dass die Sporulation durch Hemmung, aber nicht vollständige Unterdrückung der Nukleotid-Synthese auch in Gegenwart von Nährstoffen ausgelöst werden kann. Zum Beispiel konnte durch Zugabe von Decoyinin, einem Inhibitor der Synthese von Guaninmonophosphat (GMP), ein signifikanter Anteil der Zellen einer

exponentiell wachsenden Kultur von *Bacillus subtilis* zur Sporenbildung angeregt werden.

Kulturen von Sporenbildnern unterscheiden sich oft stark in ihrem Sporulationsgrad. In den meisten Fällen kann nur ein Bruchteil der Zellen zur Sporenbildung angeregt werden. Der Sporulationsgrad der Kulturen variiert dabei mit der Zusammensetzung des Mediums, unterscheidet sich aber auch stark von Art zu Art. Während zum Beispiel *Bacillus popilliae* bei optimaler Acetat-Konzentration auf Platten einen maximalen Sporulationsgrad von 0.3 % zeigte und keine Sporen in Flüssigmedium bildete (Rhodes et al., 1965), konnte bei *Bacillus subtilis* ein Sporulationsgrad von > 70 % in Flüssigkultur erreicht werden (Oh & Freese, 1976). Dieser hohe Sporenanteil wurde jedoch nur in Gegenwart ausreichender Mengen an Mangan erzielt, da dieses Spurenelement als Cofaktor für an der Sporulation beteiligte Enzyme benötigt wird (Charney et al., 1951; Oh & Freese, 1976).

Die Bildung von Endosporen ist ein energetisch aufwendiger Prozess, der etwa acht Stunden benötigt (Driks, 1999) und ab einem frühen Stadium irreversibel ist. Eine vollständige Sporulation von Populationen kann deshalb von Nachteil sein, falls nur ein kurzfristiger Nährstoffmangel überbrückt werden muss. Sporenbildner scheinen deshalb eine Taktik entwickelt zu haben, um die Sporenbildung hinauszuzögern. In Kulturen von *Bacillus subtilis* haben González-Pastor et al. (2003) ein kannibalistisches Verhalten beobachtet. Zellen, die bereits in den Sporulationszyklus eingetreten sind, produzieren ein Signalprotein und ein Toxin. Diese Substanzen unterbinden eine Sporulation von Schwesterzellen und führen zur deren Lyse. Durch die Lyse der Schwesterzellen werden Nährstoffe freigesetzt, wodurch die Sporulation verzögert werden kann. Aus diesem Grund kommt es zur Ausbildung von zwei Fraktionen innerhalb der Population. Maximal 80 % der Zellen bilden Sporen, die restlichen 20 % treten in eine stationäre Phase ein (Graumann, 2006).

Bildung und Freisetzung von Endosporen Während der Sporulation erfolgt die Umwandlung einer vegetativen Zelle in eine metabolisch inaktive und sehr widerstandsfähige Endospore. Die Sporenbildung (Sporogenese) stellt einen der kompliziertesten Prozesse der Zelldifferenzierung von Bakterien dar. Sie lässt sich in verschiedene Stadien unterteilen (siehe Abb. 1).

Die Bildung von Endosporen beginnt mit der Replikation des genetischen Materials, gefolgt von einer speziellen inäqualen Zellteilung. Durch

Einschnürung der Cytoplasmamembran wird ein Teil des Protoplasten von der Mutterzelle abgetrennt. Der sich durch die Einschnürung bildende Sporenprotoplast wird von der Cytoplasmamembran der Mutterzelle umwachsen und eingehüllt. Dies hat zur Folge, dass der Sporenprotoplast von zwei Cytoplasmamembranen umgeben ist. Zwischen beiden Membranen erfolgt die Synthese einer mehrschichtigen Ummantelung des Sporenprotoplasten (siehe Kapitel 1.2.3). Während der Sporogenese findet zudem die Synthese von sporenspezifischen Substanzen statt, die eine wichtige Rolle für die hohe Resistenz von Endosporen spielen (siehe Kapitel 1.2.5). Im Sporenprotoplasten werden große Mengen an kleinen säurelöslichen Proteinen gebildet. Gleichzeitig wird Dipicolinsäure (siehe Kapitel 1.2.4) in der Mutterzelle synthetisiert und zusammen mit Calciumionen durch die Vorspore (engl. *prespore*) aufgenommen. Am Ende der Sporogenese werden die reifen Sporen durch Lyse der Mutterzelle freigesetzt (Errington, 2003).

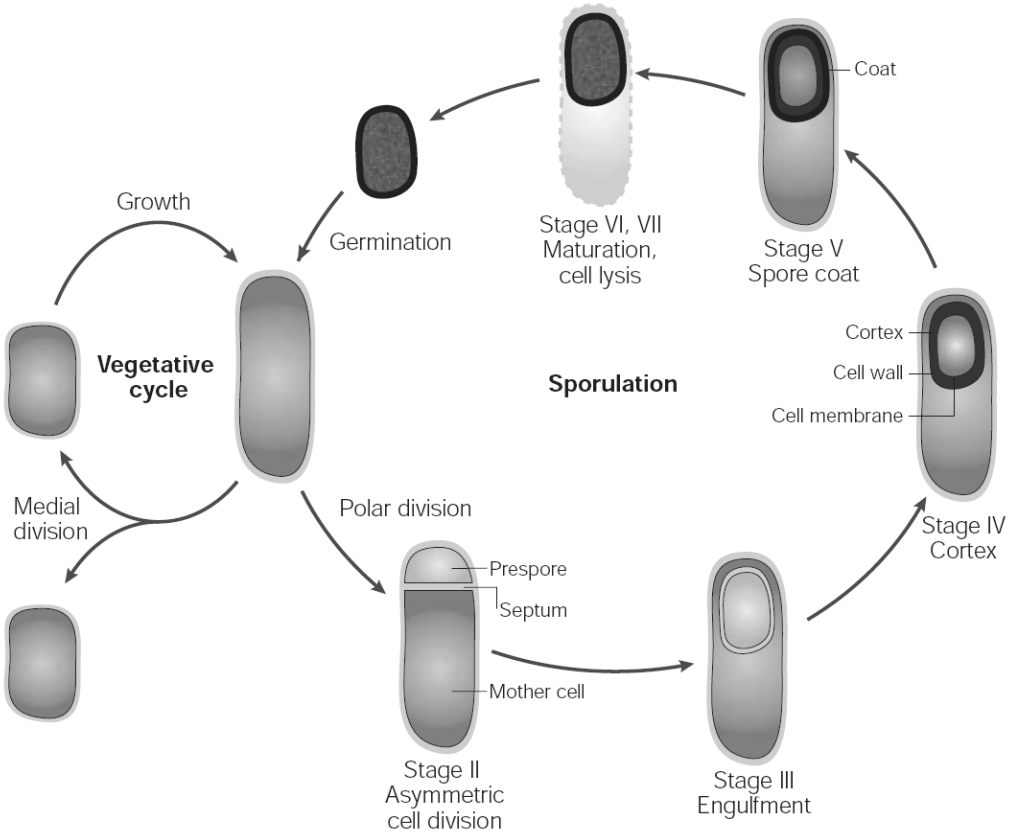

Abb. 1: Vereinfachte Darstellung des Sporulationszyklus von *Bacillus subtilis* (Errington, 2003).

Germination Als Germination (Auskeimung) bezeichnet man die Umwandlung einer resistenten inaktiven Endospore in eine neue vegetative, metabolisch aktive, aber weniger resistente Zelle, wodurch letztendlich der Sporulationszyklus (siehe Abb. 1) geschlossen wird.

Die Germination wird durch spezifische Nährstoffe (engl. *Germinants*) induziert, die an Rezeptoren auf der inneren Membran binden. Entsprechende *Germinants* müssen daher zunächst die Sporenhülle durchdringen, um mit den Rezeptoren interagieren zu können (Moir, 2006). Zur Germination ist keine Synthese makromolekularer Strukturen notwendig. Der komplette Apparat zum Auskeimen ist in der Spore schon vorhanden (Gould, 1969). Eine Metabolisierung von *Germinants* findet während der frühen Phase der Germination anscheinend nicht statt (Scott & Ellar, 1978a).

Gewöhnlich besitzen Endosporen mehrere verschiedene Rezeptoren und reagieren daher auf mehr als ein *Germinant*. Sporen von *Bacillus subtilis* besitzen beispielsweise drei verschiedene Typen von Rezeptoren (Vepachedu & Setlow, 2007). Ein Auskeimen von Endosporen kann durch die Zugabe von Aminosäuren, Kohlenhydraten und Nukleosiden induziert werden (Gould, 1969). Eine effektive Germination wird allerdings oft nur durch eine Kombination unterschiedlicher *Germinants* erreicht. Bei Verwendung von L-Alanin zusammen mit Inosin haben sich in einer Studie von Foerster & Foster (1966) die meisten Endosporen verschiedener *Bacillus*-Arten zum Auskeimen anregen lassen. Die gleichzeitige Zugabe von Glucose führte dagegen nur in wenigen Fällen zu einem höheren Anteil auskeimender Sporen.

Im Gegensatz zur Sporenbildung ist die Germination von Endosporen ein sehr schneller Prozess. Nach einer Hitzeaktivierung bei ca. 70 °C und der Zugabe von L-Alanin als germinationsinitiierendem Substrat wurde bereits nach weniger als 1 min ein Auskeimen von Sporen detektiert (Vary & Halvorson, 1965; Scott & Ellar, 1978b). Der gesamte Germinationsprozess von der Zugabe von *Germinants* bis zur ausgewachsenen vegetativen Zelle benötigte dagegen abhängig von den Wachstumsbedingungen 40 bis 100 min (Setlow, 1981).

Während der ersten Minuten der Germination geben Endosporen bis zu 30 % ihres Trockengewichts vor allem in Form von Calciumionen und Dipicolinsäure in die Umgebung ab (Powell & Strange, 1953). Die freigesetzte Dipicolinsäure wird von den auskeimenden Sporen jedoch nicht metabolisiert (Setlow, 1983).

1.2.3 Morphologie und molekularer Aufbau von Endosporen

Endosporen besitzen durch ihren Aufbau aus mehreren Schichten eine komplexere Struktur als vegetative Zellen (siehe Abb. 2).

Im Folgenden soll auf die einzelnen Komponenten eingegangen werden.

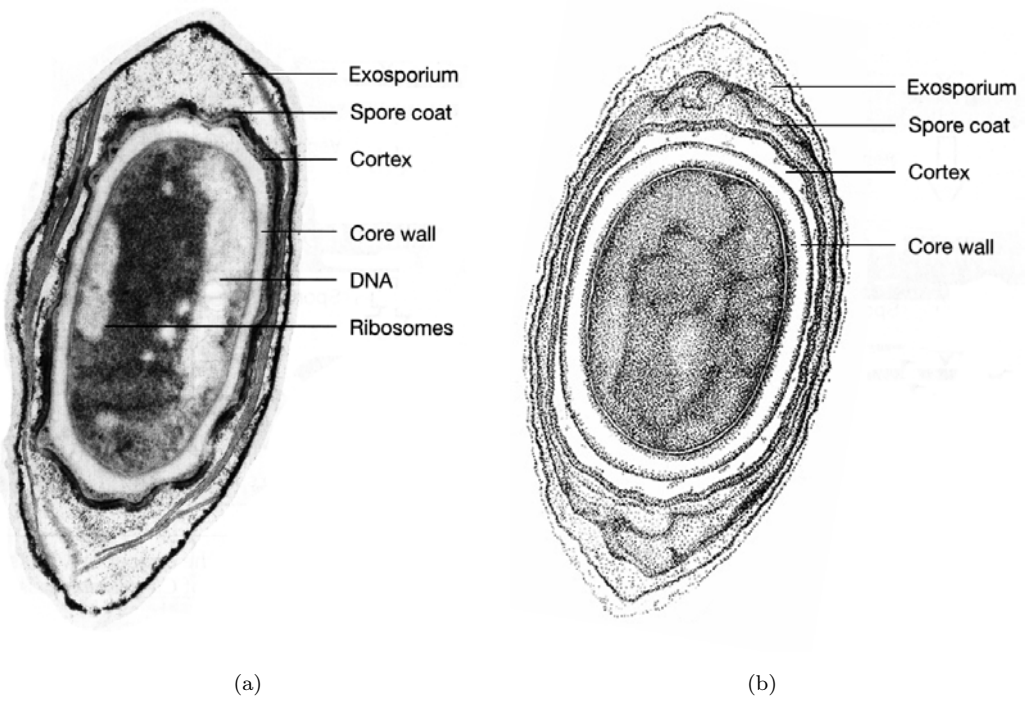

Abb. 2: Morphologie von Endosporen: a) Transmissionselektronenmikroskopische Aufnahme einer Endospore von *Bacillus megaterium* (verändert nach Madigan et al., 2000) b) Schemazeichnung einer Endospore der Gattung *Bacillus*(verändert nach Haas, 2004)

Exosporium Viele Endosporen sind von einer dünnen, locker anliegenden Proteinhülle, dem sogenannten *Exosporium* umgeben. Das *Exosporium* ist verantwortlich für die höhere Hydrophobizität von Sporen im Vergleich zu vegetativen Zellen und spielt damit eine Rolle für das Anhaften von Sporen auf Oberflächen (Koshikawa et al., 1989; Wiencek et al., 1990) Die Oberfläche des *Exosporiums* kann als Antigen für die immunologische Detektion von Endosporen bestimmter Arten über spezielle Antikörper dienen (z.B. Costa et al., 2006). Für die Bestimmung der Gesamtzahl an Endosporen unterschiedlicher Arten ist dieses Verfahren jedoch nicht geeignet, da sich Endosporen in ihren Oberflächen stark unterscheiden können und auch nicht alle Sporen ein *Exosporium* besitzen (Blake & Weimer, 1997).

Spore coat Alle Endosporen besitzen eine Sporenhülle (engl. *Spore coat*), die aus mehreren Schichten sporenspezifischer Proteine besteht und bis zu 50 % des Volumens von Endosporen ausmacht (Murrell, 1969). Die Anzahl und Struktur dieser feinen Schichten variiert zwischen verschiedenen Arten (Driks, 1999). Aufgrund ihrer Porosität dient die Sporenhülle als Molekularsieb. Nach Abschätzungen von Scherrer et al. (1971) und Nishihara et al. (1989) können nur Moleküle mit einer Molekülmasse von $\leq 10^3$ u durch die Sporenhülle diffundieren. Die Sporenhülle schützt dadurch den darunter liegenden *Cortex* vor enzymatischen Angriffen wie zum Beispiel durch Lysozym (Driks, 1999). Potentielle *Germinants* wie Aminosäuren und Monosaccharide dagegen können die Sporenhülle passieren (Black & Gerhardt, 1961; Gerhardt & Black, 1961).

Cortex Der *Cortex* besteht wie die Zellwand von vegetativen Zellen aus Peptidoglycan. Einheiten aus N-Acetylglucosamin und N-Acetylmuraminsäure bilden β-1-4-glykosidisch verknüpfte Polysaccharidstränge, die über kurze Ketten, bestehend aus L-Alanin, D-Glucose, *m*-Diaminopimelinsäure und D-Alanin, miteinander vernetzt sind. Der Unterschied liegt im Vernetzungsgrad des Peptidoglycans. Im Cortex von Endosporen sind nur etwa 3 % der N-Acetylmuraminsäure-Einheiten über Peptidbindungen verknüpft, während in vegetativen Zellen der Anteil bei über 30 % liegt. Die Peptidoglycanstruktur ist somit flexibler als die von vegetativen Zellen. Eine weitere Besonderheit sind die δ-Lactamderivate der N-Acetylmuraminsäure-Einheiten. Etwa die Hälfte der N-Acetylmuraminsäure-Einheiten im *Cortex* liegt in Form dieses zyklischen Amids vor (Atrih et al., 1996; Popham et al., 1996). Diese Modifizierung ist notwendig für die Hydrolyse des *Cortex* bei der Germination (Atrih & Foster, 1999).

Core wall Eine weitere Peptidoglycanschicht bildet die eigentliche Zellwand (*Core wall*). Diese Schicht hat einen höheren Vernetzungsgrad und gleicht in der chemischen Struktur der Zellwand von vegetativen Zellen. Sie bleibt bei der Germination erhalten und dient als Grundlage für die Bildung einer neuen vegetativen Zelle (Atrih et al., 1998; Atrih & Foster, 1999).

Innere Membran Die innere Membran grenzt den Sporenprotoplasten ab. Nach der Germination bildet sie die Cytoplasmamembran der vegetativen Zelle. Entsprechend zeigen Sporen und vegetative Zellen keine signifikanten Unterschiede im Phospholipidverteilungsmuster (Matches et al., 1964; Ma-

stroeni et al., 1968; Bertsch et al., 1969). Nur über statistische Verfahren scheint eine Unterscheidung von Sporen und vegetativen Zellen anhand ihrer Phospholipidverteilungsmuster möglich zu sein (Thompson et al., 2004). Aufgrund ihrer hohen Resistenz ist jedoch fraglich, ob Sporen bei der Analyse von Phospolipiden quantitativ erfasst werden. Macnaughton et al. (1997) haben gezeigt, dass über das übliche *Bligh and Dyer*-Extraktionsverfahren nur ein Bruchteil der Phospholipide von Endosporen extrahiert wird. Mit Hilfe der *Accelerated solvent extraction* (ASE), d.h. durch Erhöhung der Temperatur und des Drucks bei der Extraktion, wurde die dreifache Extraktionseffizienz erzielt.

Sporenprotoplast Der Sporenprotoplast (*Core*) enthält ein vollständiges Chromosom und Ribosomen. Er unterscheidet sich durch seinen Wassergehalt von zum Teil weniger als 30 % (Beaman et al., 1984; Nakashio & Gerhardt, 1985; Lindsay et al., 1985; Beaman & Gerhardt, 1986), aber einen hohen Gehalt an Dipicolinsäure (siehe Kapitel 1.2.4) deutlich vom Protoplasten vegetativer Zellen. Der dehydrierte Zustand des Cytoplasmas spielt eine wichtige Rolle für die Resistenz von Endosporen (siehe Kapitel 1.2.5).

Charakteristisch ist der hohe Gehalt an kleinen säurelöslichen Proteinen (*small acid-soluble spore proteins*, SASPs) im Sporenprotoplasten (Hathout et al., 2003). SASPs werden in der sich entwickelnden Vorspore synthetisiert und machen bis zu 15 % des Proteingehalts von Sporen aus. Diese Proteine tragen ebenfalls zur hohen Resistenz von Endosporen bei (siehe Kapitel 1.2.5) und dienen gleichzeitig als Aminosäurendepot. Während der Germination findet ein schneller Abbau von SASPs in freie Aminosäuren statt, welche zur Synthese von neuen Proteinen benötigt werden (Setlow, 1975a,b).

Endosporen zeigen keinen nachweisbaren Metabolismus (Lewis, 1969), entsprechend ist ihr RNA-Gehalt gering. Er beträgt etwa ein Zehntel des Gehalts von vegetativen Zellen (Moeller et al., 2006). Zudem wurden in Sporen nur geringe Mengen an Adenosintriphosphat (ATP) nachgewiesen. Dieses Nukleotid findet man vorwiegend in seinen dephosphorilierten Formen, d.h. als Adenosinmonophosphat (AMP) und Adenosindiphosphat (ADP).

Als Energiespeicher enthalten Endosporen große Mengen an 3-Phosphoglycerat. Diese Verbindung hat einen Anteil von bis zu 5 % am Trockengewicht von Sporen. Die im Sporenprotoplasten gespeicherten Vorräte an 3-Phosphoglycerat werden während der Germination für die anlaufende Biosynthese vollständig aufgebraucht (Setlow, 1983).

1.2.4 Dipicolinsäure

Dipicolinsäure (Pyridin-2,6-dicarbonsäure, DPA) ist ein Hauptbestandteil von Endosporen (Powell, 1953), ihr Anteil am Trockengewicht liegt bei 5 % bis 14 % (Walker et al., 1961; Murrell & Warth, 1965; Murrell, 1969). DPA wird nur in Sporen akkumuliert, in vegetativen Zellen wurde sie bisher nicht nachgewiesen. Sie ist im Sporenprotoplasten lokalisiert (Leanz & Gilvarg, 1973) und liegt dort in einem Molverhältnis von 1:1 mit Ca^{2+}-Ionen vor (Walker et al., 1961; Murrell & Warth, 1965; Murrell, 1969). Es wird angenommen, dass Ca^{2+} und DPA zum Großteil in Form von Calciumdipicolinatkomplexen assoziiert sind (Bailey et al., 1965; Huang et al., 2007). Durch Verknüpfung der Carboxylgruppen über Ca^{2+}-Ionen sind die DPA-Moleküle vermutlich zu einer linearen Struktur angeordnet (siehe Abb. 3).

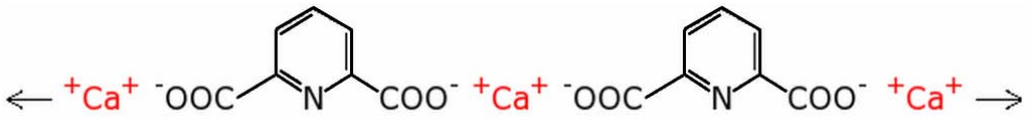

Abb. 3: Struktur des Calciumdipicolinatkomplexes, der im Sporenprotoplasten vorliegenden Form der Dipicolinsäure (DPA, Pyridin-2,6-dicarbonsäure).

Der absolute DPA-Gehalt von Endosporen liegt im Bereich von 10^{-16} bis 10^{-15} mol pro Spore und scheint mit dem Volumen der Endosporen zu korrelieren. Endosporen unterschiedlicher Arten zeigten zum Teil große Unterschiede in ihrem DPA-Gehalt, die Konzentration an DPA im Inneren der Sporen unterlag dagegen nur sehr geringen Schwankungen (siehe Kapitel 2; Fichtel et al., 2007a). Dieser Zusammenhang wird durch eine aktuelle Studie von Huang et al. (2007) bekräftigt.

Über ein spezielles Raman-Spektroskopieverfahren haben Huang et al. (2007) die DPA-Konzentrationen in Sporen verschiedener *Bacillus*-Arten bestimmt und über das Volumen der Endosporen eine durchschnittliche DPA-Konzentration von 400 bis 500 mmol DPA l^{-1} berechnet. Diese Konzentration stimmt mit der in Kapitel 2 bestimmten mittleren DPA-Konzentration von Sporen verschiedener Watt- und Referenzstämme gut überein. DPA liegt jedoch ausschließlich im Sporenprotoplasten vor, bis zu 50 % des Sporenvolumens werden durch die verschiedenen Sporenschichten eingenommen (Murrell, 1969). Im Sporenprotoplasten wird deshalb eine DPA-Konzentration von >800 mmol l^{-1} vermutet, welche deutlich über der Löslichkeit der Calciumdipicolinatkomplexe (<100 mmol l^{-1}) liegt (Huang et al., 2007).

1.2.5 Resistenz von Endosporen

Endosporen zeigen eine hohe Resistenz gegenüber Hitze, Strahlung und Chemikalien (Setlow, 2006). Aufgrund ihrer hohen Widerstandsfähigkeit und ihrer weiten Verbreitung in der Umwelt sind Endosporen oft für den Verderb von Lebensmitteln verantwortlich. Endosporen von *Bacillus sporothermodurans* beispielsweise können sogar die Ultrahochtemperaturbehandlung von Milch, d.h. kurzzeitiges Erhitzen auf 140 °C, überleben (Pettersson et al., 1996). Für die Qualitätskontrolle vieler Lebensmittel spielt daher der schnelle und empfindliche Nachweis von Endosporen eine entscheidende Rolle (siehe auch Kapitel 4; Fichtel et al., 2008).

Die Resistenz von Endosporen ist auf eine Reihe unterschiedlicher Faktoren zurückzuführen. Einen Überblick über den Einfluss der verschiedenen Schichten und molekularen Bestandteile von Endosporen auf deren Resistenz geben die Review-Artikel von Nicholson et al. (2000) und Setlow (2006). Inwieweit der DPA-Gehalt eine Bedeutung für die Resistenz von Sporen hat, ist weitestgehend unklar. Es wird aber ein Zusammenhang mit der Resistenz gegenüber feuchter Hitze und der hohen Widerstandsfähigkeit gegenüber UV-Strahlung diskutiert (Slieman & Nicholson, 2001; Nicholson et al., 2002; Setlow et al., 2006).

Für die hohe Resistenz gegenüber feuchter Hitze ist vor allem der geringe Wassergehalt des Sporenprotoplasten verantwortlich (Gerhardt & Marquis, 1989). Die starke Dehydratisierung führt höchstwahrscheinlich zu einer Inaktivierung und Stabilisierung der im *Core* enthaltenen Enzyme (Warth, 1981; Cowan et al., 2003). Der Einfluss des DPA-Gehalts auf die Resistenz gegenüber feuchter Hitze ist nicht eindeutig geklärt. Bei Sporulation von *Bacillus subtilis* bei erhöhter Temperatur (ca. 50 °C) wurde ein geringerer Wassergehalt des Sporenprotoplasten und eine höhere Resistenz der gebildeten Sporen gegenüber feuchter Hitze festgestellt. Diese Sporen unterschieden sich aber nicht im DPA-Gehalt von den Sporen, die bei etwa 20 °C gebildet wurden (Melly et al., 2002). Andererseits wiesen Endosporen von Mutanten, die keine DPA bilden können, einen erhöhten Wassergehalt und eine entsprechend niedrigere Resistenz gegenüber feuchter Hitze auf. DPA könnte folglich für die Dehydratisierung des Sporenprotoplasten von Bedeutung sein und damit indirekt einen Einfluss auf Hitzeresistenz besitzen (Paidhungat et al., 2000; Setlow et al., 2006). Die Hitzeresistenz von Endosporen wird jedoch noch von anderen Faktoren beeinflusst, wie zum Beispiel dem Mineralstoffgehalt des Sporenprotoplasten (Bender & Marquis, 1985; Gerhardt & Marquis, 1989)

und der DNA-stabilisierenden Wirkung von SASPs (Fairhead et al., 1993), und lässt sich nicht ausschließlich durch den Grad der Dehydratisierung erklären (Nakashio & Gerhardt, 1985; Beaman & Gerhardt, 1986).

Allgemein ist die Stabilität der DNA ein entscheidender Faktor für die Resistenz von Organismen. DPA könnte aufgrund ihrer Absorptionseigenschaften die DNA von Sporen vor schädigender UV-Strahlung schützen. Es wird daher vermutet, dass DPA zur UV-Resistenz von Endosporen beiträgt. Entsprechend wurde bei Sporen von Mutanten, die keine DPA bilden können, eine deutlich geringere UV-Resistenz festgestellt (Slieman & Nicholson, 2001). Diese Studie wurde allerdings mit trockenen Endosporenfilmen durchgeführt. In Sporensuspensionen wurde der gegenteilige Effekt beobachtet, hier zeigten Endosporen ohne DPA eine deutlich höhere UV-Resistenz (Setlow & Setlow, 1993; Paidhungat et al., 2000). Der Einfluss von DPA auf die UV-Resistenz erscheint daher widersprüchlich. Allerdings ist die UV-Resistenz von Endosporen auch in erster Linie auf die Stabilisierung der DNA durch sporenspezifische Proteine zurückzuführen. In Endosporen bewirken die im Sporenprotoplasten vorliegenden SASPs (siehe auch Kapitel 1.2.3) eine Sättigung von Bindungsstellen der DNA und eine Änderung der DNA-Struktur. Durch die Konformationsänderung der DNA wird eine Stabilisierung gegenüber UV-Strahlung und Hitze erzielt. Es wird angenommen, dass die SASPs dadurch DNA-Schäden deutlich reduzieren und damit die enorme Langlebigkeit von Endosporen ermöglichen (Fairhead et al., 1993).

1.2.6 Überlebensdauer von Endosporen

Die Frage, wie lange Mikroorganismen, insbesondere Endosporen, überleben können, beschäftigt die Wissenschaft schon seit Jahrzehnten. Es gibt mehrere Veröffentlichungen, die von einem Nachweis lebensfähiger Endosporen in mindestens 100 bis 10.000 Jahre alten Proben berichten (Kennedy et al., 1994).

Nach ersten Berichten über die Isolierung keimfähiger Endosporen aus Jahrzehnte alten Präparaten gelang Sneath (1962) die Isolierung von Endosporenbildnern aus über 300 Jahre alten Bodenanhaftungen von getrockneten Pflanzen eines Herbariums. Wenige Jahre später wurden von Bartholomew & Paik (1966) thermophile sporenbildende *Bacillus* sp. in marinen Sedimenten mit einer konstanten Temperatur von 4 °C und einem geschätzten Alter von 5800 Jahren nachgewiesen. Die Isolate wurden als Verwandte von *Bacillus stearothermophilus* identifiziert und hatten höchstwahrscheinlich in Form

von Endosporen Jahrtausende im Sediment überdauert. Interessanterweise benötigten die isolierten Stämme keine marinen Medien zum Wachstum und könnten folglich aus terrestrischen Quellen eingetragen worden sein. Eine Kontamination der Sedimentproben durch thermophile Bakterien im Meerwasser konnte ausgeschlossen werden.

Bei archäologischen Grabungen zur Freilegung einer ehemaligen römischen Siedlung wurden von Seaward et al. (1976) noch in 1900 Jahre alten Schichten keimfähige Endosporen der Gattung *Thermoactinomyces* identifiziert. Die höchsten Zellzahlen wurden in Schichten mit einem hohen Anteil von Pflanzenmaterial bestimmt.

Eine hohe Anzahl keimfähiger *Thermoactinomyces*-Sporen wurde auch in über tausend Jahre alten limnischen Sedimenten nachgewiesen (z.B. Cross & Attwell, 1974). Parduhn & Watterson (1985) haben festgestellt, dass das erhöhte Vorkommen von keimfähigen *Thermoactinomyces vulgaris*-Sporen in Sedimentschichten des Elk Lake (Minnesota) auf eine warme und trockene Periode zurückzuführen ist. Es wurde vermutet, dass während dieser Phase der See von ausgedehnten Grasebenen umgeben war und die Endosporen wahrscheinlich über Wind eintragen wurden. In drei verschiedenen Seen in Nordschweden wurde ein zeitlicher Zusammenhang festgestellt zwischen der Anzahl keimfähiger *T. vulgaris*-Sporen und der Anzahl an Pollen, die auf eine landwirtschaftliche Nutzung im Umfeld des Sees schließen lassen. Die ältesten keimfähigen Endosporen wurden in vor etwa 9000 Jahren abgelagerten Schichten nachgewiesen. Interresanterweise zeigten die Tiefenprofile kein Absterben von Endosporen an, sondern spiegelten sehr gut den zeitlich variierenden Eintrag von Pollen durch die landwirtschaftliche Nutzung im Umfeld wider (Nilsson & Renberg, 1990; Renberg & Nilsson, 1992).

In Auskeimungsexperimenten mit Endosporen von *Bacillus subtilis* wurde nach 16 Jahren Lagerung keine signifikante Verringerung der Keimfähigkeit festgestellt. Auf der Basis dieses Experiments haben Gest & Mandelstam (1987) eine mögliche Überlebensdauer von über 200.000 Jahren für Endosporen in Betracht gezogen. Diese theoretischen Überlegungen wurden etwa zehn Jahre später von Cano & Borucki (1995) bestätigt. Sie haben Endosporen im Hinterleib einer Biene nachgewiesen, die in einem 25 bis 40 Millionen Jahre alten Bernstein eingeschlossen war. Cano & Borucki (1995) isolierten die DNA der enthaltenen Endosporen. Es stellte sich heraus, dass diese mit der DNA des *Bacillus* übereinstimmte, der kultiviert werden konnte, nachdem die Oberfläche des Bernstein sterilisiert worden war.

Aufgrund neuer Funde wurde sogar eine Überlebensdauer von bis zu 250 Millionen Jahre in Betracht gezogen. Vreeland et al. (2000) isolierten ein endosporenbildendes Bakterium (*Bacillus* sp.) aus einem Salzlaugeneinschluss in einem Salzkristall. Das Kristallgefüge und die Sedimenttextur wiesen darauf hin, dass der Kristall seit der Bildung nicht umkristallisierte. Es gibt jedoch keinen direkten Beweis für die Existenz von Endosporen in dem untersuchten Salzlaugeneinschluss. Der Befund von Vreeland et al. (2000) wird daher von manchen Wissenschaftlern in Frage gestellt, und es werden zusätzliche Analysen zum direkten Beweis der Existenz von Sporen gefordert (Parkes, 2000).

1.3 Nachweis von Endosporen in Wattsedimenten

Um den Anteil von Endosporen an mikrobiellen Gemeinschaften in Sedimenten abschätzen zu können, werden gewöhnlich die Lebendzellzahlen von pasteurisierten und unbehandelten Kultivierungsansätzen über die MPN-Methode (*most probable number*) ermittelt (z.B. Rothfuss et al., 1997; Miskin et al., 1998; Sass et al., 2003a). Dieses Verfahren beruht auf der höheren Hitzeresistenz von Endosporen im Vergleich zu vegetativen Zellen.

Köpke et al. (2005) haben dieses Verfahren verwendet, um den Endosporenanteil an der mikrobiellen Gemeinschaft in Wattsedimenten zu bestimmen. Diese Untersuchungen waren in die Forschergruppe „Biogeochemie des Watts“ eingebunden, einem von der Deutschen Forschungsgemeinschaft geförderten Projekt zur Untersuchung der mikrobiellen und chemischen Prozesse in Sedimenten des Rückseitenwatts der ostfriesischen Insel Spiekeroog (siehe Abb. 17). Die von Köpke et al. (2005) in der ersten Phase dieses Projekts untersuchten Sedimentproben wurden auch im Rahmen dieser Arbeit analysiert (siehe Kapitel 2; Fichtel et al., 2007a).

An der Sedimentoberfläche zeigten die MPN-Ansätze von Köpke et al. (2005) nur einen Endosporenanteil von wenigen Prozent. Mit zunehmender Tiefe stieg der Anteil von Endosporen an der Lebendzellzahl jedoch stark an. Während bei oxischer Inkubation der Sporenanteil in 50 cm Tiefe bei nur 4 % lag, betrug er in 100 cm Tiefe bereits 20 %. Im Tiefenbereich von 200 cm bis 450 cm wurden sogar fast ausschließlich Endosporen nachgewiesen. Ein mit der Tiefe zunehmender Anteil von Endosporen an der mikrobiellen Gemeinschaft lässt sich auch aus den Ergebnissen der Analyse intakter Phospholipide ableiten. Der Phospholipidgehalt nahm an dem untersuchten

Standort zwischen 5 cm und 50 cm Tiefe um das 13- bis 20-fache ab, während die über Färbung mit AO bestimmten Gesamtzellzahlen nur eine drei- bis siebenfache Abnahme zeigten (Rütters et al., 2002). Da Endosporen mit dem für die Phospholipidanalytik verwendeten Extraktionsverfahren nicht quantitativ erfasst werden (Macnaughton et al., 1997), sich jedoch auch mit AO anfärben lassen, wäre ein mit der Tiefe zunehmender Sporenanteil eine plausible Erklärung für die deutlich geringere Abnahme der Gesamtzellzahlen (Rütters et al., 2002). Über den Phospholipidgehalt des Sediments ist allerdings nur eine grobe Abschätzung der Anzahl vegetativer Zellen möglich (z.B. Zink et al., 2008). Auch auf der Grundlage der MPN-Zahlen lässt sich keine exakte Aussage über den Anteil von Endosporen an der Gesamtzellzahl ableiten. Lebendzellzahlen liegen oft einige Größenordnungen unter den Gesamtzellzahlen, da über die Medien der MPN-Ansätze immer nur ein Bruchteil der natürlichen Bakterienpopulation kultiviert werden kann (Brock, 1971; Ritz, 2007). Nur durch sorgfältige Wahl der Kultivierungsbedingungen wird eine Kultivierungseffizienz im ein- bis zweistelligen Prozentbereich erreicht (z.B. Süß et al., 2004; Köpke et al., 2005).

Aufgrund der bisherigen Ergebnisse konnte deshalb nur spekuliert werden, wie hoch der Anteil von Endosporen an der mikrobiellen Gemeinschaft in den untersuchten Sedimenten ist. Für eine genauere Bestimmung von Endosporenabundanzen im Sediment, über die auch die Anzahl *in situ* aktiver Prokaryonten genauer abgeschätzt werden könnte, fehlte bisher eine geeignete Methode. Die Entwicklung einer Methode zur Quantifizierung von Endosporen (siehe auch Kapitel 2 und 3; Fichtel et al., 2007a,b) ist deshalb von großer Bedeutung, insbesondere auch im Hinblick auf die enorme Anzahl an Prokaryonten in der tiefen Biosphäre (siehe Kapitel 1.1). Derzeit kann nicht ausgeschlossen werden, dass ein signifikanter Anteil dieser Mikroorganismen und damit ein Großteil der Prokaryonten auf der Erde in Form von Endosporen überdauert.

1.4 Zielsetzung und Gliederung der vorliegenden Arbeit

Ziel der vorliegenden Arbeit war es, eine geeignete Methode zur quantitativen Bestimmung von DPA in marinen Sedimenten zu entwickeln. Über die Konzentration dieses Biomarkers im Sediment sollte eine kultivierungsunabhängige Bestimmung der Endosporenzahl sowie des Endosporenanteils an der mikrobiellen Gemeinschaft erfolgen.

Außerdem sollten die DPA-Gehalte von Endosporen verschiedener Arten – insbesondere von Isolaten aus den entsprechenden Wattsedimenten – untersucht werden. Über diese Untersuchungen sollte geklärt werden, inwieweit eine quantitative Umrechnung über den DPA-Gehalt auf die Endosporenzahl möglich ist oder ob große Unterschiede im DPA-Gehalt von Endosporen unterschiedlicher Arten eventuell nur eine grobe Abschätzung der Endosporenabundanz im Sediment zulassen. Die Quantifizierung von DPA im Sediment sollte mit Hilfe von dotierten Proben überprüft werden, um die Extraktionseffizienz abzuschätzen und eine Unterschätzung der Sporenzahl z.B. durch Absorption von DPA an die Sedimentmatrix auszuschließen.

Über diesen Ansatz sollte die quantitative Bedeutung von Endosporen in Wattsedimenten bestimmt werden. Insbesondere sollte überprüft werden, ob sich der in den Kultivierungsexperimenten abzeichnende hohe Endosporenanteil (Köpke et al., 2005) über dieses kultivierungsunabhängige Verfahren bestätigen lässt.

1. Publikation (Fichtel et al., 2007a) Diese Veröffentlichung beschreibt die Entwicklung einer empfindlichen Methode zur Bestimmung von DPA in Wattsedimenten. Das Verfahren beruht auf der fluorimetrischen Bestimmung von DPA nach Komplexierung mit Terbium. Aufgrund von *Quenching*-Prozessen und der Eigenfluoreszenz des im Sediment enthaltenen organischen Materials stellte diese fluorimetrische Methode hohe Anforderungen an die Probenaufarbeitung. Aus diesem Grund wurde ein Flüssig-Flüssig-Extraktionsverfahren zur Aufreinigung von Sedimentextrakten entwickelt. Über dieses Verfahren konnte erstmals der DPA-Gehalt von Wattsedimenten bestimmt werden. Auf der Basis der DPA-Gehalte wurde die Anzahl an Endosporen im Sediment abgeschätzt. Die Umrechnung erfolgte auf der Grundlage der DPA-Gehalte von Endosporen verschiedener Wattstämme, die von Köpke et al. (2005) isoliert worden waren. Zusätzlich enthält die Veröffentlichung eine Übersicht über bisher bestimmte DPA-Gehalte verschiedener Arten von Endosporen. Außerdem wird eine mögliche Beeinflussung des DPA-Gehalts von Endosporen durch Kultivierungsbedingungen diskutiert.

2. Publikation (Fichtel et al., 2007b) In der zweiten Publikation wird eine sehr empfindliche HPLC-Methode (*high-performance liquid chromatography*) zur Bestimmung von DPA beschrieben. Diese Methode kombiniert die Trennleistung eines HPLC-Systems mit der hohen Empfindlichkeit der in der ersten Publikation beschriebenen Tb-DPA-Fluoreszenzmethode. Über

diese HPLC-Methode wurde eine höhere Empfindlichkeit und bessere Reproduzierbarkeit erreicht. Durch die Möglichkeit der externen Kalibrierung wurde außerdem der Zeitaufwand für die Probenaufarbeitung deutlich reduziert. Im Gegensatz zum erstgenannten Verfahren benötigt diese Methode ein spezielles HPLC-System, das eine *Post-Column*-Komplexierung von DPA mit Terbium und anschließende fluorimetrische Detektion der gebildeten Komplexe ermöglicht.

3. Publikation (Fichtel et al., 2008) Diese Veröffentlichung beschreibt eine mögliche Anwendung der HPLC-Methode im Bereich der Lebensmittelmikrobiologie. Endosporen können eine Gefahr für den Verderb von Lebensmitteln darstellen, da sie aufgrund ihrer hohen Resistenz durch das Kochen von Nahrungsmitteln nicht vollständig abgetötet werden. Ein schneller, kultivierungsunabhängiger Nachweis von Sporenkontaminationen ist deshalb von großer Bedeutung und wird in dieser Publikation am Beispiel von Pfefferproben aufgezeigt. Für die Aufreinigung von stark fluoreszierenden Pfefferextrakten wurde ein geeignetes Extraktionsverfahren entwickelt. Außerdem wurde ein erweitertes Detektionsverfahren angewendet, um im Fall von Koelutionen eine bessere Auswertung von Fluoreszenzchromatogrammen zu ermöglichen.

4. Publikation (eingereicht bei *Geomicrobiology Journal*) Dieses Manuskript beinhaltet die Ergebnisse einer umfangreichen Analyse von Sedimentkernen aus dem Rückseitenwatt der Insel Spiekeroog. Die in dieser Studie analysierten, bis zu 5.5 m langen Kerne stammten von einem Sand- und einem Mischwattbereich. Aufgrund des geringeren Zeitbedarfs für die Probenaufarbeitung und die Möglichkeit der externen Kalibrierung gestattete die in der zweiten Publikation beschriebene HPLC-Methode (Fichtel et al., 2007b) einen höheren Probenumfang als das in Kapitel 2 (Fichtel et al., 2007a) verwendete Verfahren. Dadurch konnte erstmals eine lithologieabhängige, hochaufgelöste Untersuchung von Sedimentkernen auf ihren DPA-Gehalt erfolgen. Die gewonnenen Ergebnisse geben einen guten Einblick in die quantitative Bedeutung von Endosporen in Wattsedimenten und lassen eine Abschätzung des Sporenanteils an der Gesamtzellzahl zu. Außerdem lassen die Ergebnisse vermuten, dass der Endosporengehalt des Sediments durch sedimentologische Faktoren beeinflusst wird.

2 Spore dipicolinic acid contents used for estimating the number of endospores in sediments

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2.1 Abstract

Endospores are heat-resistant bacterial resting stages that can remain viable for long periods of time and may thus accumulate in sediments as a function of sediment age. The number of spores in sediments has only rarely been quantified, because of methodological problems, and consequently little is known about the quantitative contribution of endospores to the total number of prokaryotic cells. We here report on a protocol to determine the number of endospores in sediments and cultures. The method is based on the fluorimetric determination of dipicolinic acid (DPA), a spore core-specific compound, after reaction with terbium chloride. The concentration of DPA in natural samples is converted into endospore numbers using endospore-forming pure cultures as standards. Quenching of the fluorescence by sediment constituents and background fluorescence due to humic substances hampered direct determination of DPA in sediments. To overcome those interferences, DPA was extracted using ethyl acetate prior to fluorimetric measurements of DPA concentrations. The first results indicated that endospore numbers obtained with this method are orders of magnitude higher than numbers obtained by cultivation after pasteurization. In one of the explored sediment cores, endospores accounted for 3 % of all stainable prokaryotic cells.

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2.2 Introduction

Numbers of endospores in sediments are typically estimated by viable counts obtained after pasteurization (Isaksen et al., 1994; Rothfuss et al., 1997). Cultivation-based methods applied to marine sediments have demonstrated the presence of at least 10^5 spores g^{-1} sediment (Isaksen et al., 1994; H. Sass et al., unpublished data). It was found for freshwater as well as for marine sediments that, in depths of tens of centimeters to some meters, viable counts for spores outnumber those for vegetative cells (Rothfuss et al., 1997; Miskin et al., 1998; Sass et al., 2003a; Köpke et al., 2005). These results suggest that the majority of bacterial cells in these deeper layers are present as heat-resistant endospores. However, as endospore-forming bacteria represent a variety of different physiological groups, including aerobes, anoxygenic phototrophs, and sulfate-reducing bacteria, it is obvious that cultivation methods underestimate the actual in situ numbers of endospores, as reflected by their relatively low absolute numbers in most studies so far (e. g. Köpke et al., 2005).

To circumvent these shortcomings, a new cultivation-independent approach for the detection and quantification of bacterial endospores in sediments was sought. In the present study, a protocol for the quantification of dipicolinic acid (DPA; pyridine-2,6-dicarboxylic acid) was developed. DPA is a universal, but specific, component of bacterial endospores (Powell, 1953). It has been shown that DPA is located in the core, representing 5–14 % of endospore dry weight (Murrell, 1969). DPA is only preserved within intact endospores, and can be easily degraded under oxic (Arima & Kobayashi, 1962; Taylor & Amador, 1988; Amador & Taylor, 1990) and strictly anoxic (Seyfried & Schink, 1990) conditions after its release (e. g. during spore germination). Therefore, this compound appears to be a suitable biomarker for the estimation of endospore numbers.

DPA can be detected by a range of analytic techniques (Janssen et al., 1958; Lewis, 1967; Tabor et al., 1976; Scott & Ellar, 1978b; Warth, 1979; Paulus, 1981; Beverly et al., 1996, 1999, 2000; Goodacre et al., 2000; He et al., 2003), with the terbium dipicolinate fluorescence method (Rosen et al., 1997, especially Hindle & Hall, 1999) having been described as the most sensitive technique, allowing the determination of nanomolar concentrations. In the present study, a protocol for the extraction and fluorimetric quantification of DPA from endospores in sediment samples was developed and used to estimate endospore numbers in situ.

2.3 Materials and methods

2.3.1 Sediment samples

Sediment cores were collected at sites Neuharlingersieler Nacken (NSN; 53°43.270'N, 7°43.718'E) and Gröninger Plate (GP; 53°43.638'N, 7°43.718'E) in the backbarrier tidal flat of the island of Spiekeroog in the southern North Sea (Köpke et al., 2005). Aluminum tubes (8 cm in diameter) were driven into the sediment using a vibrating unit. The cores were recovered by the use of a tripod with a hoist, and cut longitudinally for subsampling. The lithologic structure of the cores was determined by visual inspection. Sediment samples for DPA analysis were taken from the innermost part of the core to avoid cross-contamination due to smearing along the aluminum tube. Sediment samples were freeze-dried and stored at −20 °C until processing.

2.3.2 Bacterial growth and induction of sporulation

For converting DPA concentrations into endospore numbers, 10 bacterial strains were examined for their DPA content per endospore (Table 1). Six strains were isolated from the same cores that were investigated in the present study (*Bacillus* sp. G50II, *Bacillus* sp. G400I, *Bacillus* sp. N300I, and *Clostridiales* bacterium strain G100VIII) (Köpke et al., 2005) or from an adjacent tidal flat (*Bacillus* sp. NA402 and *Oceanobacillus* sp. NC302) (H. Sass and H. Rütters, unpublished data). These tidal flat strains were grown in a dilute marine yeast extract peptone glucose medium as described by Köpke et al. (2005). The other four strains comprised two well-described *Bacillus* species from a culture collection (*B. megaterium* DSM 32^T and *B. subtilis* ssp. *subtilis* DSM 10^T) and two sulfate-reducing bacteria, *Desulfosporosinus orientis* DSM 765^T and *Desulfotomaculum* sp. B2T (Sass & Cypionka, 2004). The two *Bacillus* strains were grown in a freshwater medium (5 g of peptone, 3 g of meat extract and 10 mg of $MnSO_4 \cdot 2H_2O$ per liter, pH 7.0). The growth media for the two sulfate reducers were supplemented with 20 mmol l^{-1} sodium lactate and 10 mmol l^{-1} sodium sulfate. *Desulfosporosinus orientis*^{T} was grown in a freshwater medium as described by Sass et al. (1997), and *Desulfotomaculum* sp. B2T was grown in a marine mineral medium (Köpke et al., 2005).

Sporulation in the tidal flat strains and the two sulfate reducers was induced by allowing cells to exhaust nutrients in their growth medium (exhaustion method), for *B. megaterium*^{T} and *B. subtilis*^{T} by transferring the

cells to a nutrient poor resuspension medium (resuspension method). *Bacillus subtilis*^{T} was resuspended in pure water, and *B. megaterium*^{T} in resuspension medium (Sterlini & Mandelstam, 1969).

2.3.3 Purification of spores

Strains used in this study were chosen for their spore yield (generally $>10\,\%$). Nonetheless, in order to obtain pure spore suspensions, a purification step to separate the spores from vegetative cells and cell debris was required. This was done by density gradient centrifugation adapted from a method described by Nicholson & Setlow (1990). Instead of Urugrafin, the X-ray contrast agent Gastrografin [76 %,(w/v), Schering, Berlin, Germany] was used; this contains almost the same compounds and has a similar density.

Sporulated cultures were centrifuged (17400 *g*, 4 °C, 15 min), and the obtained pellet was suspended in 5 ml of 20 % Gastrografin. This suspension was taken up with a syringe and layered gently on top of 25 ml of 50 % Gastrografin in a 40 ml Nalgene centrifuge tube. For some strains, the concentration of the lower Gastrografin phase was reduced to 45 % to obtain better separation of spores and vegetative cells. After centrifugation (14600 *g*, 4 °C, 30 min), the pellet contained only free endospores. Vegetative cells, debris and cells with enclosed immature spores accumulated in the upper phase and at the phase interface, and were removed with a disposable pipette. The spore pellet was washed at least three times to remove residual Gastrografin. Spores of marine strains were washed and resuspended in artificial seawater, and other strains in 0.9 % NaCl solution. Purity was checked microscopically by phase contrast. If necessary, density gradient separation was repeated to achieve a purity of $>99\,\%$ spores. Endospore suspensions were stored at 4 °C.

2.3.4 Determination of spore counts and volume

Spore counts were determined by phase contrast microscopy using a Thoma counting chamber. At least 24 randomly chosen squares with about 100 cells were counted. Suspensions were diluted to an appropriate spore density of about 10^8 spores ml^{-1}.

For volume determination, the shape of the endospores was approximated as a cylinder with hemispherical ends. Volume (V) was calculated from the length (l) and width (w) of the spores using the following formula:

$$V = [(w^2\pi/4)(l - w)] + (\pi w^3/6)$$

(Norland et al., 1987; Loferer-Krossbacher et al., 1998). This formula can be used for rod-shaped as well as coccoid spores, as for cocci the term $(l - w)$ becomes zero. For calculation of spore volume, the length and width of at least 100 spores were determined. Associated errors were estimated by consideration of error propagation on the basis of SDs of length and width measurements.

2.3.5 Extraction of DPA from endospores

Spore suspensions were extracted immediately after determination of spore counts to avoid possible alteration of DPA content due to germination of spores and possible subsequent microbial degradation of DPA. For extraction of DPA from endospores, c. 5 ml volumes of endospore suspension (with at least 10^8 spores ml^{-1}, necessary for quantification of DPA via HPLC) were placed into autoclavable 15 ml polypropylene tubes with screw caps (Sarstedt, Nümbrecht, Germany) and autoclaved at 121 °C for 30 min to completely release DPA from endospores (Pellegrino et al., 2002). Impermeability of the tubes was confirmed by weighing all samples before and after autoclaving. After cooling, the samples were centrifuged (4000 *g*, 5 min, 15 °C), and the supernatant was sterile-filtered (0.2 µm pores, cellulose acetate) to remove spore debris.

2.3.6 Determination of DPA from cultivated spores by reversed-phase (RP)-HPLC

Spore DPA contents were determined by RP-HPLC as described by Warth (1979) on a 4.6×250 mm RP-8 column (Nucleosil 100-5C8; Macherey-Nagel, Düren, Germany). The liquid chromatograph comprised a gradient pump (Waters 600 E Multisolvent Delivery System; Eschborn, Germany), an autosampler (Waters 717plus), and a photodiode array detector (Waters 996). All cultures were analyzed in triplicate.

2.3.7 Determination of DPA in sediment samples

For the determination of DPA in sediment samples, 2.0 g of freeze-dried sediment was weighed into 10 ml glass ampoules and suspended in 5 ml of 0.5 M HCl for extraction of DPA. Standard addition was used for quantification, taking into account background and possible quenching of terbium dipicolinate fluorescence, but also potential loss of DPA during extraction. Therefore, five additional samples were spiked with different amounts of DPA by suspending the sediment aliquots in standard solutions with defined DPA concentrations. DPA standard solutions (in the concentration range from 100 nmol l^{-1} to 2 µmol l^{-1}, corresponding to 0.25–5.0 nmol DPA g^{-1} sediment) were also prepared in 0.5 M HCl. For release of DPA from endospores in the sediment, samples were autoclaved and filtered as described for the spore suspensions.

Because of the high background fluorescence, presumably caused by humic material in the sediment, DPA had to be extracted from the filtrate. After cooling, the samples were transferred to 40 ml centrifugation tubes, and the glass ampoules were rinsed with 5 ml of 0.5 M HCl to quantitatively transfer DPA. The pH was checked and adjusted to 0.5 by adding HCl (35 %), if necessary. The aqueous solution was extracted three times with 20 ml of ethyl acetate. In each extraction step, the sample was vortexed for about 1 min and centrifuged (4000 *g*, 10 min, 15 °C), and the added ethyl acetate was removed with a disposable pipette. The three ethyl acetate phases obtained were transferred into the same 100-ml round-bottomed flask. The solvent was evaporated, and the extract was redissolved in 5 ml of sodium acetate buffer (1 mol l^{-1}, pH 5.6) and subsequently washed with diethyl ether (10 ml) and ethyl acetate (5 ml).

To the sodium acetate fraction containing the DPA, 100 µl of an $AlCl_3$ solution (2 mmol l^{-1}) was added to minimize quenching of terbium dipicolinate fluorescence by phosphate-containing substances (Fell et al., 2001). As organophosphates, e. g. NAD, have been shown reduce terbium dipicolinate fluorescence, presumably by complexation of terbium (Pellegrino et al., 1998), addition of Al^{3+} was expected to saturate the Tb^{3+}-binding sites and reduce this effect. After addition of $AlCl_3$, the samples were passed through a 0.2 µm cellulose acetate filter. For fluorescence measurements, 3 ml of the filtrate was transferred to a 3.5 ml quartz cuvette. First, emission spectra of the samples without addition of terbium were recorded to obtain background spectra for baseline correction. For the fluorimetric detection of DPA, 30 µl

of a 1 mmol l^{-1} $TbCl_3$ solution was added, and the samples were mixed in the quartz cuvette by pipetting up and down several times before measurement.

To check for extraction efficiency, the residues of the diethyl ether and ethyl acetate washing fractions were also dissolved in sodium acetate buffer and subjected to fluorimetric detection of DPA as described above.

2.3.8 Fluorimetric determination of DPA

Fluorimetry was performed using a Shimadzu RF-1501 fluorimeter (Shimadzu, Kyoto, Japan). The light output was given in arbitrary units on a scale from 0 to 1000. For data interpretation and control of measurement parameters, SHIMADZU HYPER RF software (Version 1.57) was used. Emission spectra (270 nm excitation) were recorded from 450 nm to 650 nm with a resolution of 1 nm. The spectral bandwidth was 10 nm on both the excitation side and the emission side. Spectra were collected at scan speed "SLOW" with a 0.1 s response time. For quantification, the strongest emission maximum at 548 nm was used. A 420-nm-long pass filter (GG-420; Schott, Mainz, Germany) was inserted between cuvette and emission monochromator to eliminate second order diffraction of elastically scattered light.

2.4 Results and Discussion

2.4.1 Variations in endospore DPA content and volume among different bacterial strains

DPA contents of endospores of the investigated strains ranged from 1.4×10^{-16} mol cell^{-1} (*Bacillus* sp. G400I) to 1.3×10^{-15} mol cell^{-1} (*Desulfosporosinus orientis* DSM765^{T}). The spores of the tidal flat isolates showed little variation in size and DPA content, and generally contained less DPA than endospores of the two sulfate reducers or of *B. megaterium*^{T} (Table 1). Average spore volumes determined ranged from 0.40 to 2.43 µm^3, with spores of *Desulfotomaculum* sp. B2T being largest and having six times the volume of *Oceanobacillus* sp. NC302 spores.

A correlation of DPA content and volume was indicated by linear regression using all data points, resulting in an average DPA concentration of 0.47 mol DPA l^{-1} of spore volume (Fig. 4). Calculated DPA concentrations in the spore interior were in the range from 0.19 to 0.54 mol DPA l^{-1}, with the exception of *Desulfosporosinus orientis* DSM 765^{T}, which formed spores with more than twice this DPA concentration (Table 1). Owing to this outlier, the correlation between DPA content and volume was low (r^2=0.43). Generally, a correlation between DPA content and volume can be expected, as DPA is necessary to lower the core water content, being responsible for spore resistance and for maintenance of dormancy (Lewis, 1969; Gould, 1983; Paidhungat et al., 2000). Moreover, determinations of DPA content relative to spore dry weight showed only minor variations, in the range of 5–14 % (Murrell & Warth, 1965; Murrell, 1969), suggesting that an increase in spore size and weight is accompanied by an increase in DPA content.

DPA contents determined for the isolates from the tidal flat and the reference strains were in an order of magnitude that agrees with published data, although the range of volumes and DPA contents was slightly smaller than reported in the literature. Carrera et al. (2007) found variations of a factor of 5.5 when analyzing the volume of endospores of 14 *Bacillus* strains. Aronson & Fitzjames (1976), Sojka & Ludwig (1997), Hindle & Hall (1999), He et al. (2003), Kort et al. (2005) and Shafaat & Ponce (2006) reported DPA contents ranging from 0.83×10^{-16} to 10.5×10^{-16} mol for different *Bacillus* strains, with the highest content being found in *B. megaterium* (Table 2). Unfortunately, analysis of spore DPA contents of different strains has not been intensely investigated so far. DPA contents published in the literature

were determined with several different methods for preparation of spore suspensions, and extraction and quantification of DPA. Therefore, we can only speculate about the differences between the literature data and the contents determined in this study, e. g. for *B. megaterium* and *B. subtilis*. On the other hand, significant variations in DPA content appear to occur among strains belonging to the same species, as observed for *B. subtilis*, with values ranging from 4.3×10^{-16} to 8.5×10^{-16} mol (Kort et al., 2005). These differences might be explained by variations in spore volume, as this has been suggested to correlate with DPA content.

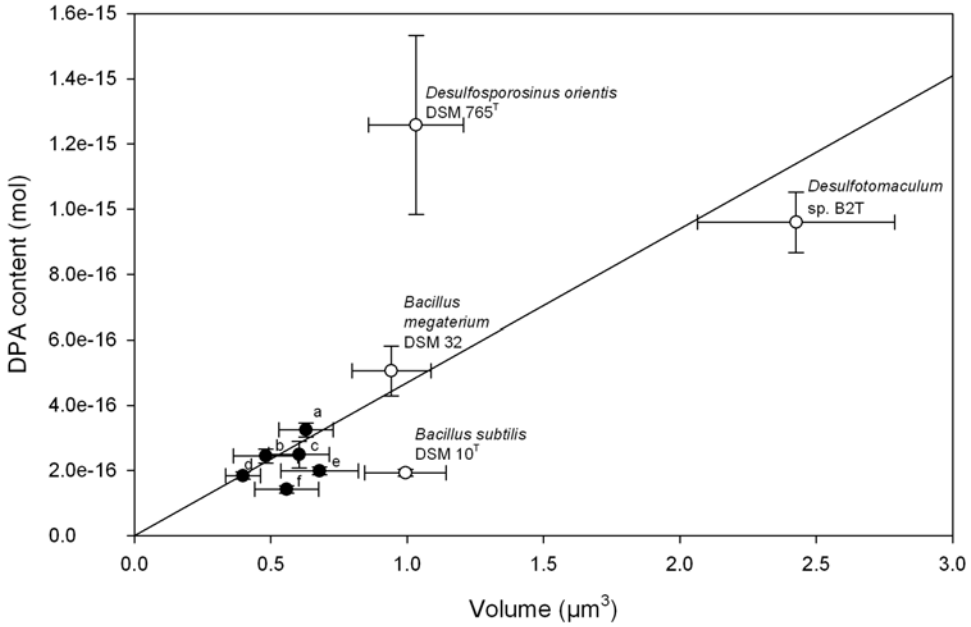

Fig. 4: Plot of DPA content vs. volume of the endospores analyzed in this study. The regression line (including all data points) represents 0.47 mol DPA l^{-1} spore volume. Closed circles, tidal flat isolates: (a) *Bacillus* sp. NA402; (b) strain G100XIII; (c) *Bacillus* sp. N300I; (d) *Oceanobacillus* sp. NC302; (e) *Bacillus* sp. G50II; (f) *Bacillus* sp. G400I. Open circles, sulfate reducers and aerobic freshwater bacilli.

Table 1: Bacterial strains investigated for endospore DPA content and volume, as well as estimated DPA concentrations in the core of the spores

Strain	Closest relative	Endospore volume (μm^3)	DPA content (10^{-16} mol)	DPA concentration (mol l^{-1})
Tidal flat isolates				
Bacillus sp. G400I (AM494003)	*B. aquimaris*^{T} (98.5)	0.56 ± 0.12	1.42 ± 0.11	0.25 ± 0.06
Bacillus sp. G50II (AM494002)	*B. aquimaris*^{T} (97.5)	0.68 ± 0.14	1.99 ± 0.12	0.29 ± 0.06
Bacillus sp. N300I (AJ786037)	*B. indicus*^{T} (98.0)	0.60 ± 0.11	2.49 ± 0.41	0.42 ± 0.10
Bacillus sp. NA402 (AJ866951)	*B. firmus*^{T} (98.1)	0.63 ± 0.10	3.24 ± 0.21	0.51 ± 0.09
Oceanobacillus sp. NC302 (AJ866952)	*O. picturae*^{T} (97.2)	0.40 ± 0.06	1.84 ± 0.11	0.46 ± 0.07
Strain G100VIII (AJ786046)	*Psb. paucivorans*^{T} (93.4)	0.48 ± 0.12	2.45 ± 0.21	0.51 ± 0.13
Reference strains				
Bacillus megaterium DSM 32^T		0.94 ± 0.14	5.05 ± 0.76	0.54 ± 0.11
Bacillus subtilis DSM 10^T		0.99 ± 0.15	1.93 ± 0.10	0.19 ± 0.03
Desulfosporosinus orientis DSM 765^T		1.03 ± 0.17	12.6 ± 2.7	1.22 ± 0.33
Desulfotomaculum sp. B2T	*D. geothermicum*^{T} (98.3)	2.43 ± 0.36	9.59 ± 0.93	0.39 ± 0.07

Psb., *Parasporobacterium.*
GenBank accession numbers for the tidal flat strains are given in parentheses. Sequence similarities (%) to the closest related species are given in parentheses.

Table 2: Endospore DPA contents published in the literature

Strain	Reference	DPA content (10^{-16} mol)
Bacillus atrophaeus ATCC 9372	Shafaat & Ponce (2006)	2.8
Bacillus atrophaeus ATCC 9372	Sojka & Ludwig (1997)	2.3
Bacillus cereus	Aronson & Fitzjames (1976)	6.1–7.1
Bacillus megaterium	Cited in Hindle & Hall (1999)	10.5
Bacillus sporothermodurans IC4	Kort et al. (2005)	8.2 ± 0.3
Bacillus subtilis 168	Kort et al. (2005)	4.3 ± 0.2
Bacillus subtilis A163	Kort et al. (2005)	8.5 ± 0.4
Bacillus subtilis NCTC 3610^{T}	Hindle & Hall (1999)	3.65
Bacillus thuringiensis 833-2-1	He et al. (2003)	0.83

It has been indicated that DPA content as well as volume might be affected by growth conditions (Hitchins et al., 1972). In contrast to this, a more detailed study on the spore size distribution of *Bacillus* species by Carrera et al. (2007) showed that the choice of medium seems to have a relatively small impact on spore volume. Also, temperature does not seem to affect the DPA content of spores, as *B. subtilis* spores prepared at temperatures from 22 to 48 °C by Melly et al. (2002) contained almost identical amounts of DPA (variations $< 10\,\%$). However, endospores are formed in a state of starvation, and the cell is constrained to keep the size and content of proteins and other components at an absolute minimum; therefore, little variation is to be expected.

It has been reported that the choice of substrate has a significant influence on the extent of spore formation in a culture (e. g. Widdel & Pfennig, 1981). However, if spores are not separated from vegetative cells and immature spores, an accurate determination of spore counts is difficult and is often associated with high SDs, particularly if spore counts are low. In addition, in some cases, aggregation of spores and vegetative cells does occur, rendering the determination of reliable spore numbers almost impossible. To circumvent these problems, we used density gradient centrifugation to separate spores and vegetative cells, and obtained spore suspensions with a purity of $> 99\,\%$. This high purity of spore suspensions is reflected in the low variations of spore DPA contents and volumes of the tidal flat strains and the two *Bacillus* species. This provides confidence as to the reliability of the data.

2.4.2 Implications for the determination of DPA in sediment samples

For the determination of DPA in endospore-containing cultures, the liquid chromatographic method described by Warth (1979) required minimal sample preparation, and was rapid, free of interferences and characterized by high reproducibility ($\mathrm{SD} < 2\,\%$). However, determination of DPA in sediments required a more sensitive approach. With HPLC analysis, DPA was not detected in any of the sediment samples, indicating that 1 g of sediment contained < 10 nmol of DPA. Considering the average DPA content of the tidal flat isolates, this would correspond to c. 10^8 endospores g^{-1} sediment. Taking into account the total cell counts in the respective sediments, which range from about 10^9 cells g^{-1} sediment at the sediment surface to about 10^7 cells g^{-1} at 300 cm depth (Köpke et al., 2005), it is obvious that endospores

would be detectable by HPLC only in the upper sediment layers and only if they represented more than 10 % of the total cell count.

The terbium dipicolinate-fluorescence method described by Hindle & Hall (1999) has a three orders of magnitude lower detection limit of 2 nmol DPA l^{-1} and theoretically allows detection of 10^5 spores g^{-1} sediment. A similar assay has recently been successfully applied to follow the germination of endospores from a Greenland ice core (Shafaat & Ponce, 2006; Yung et al., 2007). However, when sediment samples are used, potential interference by humic substances can be expected in terms of background fluorescence [examples of fluorescence spectra of humic substances are given in Ewald et al. (1983)] or quenching of fluorescence. Furthermore, Lippold et al. (2005) found almost complete complexation of Tb^{3+} by humic acids at pH > 6, whereas acidification to pH 3 reduced complexation to about 10 %, due to protonated carboxyl groups. However, as pH also influences the complexation of Tb^{3+} with DPA, the fluorimetric determination of DPA limits the choice of pH level. Therefore, a method for the separation and enrichment of DPA from crude sediment extracts was developed.

2.4.3 Extraction of DPA with ethyl acetate

On the basis of literature data (Warth, 1979), DPA can be extracted with ethyl acetate from the aqueous phase after adjustment of the solution to pH 1.8. However, our own experience from experiments with internal DPA standards showed that the samples had to be acidified to as low as pH 0.5, which was achieved by resuspending the sediment in 0.5 M hydrochloric acid prior to autoclaving. Extraction efficiency is strongly influenced by pH. Therefore, it can be necessary to adjust the pH after autoclaving, particularly if carbonate-rich sediments are being analyzed. Three times extraction with twice the volume of ethyl acetate at pH 0.5 generally resulted in an extraction efficiency of more than 96 %.

Further purification was achieved by redissolving the extract in 5 ml of sodium acetate buffer (pH 5.6) and then washing with diethyl ether and ethyl acetate. At this pH, a loss of DPA caused by the washing procedure was not observed, whereas some originally coextracted fluorescent compounds were removed, as indicated by the spectra of the washing fractions (data not shown).

2.4.4 Determination of DPA in sediment samples

The additional extraction step for separation of DPA from interfering and fluorescent compounds allowed the determination of a DPA depth profile along the cores taken in the backbarrier tidal flat of Spiekeroog Island. Figure 5 shows the emission spectra of purified sediment extracts from a depth of about 4 m at site GP. Five sample aliquots were spiked with different amounts of DPA (0.25–5.0 nmol DPA g^{-1} sediment). Extraction with ethyl acetate at pH 0.5 and washing with diethyl ether and ethyl acetate at pH 5.6 did not result in a complete reduction of background fluorescence, as can be seen from the slope of the baseline. However, quenching was significantly reduced, so that DPA could be quantified. Standard addition was used for quantification, as each sediment sample had its specific background fluorescence. Hence, quantification using an external calibration curve was not possible. In the tidal flat sediments used in this study, the increase in fluorescence intensity of the samples was generally linear with respect to the added DPA concentration. In the case of the sediment extract shown in Fig. 5, linear regression resulted in a DPA concentration of 37 nmol l^{-1}, corresponding to 0.09 nmol DPA g^{-1} sediment (dry weight).

At site NSN, between 0.26 and 1.29 nmol DPA g^{-1} sediment was found (Fig. 6). In the upper 40 cm of the sediment, the amounts of extracted DPA were relatively constant, with values around 0.6 nmol DPA g^{-1} sediment. The highest amounts were obtained between depths of 50 and 100 cm. Beneath this, DPA contents decreased again to values half as high as at the surface. The two deepest samples (250 and 300 cm below the surface) did not yield reliable results (see below). At site GP, significantly lower amounts of DPA were determined, ranging from 0.09 to 0.17 nmol DPA g^{-1} (Fig. 7). At this site, only slight variations with depth were found, and DPA contents in the deepest sample at a depth of 500 cm and in the surface sample did not differ significantly (Fig. 7).

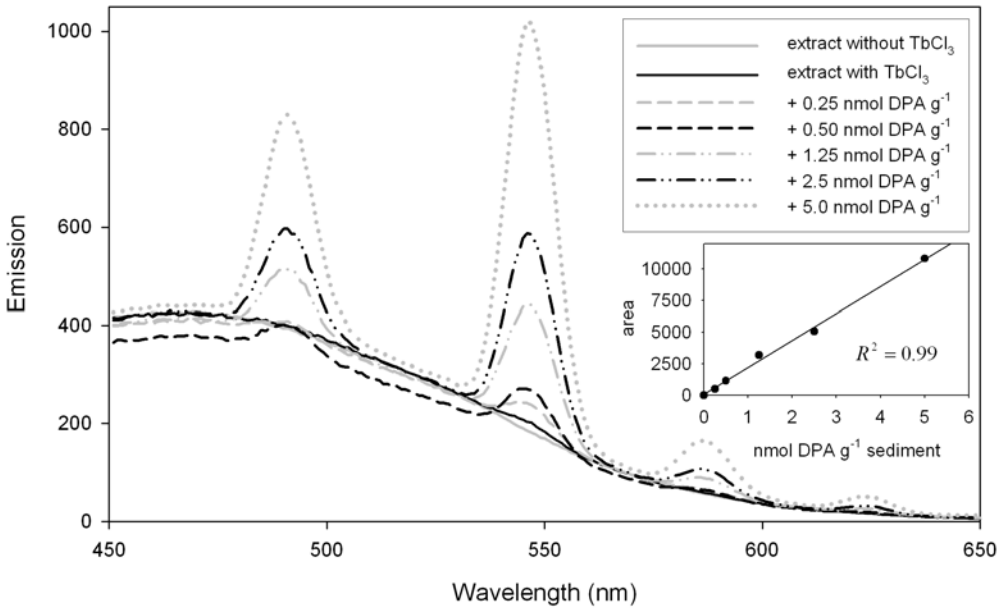

Fig. 5: Emission spectra (270 nm excitation) of purified sediment extracts from a depth of about 4 m at site GP. First, an emission spectrum of the sample without addition of terbium was recorded to perform a baseline correction (gray solid line). Then, terbium was added for complexation and fluorimetric detection of DPA in the purified sediment extract (black solid line). For quantification of DPA, a standard addition approach was applied. Therefore, five sample aliquots were spiked with different amounts of DPA (0.25–5.0 nmol DPA g^{-1} sediment) before being autoclaved and processed as the untreated sample for purification and quantification of DPA (dashed and dotted lines).

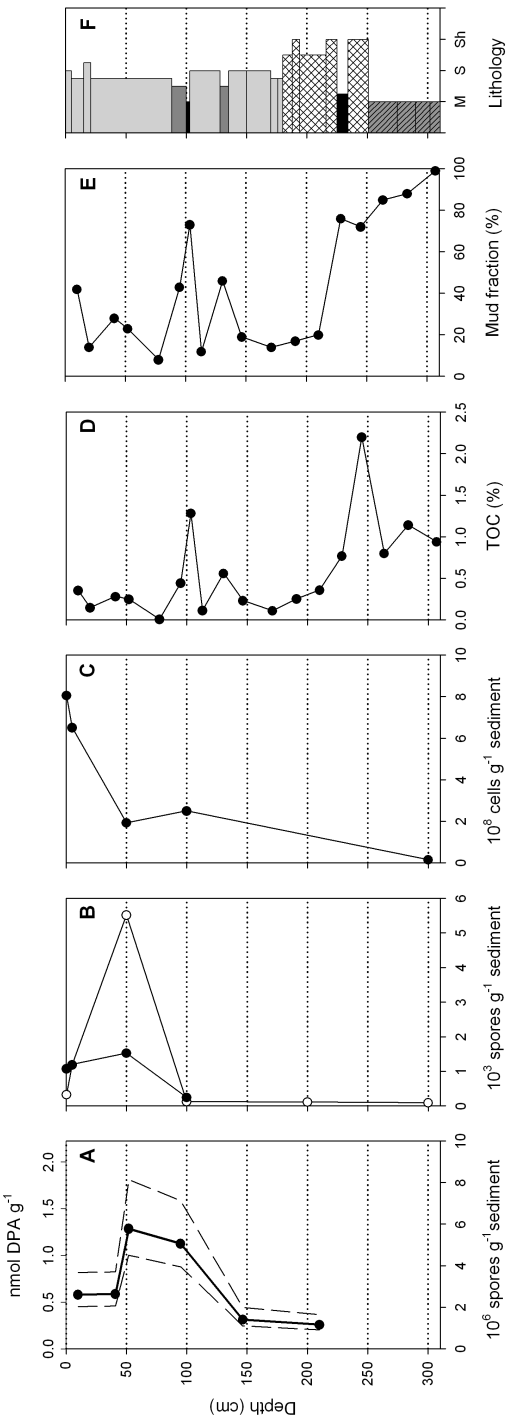

Fig. 6: Depth profiles of microbiological and sedimentological parameters in a core taken from site NSN. (a) Endospore numbers estimated from sediment DPA contents (solid line). For conversion, an average of 2.24×10^{-16} mol DPA spore^{-1} was assumed. Dashed lines indicate the SD. (b) MPN counts of spores obtained after pasteurization. Open circles, after oxic incubation. Closed circles, after anoxic incubation (from Köpke et al., 2005). (c) Total cell counts (from Köpke et al., 2005). (d) Total organic carbon (TOC) content. (e) Relative contribution of mud (< 63 µm) in the sediment after passage through a 500 µm mesh sieve. (f) Lithological profile of the core (simplified after Chang et al., 2006). The width of the bars represents the grain size (M, mud; S, sand; Sh, shells). Black: black mud. Dark gray, hatched: gray mud containing abundant plant remains. Dark gray: mud-sand mixed sediments. Light gray: sand. Cross-hatching: shells.

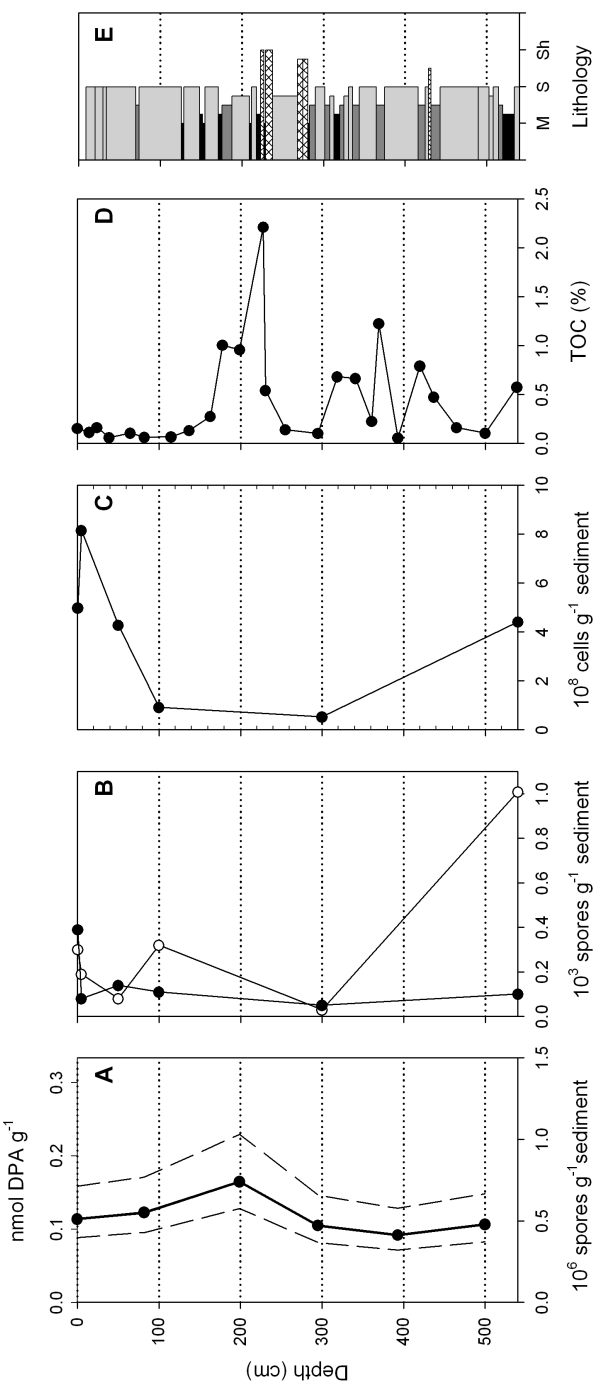

Fig. 7: Depth profiles of microbiological and sedimentological parameters in a core taken from site GP. (a) Endospore numbers estimated from sediment DPA contents (solid line). For conversion, an average of 2.24×10^{-16} mol DPA spore^{-1} was assumed. Dashed lines indicate the SD. (b) MPN counts of spores obtained after pasteurization. Open circles, after oxic incubation. Closed circles, after anoxic incubation (from Köpke et al., 2005). (c) Total cell counts (from Köpke et al., 2005). (d) Total organic carbon (TOC) content. (e) Lithological profile of the core (simplified after Chang et al., 2006). The width of the bars represents the grain size (M, mud; S, sand; Sh, shells). Black: black mud. Dark gray: mud-sand mixed sediments. Light gray: sand. Cross-hatching: shells.

2.4.5 Influence of lithology on DPA extraction

DPA contents were analyzed in sediments down to 5 m below the surface with ages of up to 1500 years (D. Ziehe, 2007, pers. commun.). Generally, DPA was detectable in sediments irrespective of lithology. DPA was extracted from and quantified in the three major sediment types: sand, sediments dominated by calcareous shells, and organic matter-rich mud layers (e. g. 100 cm below the surface, site NSN). Remarkable exceptions were the two samples from depths of about 250 and 300 cm at site NSN. This fine-grained sediment type, which was only found at depths of more than 2.5 m at site NSN, was deposited in a saltmarsh/mudflat environment. It was characterized by greenish-gray or pale olive mud containing abundant plant remains (Chang et al., 2006). Reliable quantification of DPA in this type of sediment was not feasible, due to almost complete adsorption of DPA, as was inferred from the low recovery of DPA added as an internal standard before extraction. Quenching of terbium dipicolinate fluorescence being responsible for the weak fluorescence intensity could be ruled out, as subsequent addition of a defined amount of DPA to the final extract resulted in an appropriate signal. Thus, there is circumstantial evidence that DPA is retained in this type of sediment and resists extraction. Notably, the high mud fraction (85–99 %) in these saltmarsh/mudflat deposits also provided a large specific surface area for adsorption (Fig. 6).

2.4.6 Estimation of endospore numbers from DPA contents

No DPA was found in the pore waters of the sediments. Therefore, it can be supposed that the detected DPA originated from intact endospores only. Calculation of endospore numbers was based on the assumption that the isolated and investigated spores adequately represent the DPA contents of the in situ spore community. For the investigated tidal sediment, the spore DPA contents of the isolated strains were very similar (Fig. 4), from our point of view providing a suitable basis for an estimation of endospore numbers in the respective tidal flat sediments. Therefore, for the conversion of sediment DPA contents into endospore numbers, an average DPA content of 2.24×10^{-16} mol spore^{-1} (SD: 0.63×10^{-16} mol) was assumed.

Estimated spore numbers at site NSN were in the range from 1.2×10^{6} spores g^{-1} sediment (210 cm depth) to 5.8×10^{6} spores g^{-1} sediment (50 cm depth; Fig. 6). Considering that total cell counts decreased by almost two

orders of magnitude from 8.1×10^8 g^{-1} at the surface to 1.5×10^7 g^{-1} at a depth of 3 m (Köpke et al., 2005), calculated endospore numbers correspond to about 0.4 % (at the surface) to 3 % (at 50 cm depth) of total cell counts. At site GP, endospore numbers were clearly lower, with estimates ranging from 4.1×10^5 to 7.4×10^5 spores g^{-1} (Fig. 7), corresponding to 0.1 % (at the surface) to about 1 % (at 300 cm depth) of the total cell counts. The errors of this approach were estimated by including the SD of DPA contents of the tidal flat strains for estimation of endospore numbers (Figs 6 and 7).

Only a limited number of strains was used for determining the conversion factor applied to convert sediment DPA contents into endospore numbers, although Köpke et al. (2005) isolated a larger variety of spore formers from the respective sediments. Most of them, as well as some spore formers from other sources, were also tested, but generally showed only very low spore yields, making purification by density gradient centrifugation almost impossible. For accurate determination of spore DPA content, however, the use of pure spore suspensions is crucial. Cells in an early stage of sporulation or containing immature spores are not contributing to the spore counts but may already contain DPA, which leads to an overestimation of the actual spore DPA contents. For most of the literature, we do not know whether the spores were separated from the vegetative cells, but this might explain the higher DPA contents found in comparison to our study, e. g. for *B. subtilis*^{T} (Table 1 and 2). The tidal flat strains and the two *Bacillus* type strains analyzed in this study, however, showed little variation in spore DPA content (approximately by a factor of 3.5). Only our two sulfate reducers strongly diverged. *Desulfotomaculum* sp. B2T had extraordinarily large spores, and the DPA concentration in *Desulfosporosinus orientis*^{T} spores was approximately twice as high as for the other strains. Whether these values are only outliers or are within the normal range of variation remains unclear, and should be the subject of future investigations.

Other potential DPA sources in sediments appear to be very unlikely. It was reported that DPA is produced and secreted as a secondary metabolite by a few molds and entomopathogenic fungi (e. g. Claydon & Grove, 1982; Kalle & Deo, 1983; Asaff et al., 2005), but these organisms do not generally thrive in marine sediments, and nor was DPA detected in spores of these fungi (Kalle & Khandekar, 1983). Furthermore, it can be expected that excreted DPA will be readily degraded even under strictly anoxic conditions (Seyfried & Schink, 1990).

2.4.7 Contribution of endospores to sediment microbial communities

Huge differences in endospore numbers estimated from DPA contents and obtained via most probable number (MPN) series were found. The conversion of sediment DPA contents resulted in at least three orders of magnitude higher endospore numbers than the MPN counts. In the cores, a maximum of about 5000 spores g^{-1} sediment was obtained after oxic incubation at site NSN (Köpke et al., 2005). There are several reasons that may explain why only a minor fraction of the present endospores was detected by cultivation-dependent approaches. Owing to the high metabolic diversity of spore-forming bacteria, and as media are generally selective for certain physiological groups, quantification of the total number of spores via MPN series is virtually impossible. In addition, germination of spores often only occurs in the presence of certain germinants, and these may vary between different strains (Gould, 1969). Endospores of different species may also vary with respect to heat sensitivity; some may already be inactivated at temperatures around 70 °C (Slepecky, 1972), i. e. the temperature used for pasteurization (Köpke et al., 2005).

Despite their potential longevity (Gest & Mandelstam, 1987; Cano & Borucki, 1995), spores may become unculturable with time or even die off (nongerminable), becoming undetectable by cultivation-based methods. Such a decrease of culturable spores with depth (and age) was observed in freshwater and marine sediments (Rothfuss et al., 1997; Köpke et al., 2005). However, in the sediments investigated in this study, spore numbers estimated from DPA contents showed a less steep decline (Figs 6 and 7). It could be argued that part of this DPA originated from dead spores. However, as long as these spores contain nucleic acids, they are likely to be stained by the commonly used fluorescent dyes, and may still contribute to the total cell counts. On the other hand, we do not know whether endospores would lyse upon death and release DPA. Released DPA, however, can be expected to be degraded rapidly (e. g. Seyfried & Schink, 1990).

Whereas total cell counts continually decreased by almost two orders of magnitude along the upper 3 m of the sediment core at site NSN (Fig. 6), only a weak decrease in endospore numbers was found. At site GP, endospore numbers remained almost constant with depth. This observation indicates that the contribution of endospores to total cell counts increases with depth. Considering the vast vertical extension of the marine subsurface and the general decrease of total cell counts with increasing sediment depth (Parkes

et al., 2000), one can expect that endospores may contribute substantially (some tens of per cent) to the deep biosphere. However, as the marine subsurface represents the largest microbial habitat in the world and harbors more than 50 % of the prokaryotic cells on earth (Whitman et al., 1998), it might be that endospores represent a major part not only of the global prokaryotic community, but also of the global biomass. Answering these questions will require future research.

2.5 Conclusions

Numbers of endospores in sediments are generally quantified using cultivation-based methods with pasteurized samples. In deeper sediment layers, MPN counts targeting endospores often outnumber those for vegetative cells (Köpke et al., 2005), suggesting that the majority of cells in the layers are in fact endospores. On the other, hand viable endospore counts were very low. This discrepancy emphasized the need for a more accurate method. In the present study, an assay based on the fluorimetric determination of DPA after reaction with terbium chloride was applied. This method discriminates neither between viable and nonviable spores nor between different physiological groups. It is therefore not surprising that it yielded higher endospore numbers than the MPN method, and apparently provided for the first time a more realistic estimate of the contribution of endospores to the sediment microbial community. Currently, the conversion of sediment DPA contents into endospore numbers relies on a limited number of strains, due to low spore yield in most species. Future research should therefore focus on analyzing a wider range of strains for spore DPA contents, to provide a more reliable conversion factor. Furthermore, sediments representing different lithological units and, in particular, deeper layers should be investigated to determine whether endospores represent a major part of the deep biosphere.

2.6 Acknowledgements

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3 A highly sensitive HPLC method for determination of nanomolar concentrations of dipicolinic acid, a characteristic constituent of bacterial endospores

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3.1 Abstract

A high-performance liquid chromatographic method with indirect fluorescence detection has been developed for quantification of dipicolinic acid (DPA), a major constituent of bacterial endospores. After separation on a reversed-phase column, a post-column reagent of sodium acetate at 1 mol l^{-1} with 50 µmol l^{-1} terbium chloride was added for complexation of dipicolinic acid. Terbium monodipicolinate complexes formed were quantified by measuring the fluorescence emission maximum at 548 nm after excitation with UV light at 270 nm wavelength. Parameters of post-column complexation were optimized to achieve a detection limit of 0.5 nmol DPA l^{-1}, corresponding to about 10^3 *Desulfosporosinus orientis* endospores per ml. The method was applied to the analysis of spore contamination in tuna and for estimating the endospore numbers in marine sediments.

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3.2 Introduction

Detection of bacterial endospores is of great importance in food and medical microbiology. Endospores are highly resistant to heat, radiation and chemicals, and are ubiquitous in the environment. This makes them major agents of food spoilage and food-borne diseases (Setlow, 2006). Rapid detection of *Bacillus anthracis* spores, in turn, is essential to prevent extended exposure and is a prerequisite to apply effective medication after anthrax outbreaks for example in the case of terrorist attacks (Atlas, 1999).

In environmental microbiology, quantification of endospores may prove to be a crucial factor in understanding the community structure of sedimentary microorganisms. Marine sediments harbour over half of all prokaryotic cells on earth (Whitman et al., 1998). Typical total cell counts, determined by epifluorescence microscopy, decrease from up to 10^9 cm^{-3} near the sediment surface to 10^5–10^6 cm^{-3} at about 800 m depth (Parkes et al., 2000). But these counts do not imply that the prokaryotes are active, since fluorescence dyes like acridine orange or DAPI generally do not discriminate between metabolically active, dormant or dead cells (Kepner & Pratt, 1994; Luna et al., 2002), and even stain endospores (Cragg and Sass, unpublished). Therefore, up to now, endospores were not distinguished from vegetative cells. However, due to their potential longevity of up to 25 million years (Cano & Borucki, 1995), it is likely that their relative abundance within the microbial community increases during sediment burial and with age, as it was suggested by cultivation-dependent approaches (Rothfuss et al., 1997; Köpke et al., 2005).

Various methods have been applied for the determination of endospore numbers. Standard plate counts on selective media are generally used in food industry to check processing parameters and to control food quality. This technique is sensitive, but often requires several days of incubation and sometimes results in considerable variability and false positives (Newsome, 2003). In environmental microbiology, cultivation-dependent approaches like most probable number (MPN) counts are applied to determine total spore counts (Köpke et al., 2005). These methods, however, are biased towards certain physiological groups. Moreover, germination of spores only occurs in the presence of appropriate germinants, varying between different strains (Gould, 1969). For selection of endospores, samples are generally pasteurised. But since endospores of different species may vary with respect

to their heat sensitivity, some may already be irreversibly inactivated at pasteurisation temperatures of around 70 °C (Slepecky, 1972).

For highly sensitive detection of endospores several immunological methods were developed, which take advantage of specific binding of fluorescence-labelled antibodies to spore surfaces. For separation and detection of endospores, antibodies can be attached to magnetic beads. In the study of Blake & Weimer (1997), these so called immunomagnetic approaches permitted the detection of about 10^4 *Bacillus stearothermophilus* spores ml^{-1}, but not of other *Bacillus* sp. tested. For the detection of certain bacterial target species the high specificity of antibodies due to heterogeneous spore surfaces is an advantage (Costa et al., 2006), but for determination of total spore counts a universal surface antigen would be required. Since spores differ strongly in their surface properties – for instance not all spores have an exosporium – the development of an appropriate antibody appears to be almost impossible. Furthermore, immunomagnetic detection of endospores may be adversely affected by the matrix. Bruno & Yu (1996) reported a decrease in sensitivity of up to three orders of magnitude in soil suspensions.

Chemical techniques for detection of bacterial endospores are generally based on the determination of dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid). DPA is a universal and specific component of bacterial endospores (Powell, 1953), representing 5–14 % of endospore dry weight (Murrell & Warth, 1965; Murrell, 1969). Some mutants that do not produce DPA were isolated, but these are rare and usually less robust. Paidhungat et al. (2000) showed that the lack of DPA, which is normally accompanied by increased core hydration, results in reduced spore wet heat resistance. Furthermore, DPA is apparently needed in some fashion for maintaining spore dormancy, since DPA-free endospores were extremely unstable and germinated spontaneously (Lewis, 1969). Therefore, DPA appears to be a useful analytical indicator of the presence of bacterial endospores and permits to estimate total spore numbers, provided that spore DPA contents are known.

In a previous study, Fichtel et al. (2007a) determined endospore DPA contents of different strains that were isolated from North Sea tidal flat sediments and used these data for conversion of sediment DPA contents into spore numbers. For separation and quantification of DPA in sediment samples, a protocol was developed which is based on the terbium dipicolinate fluorescence method described by Hindle & Hall (1999). The terbium

fluorescence approach was chosen due to its high sensitivity, which is necessary for detection of the relatively low natural spore contents in sediments. On the other hand, analytical problems arose, since terbium dipicolinate fluorescence can be completely quenched and masked by the sediment matrix. Pellegrino et al. (1998) showed that the presence of significant amounts of phosphate-containing substances can result in a suppression of terbium monodipicolinate fluorescence, presumably due to the high affinity of terbium for phosphate. This problem can be mitigated by addition of $AlCl_3$ to remove phosphate via precipitation (Fell et al., 2001), but it still affects the determination of dipicolinic acid in the presence of biological materials containing high proportions of organophosphates. Furthermore, humic acids can contribute to quenching of terbium dipicolinate fluorescence due to complexation of added Tb^{3+} (Lippold et al., 2005). To overcome these problems, quantification of DPA in sediment samples required a separation of DPA prior to fluorimetric detection of its terbium chelates. By using an ethyl acetate extraction procedure, Fichtel et al. (2007a) were able to detect DPA at concentrations of less than 0.1 nmol DPA g^{-1} sediment. The quantification of DPA in these purified sediment extracts still required a standard addition procedure, since quenching was not completely inhibited and the signal was still affected by background fluorescence. Furthermore, purification and standard addition procedures were labour-intensive and time-consuming, emphasizing the need for a fast, simple and sensitive approach.

In this study an HPLC method for separation of DPA from interfering substances is presented. To improve the detection limit, the HPLC system was directly coupled with post-column complexation and fluorimetric detection of DPA via terbium dipicolinate fluorescence. This specific set-up required technical adaptations of chromatography and post-column complexation.

3.3 Experimental

3.3.1 Reagents

Dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid) was obtained from Sigma (Taufkirchen, Germany), chelidamic acid (ChA, 4-hydroxypyridine-2,6-dicarboxylic acid) from Acros Organics (Geel, Belgium). Sodium bisulphate monohydrate (BioChemika Ultra, $\geq 98.0\,\%$) was purchased from Fluka (Taufkirchen, Germany), methanol used for HPLC was gradient grade (VWR International, Darmstadt, Germany). For the post-column rise of pH, sodium acetate of luminescence grade with a purity of $\geq 99.5\,\%$ (Fluka) was used. Terbium(III) chloride hexahydrate added for post-column complexation of DPA had a purity of 99.999 % (Aldrich, Taufkirchen, Germany).

3.3.2 Spores

Bacillus subtilis ssp. *subtilis* DSM 10^{T} was grown in a freshwater medium (5 g peptone, 3 g meat extract and 10 mg $MnSO_4{\cdot}2\ H_2O$ per litre, pH 7.0). Sporulation of *B. subtilis*^{T} was induced by transferring the cells from the nutrient-rich growth medium to pure water. The strictly anaerobic *Desulfosporosinus orientis*^{T} was grown in a freshwater medium as described by Sass et al. (1997). The medium was supplemented with 20 mmol l^{-1} sodium lactate and 10 mmol l^{-1} sodium sulphate. Sporulation of the sulphate reducer was achieved by allowing the cells to exhaust nutrients in their growth medium. This approach avoided methodological problems associated with maintaining anoxic conditions during centrifugation and resuspension of the cells.

Separation of spores from vegetative cells was done by density gradient centrifugation with gradients of the X-ray contrast agent Gastrografin (Schering) as described by Fichtel et al. (2007a). Spores were washed and stored in 0.9 % NaCl solution at 4 °C. Spore counts were determined by phase contrast microscopy using a Thoma counting chamber. At least 24 randomly chosen squares with about 100 endospores were counted. If necessary, suspensions were diluted to an appropriate density of about 10^8 endospores ml^{-1}.

3.3.3 Apparatus

Chromatography and detection of DPA were carried out on a Waters HPLC system (Eschborn, Germany) consisting of a gradient pump (model 600 E Multisolvent Delivery System), an HPLC pump (model 515) for adding the post-column complexation reagent, and an autosampler (model 717 plus) with 2000 µl sample loop and 2500 µl syringe. The use of polypropylene vials is recommended, since adsorption of DPA to glass surfaces was observed at low pH values. Furthermore, only plastic pipette tips should be used for handling extracts and standard solutions. Mobile phases and complexation reagent were degassed by an in-line degasser (In-Line Degasser AF). Absorbance in the range from 210 nm to 400 nm was measured using a photodiode array detector (model 996). A multiwavelength fluorescence detector (model 2475) was set up to excite at 270 nm and measure the resulting emission at 548 nm. The software Waters Empower 2 was used to control the operation of the system, for data collection and analysis.

3.3.4 Chromatography

A Phenomenex Gemini C18 110A HPLC column (5-µm particle diameter) with 150 mm length and 4.6 mm inner diameter (Phenomenex, Aschaffenburg, Germany) was used for separation. The analytical column was protected with a Phenomenex Gemini C18 guard column (4 mm × 3.0 mm). A column temperature of 20 °C was maintained by means of a column thermostat (BFO-04 svnl, W.O. Electronics, Langenzersdorf, Austria). The mobile phase consisted of 50 mmol l^{-1} sodium bisulphate. It was adjusted to pH 1.2 with sulphuric acid and passed through HPLC certified GH Polypro membrane disc filters (Pall, Dreieich, Germany) with 0.45 µm pore size to remove particles. A multistep binary gradient programme with a methanol ratio of 20 % to 40 % was used at a flow rate of 2.0 ml min^{-1} (Fig. 8).

3.3.5 Post-column complexation

Pure sodium acetate solution (1 mol l^{-1}, about pH 9) with 50 µmol l^{-1} $TbCl_3$ was degassed by means of an in-line degasser and added to the eluent at a flow rate of 0.5 ml min^{-1}. To prevent pulsations in the fluorescence signal derived from the Waters 515 HPLC pump, a pulse damper (Model LP-21, SSI-LabAlliance, Pa., USA) was installed together with a backpressure regulator (part no. 39024, 100–1500 psi, Alltech, Worms, Germany) set to

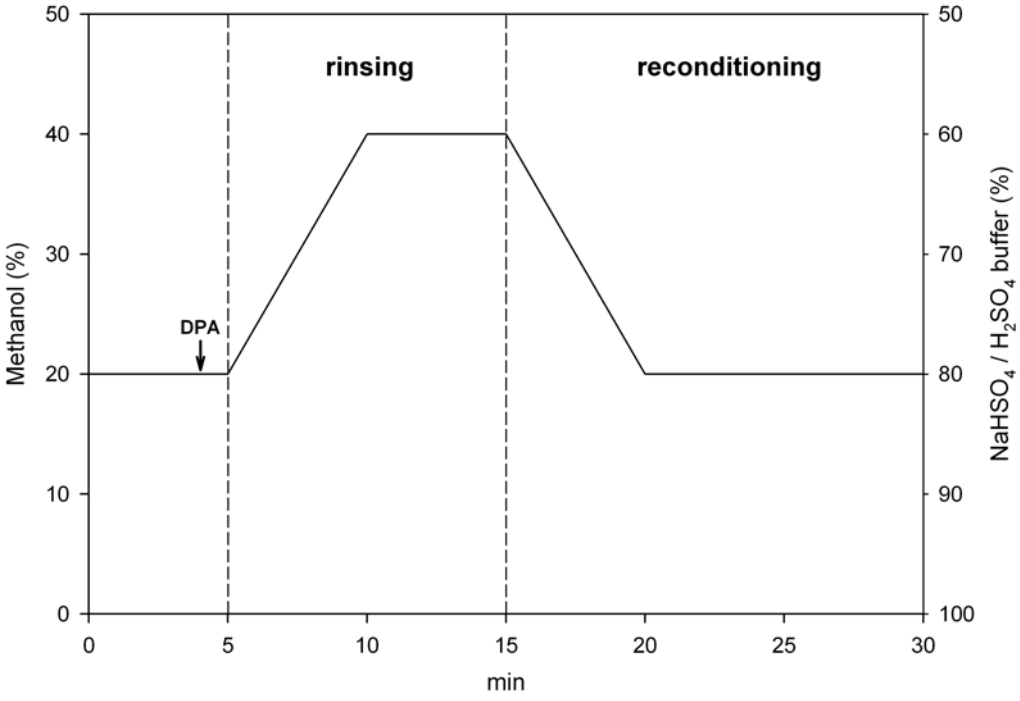

Fig. 8: Graphical representation of the multistep binary gradient programme applied for chromatographic determination of DPA. Y-axes display the relative proportions of methanol and 50 mmol l^{-1} sodium bisulphate buffer (adjusted to pH 1.2 with sulphuric acid) for a complete sample run. Due to necessary rinsing and reconditioning of the column, complex samples require 30 min analysis time. For standard runs isocratic elution with 20 % methanol is sufficient and analysis time can be reduced to 5 min.

1000 psi (Fig. 9). Complexation reagent was added by means of a post-column reactor (Metrohm, Herisau, Switzerland) with a built-in pulsation-absorbing silicone capillary. By default, a PEEK capillary (part b in Fig. 9) with smaller inner diameter (0.25 mm inner diameter, 3 m length) was installed in line with the pulsation-absorbing silicone capillary to increase the backpressure and maximize the damping. Due to the high flow rates in our application, this PEEK capillary had to be shortened to about 10 cm length to prevent bursting of the silicone capillary. Furthermore, sudden increase of pressure at system start-up should be avoided, and flow rates should rather be increased gently.

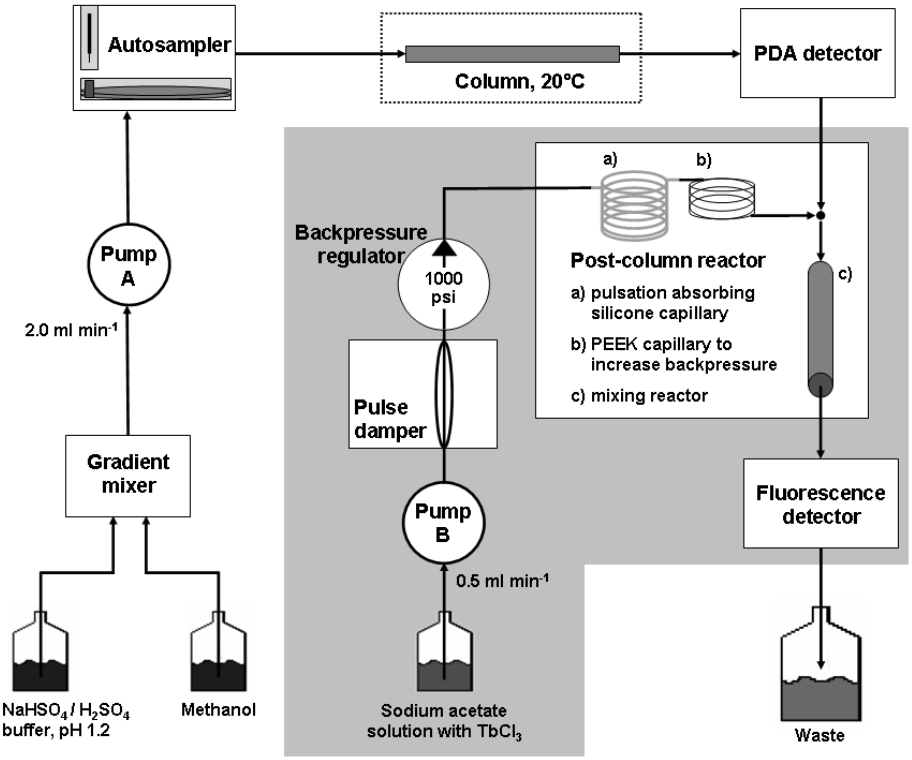

Fig. 9: Schematic diagram of the HPLC system set up for quantification of DPA. The grey shaded area represents the modules installed and modified especially for post-column complexation and fluorimetric detection of DPA.

3.3.6 Fluorimetric detection of DPA

In order to eliminate second-order diffraction of elastically scattered light, a circular long-pass filter N-WG320 (Schott, Mainz, Germany) with 7 mm diameter (1 mm thickness) was inserted between flow cell and emission

monochromator. This modification of the detector optics increased the sensitivity, since the strongest emission maximum of terbium dipicolinate complexes at 548 nm can be used. In normal operation mode (without filter) setting up excitation to 270 nm wavelength and emission to approximately twice the excitation wavelength exceeds the saturation limit of the detector's photomultiplier due to second order diffraction effects. In this case, only the about 50 % weaker emission maximum at 490 nm can be used for quantification.

3.3.7 Determination of DPA in bacterial endospores

For extraction of DPA from endospores 0.2 ml of endospore suspension (about 2.7×10^7 spores ml^{-1}) were transferred into autoclavable 15-ml polypropylene tubes with screw caps (Sarstedt, Nümbrecht, Germany) and filled up with 50 mmol l^{-1} sodium bisulphate buffer (pH 1.2) to 5 ml. DPA was completely released from endospores by autoclaving the suspensions at 121 °C for 30 min (Pellegrino et al., 2002). Impermeability of the tubes was confirmed by weighing before and after autoclaving. After cooling, the samples were centrifuged (4000 *g*, 5 min, 15 °C), and the supernatant (containing the released DPA) was sterile-filtered to remove spore debris. The cellulose acetate syringe filters (0.2 µm pore size, Nalgene Nunc International, Rochester, NY, USA) were proven not to adsorb DPA. For fluorimetric determination of DPA the extract was further diluted 1:10 (corresponding to a spore density of 1.1×10^5 endospores ml^{-1}) and spiked with 227 nmol l^{-1} ChA used as internal standard.

3.3.8 Determination of DPA from endospores added to canned tuna

As an example for application in food safety analysis the post-column complexation HPLC method was tested in the detection of spores added to canned tuna. For that purpose, 2.0 g of homogenised tuna (canned in water) were weighed into autoclavable 15-ml polypropylene tubes with screw caps and filled up to 5 ml with *B. subtilis* spore suspension (1.2×10^6 spores per ml of 50 mmol l^{-1} sodium bisulphate buffer, pH 1.2) corresponding to about 3×10^6 spores per gram tuna. Samples were vortexed and autoclaved as described above for extraction of DPA from spores. After cooling, as described above, the tuna sample was centrifuged and the supernatant filtered through cellulose acetate syringe filters.

3.3.9 Determination of DPA in sediment samples

Sediment samples from about 1 m depth were collected at site Neuharlingersieler Nacken (53°43.270'N, 7°43.718'E) in the tidal flats of the East Frisian Wadden Sea, Germany (Köpke et al., 2005). An aluminium tube (8 cm diameter) was driven into the sediment using a vibrating unit. The core was recovered by the use of a tripod with a hoist and cut longitudinally for subsampling. The sediment sample for DPA analysis was taken from the innermost part of the core. It was freeze-dried and stored at −20 °C until processing.

For determination of DPA, 2.0 g freeze-dried sediment were weighed into autoclavable 15-ml polypropylene tubes with screw caps. Duplicates were prepared for each sample to determine recovery: One of the sediment aliquots was suspended in 5 ml sodium bisulphate buffer (50 mmol l^{-1}, pH 1.2), the other one was spiked by suspending in 5 ml buffer with 100 nmol DPA l^{-1}. Both suspensions were autoclaved for release of DPA from the endospores of the sediment. After cooling, as described above, the samples were centrifuged and the supernatants filtered. For calculation of recovery, the difference in DPA concentrations of the duplicates was related to the concentration of the standard solution used for spiking.

Extraction efficiency was checked by multiple successive extraction of the sediment samples. After centrifugation and separation of the supernatant of the first extraction, the weight increase of the sediment samples was determined to estimate carryover of DPA-containing extract in the pore space of the sediment matrix. Then, the sediment samples were resuspended in 5 ml sodium bisulphate buffer, autoclaved and prepared for determination of DPA once again. The additionally extracted DPA was calculated with respect to expected carryover effects. For this purpose, weight loss of the sediment samples due to decomposition of carbonate ($< 10\,\%$ sediment dry weight) was neglected.

3.4 Results and discussion

3.4.1 Separation conditions

For high-performance liquid chromatography separation conditions had to be developed, which favour post-column complexation and fluorimetric detection of DPA. While a low pH value of the mobile phase is necessary for protonation of DPA to give adequate retention on reversed-phase HPLC columns, it also ensures dissociation of DPA chelates which otherwise could affect the separation. For chromatographic determination of DPA, Warth (1979) found phosphate buffer at pH 1.8 most suitable as the aqueous component of the eluent. However, for fluorimetric determination of DPA via post-column complexation with terbium an alternative buffer was necessary. Barela & Sherry (1976) showed that phosphate buffer reduces the fluorescence signal by more than 50 % relative to the signal of an unbuffered solution.

Sulphate buffer as an alternative turned out to be suitable concerning its fluorescence properties. Chromatograms shown in Figs. 11–13 were recorded with a Phenomenex Gemini C18 column. This type of stationary phase has a recommended operating range of pH 1–12 and allowed the use of 50 mmol l^{-1} sodium bisulphate buffer being adjusted to pH 1.2 as the aqueous component of the mobile phase. In comparison to other C18 columns initially tested in our study and to the method of Warth (1979), this stationary phase did not show binding of DPA and did not require conditioning with DPA before each analysis. For determination of DPA in complex samples, a methanol gradient of 20 % to 40 % at a flow rate of 2.0 ml min^{-1} was used (Fig. 8). For standard runs isocratic elution with 20 % methanol was sufficient and analysis time could be reduced to 5 min, since the gradient programme is not required for elution of DPA, but for rinsing of the column and subsequent reconditioning.

3.4.2 Post-column complexation

Liquid chromatographic separation of DPA required a low pH value, whereas for post-column complexation with terbium a rise in pH to at least 5 was necessary. Therefore, optimization of both, chromatography and post-column complexation, represented a compromise concerning pH values and flow rates of mobile phase and complexation reagent. Furthermore, the buffer system

itself has a great influence on fluorescence intensity (Barela & Sherry, 1976; Hindle & Hall, 1999). Hindle & Hall (1999) showed that fluorescence intensity is highest for acetate buffer at pH 6.0 and increases with acetate concentration probably by shielding from quenching due to exclusion of water from coordination sites.

However, for post-column complexation sodium acetate solution (1 mol l^{-1}, about pH 9) was used instead of buffer with defined pH value. This set-up minimized the dilution of the eluent and resulted in a higher sensitivity of the system. Experiments showed that a flow rate of 0.5 ml acetate solution min^{-1} was sufficient for the desired rise in pH. Considering an optimal terbium concentration of about 10 µmol l^{-1} (Hindle & Hall, 1999), a concentration of 50 µmol l^{-1} $TbCl_3$ was chosen for the post-column complexation reagent.

3.4.3 Prevention of pulsations in the fluorescence signal

Determination of DPA in the nanomolar range was initially hampered by periodic baseline fluctuations in the fluorescence signal. The observed periodicity was attributed to pressure fluctuations of the Waters 515 HPLC pump, even though the sensor of the pump indicated constant pressure. An improvement was achieved by means of a pulse damper and a pressure regulator to support valve performance of the pump and to optimize damping (Fig. 9). Residual periodic fluctuations affecting detection of DPA at concentrations of less than 10 nmol DPA l^{-1} were overcome by integrating the post-column reactor into the flow line instead of using a common T-piece. However, high flow rates of mobile phase and complexation solution required a modification of the post-column reactor as described in the methods section.

3.4.4 Quantification

The measurement range extended from 0.5 nmol DPA l^{-1} to 500 nmol DPA l^{-1}. Calibration curves showed a slight deviation from linearity for concentrations below 10 nmol l^{-1} (Fig. 10). It is not clear, whether this deviation is a result of the detector optics (non-linear behaviour of the photomultiplier) or a consequence of the increase in Tb^{3+}/DPA concentration ratio. With terbium being added in excess, the formation of terbium monodipicolinate $[Tb(DPA)]^+$ was strongly favoured, resulting in a maximum intensity of the fluorescence signal (Rosen & Niles, 2001). In contrast, unchelated ter-

bium ions yield higher background fluorescence and increase the detection limit. However, an adjustment of terbium concentration to the analysis of samples with less than 10 nmol DPA l^{-1} did not significantly increase the sensitivity, since terbium fluorescence is enhanced by more than four orders of magnitude by complexation with DPA (Hindle & Hall, 1999).

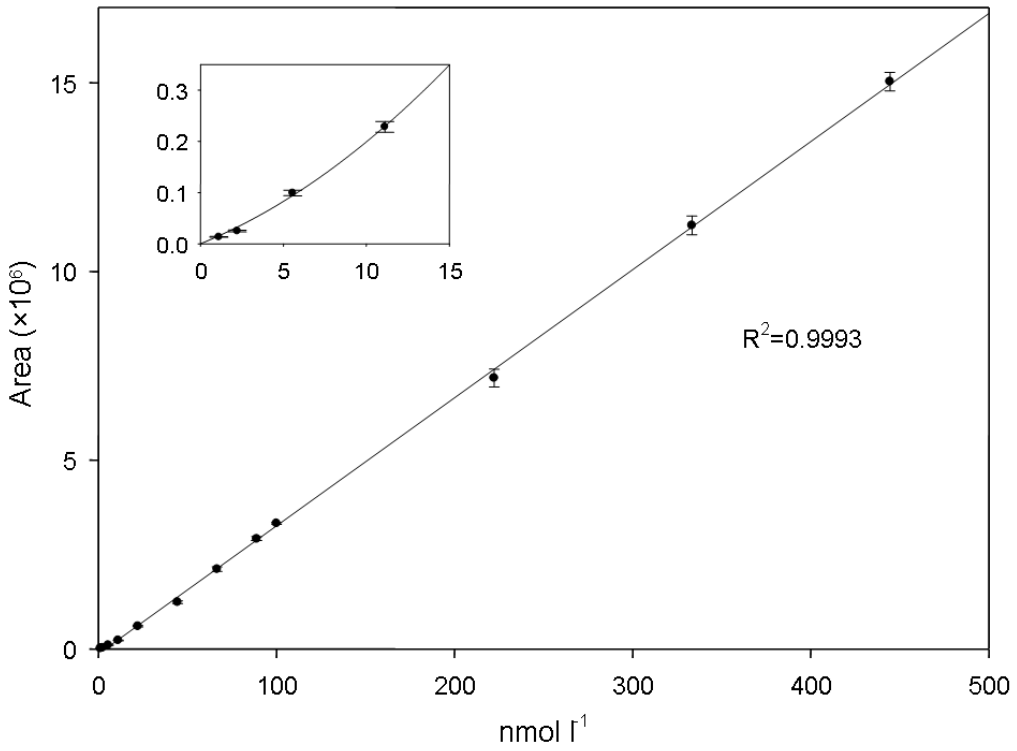

Fig. 10: Calibration curve for quantification of DPA via post-column complexation with 50 µmol l^{-1} $TbCl_3$ in sodium acetate solution (1 mol l^{-1}). The data points give the averages and standard deviations of two standard sets, which bracketed 24 injections of sediment extracts with a total running time of 12 h. The inset shows the curve for low DPA concentrations. For concentrations < 10 nmol l^{-1} a slight deviation from linearity was observed.

3.4.5 Determination of DPA extracted from endospore suspensions

Fluorimetric determination of DPA via post-column complexation with terbium verified the results previously obtained by measuring, in that case less diluted, samples with a photodiode array (PDA) detector (Fichtel et al., 2007a). The fluorescence chromatogram of an extract of 1.1×10^5 *D. orientis* endospores per ml showed a baseline-separated DPA peak of high symmetry and no interfering compounds (Fig. 11). The high sensitivity of this approach even permitted the determination of about 10^3 *D. orientis* endospores per ml.

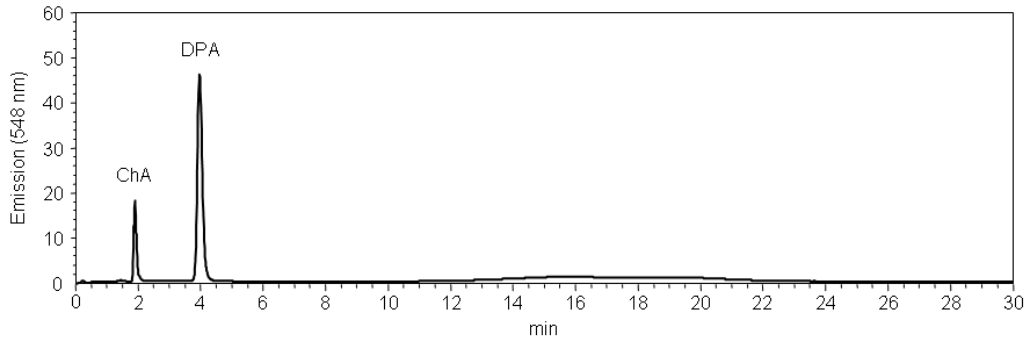

Fig. 11: Chromatogram of an extract of *Desulfosporosinus orientis* spores (about 1.1×10^5 endospores ml^{-1}, corresponding to 130 nmol l^{-1} DPA) with 227 nmol l^{-1} chelidamic acid (ChA) used as internal standard.

Application of chelidamic acid (ChA) as internal standard turned out to be suitable, even though with some restrictions. Due to the additional hydroxyl group, ChA eluted earlier than DPA, but also showed a markedly lower fluorescence response (Fig. 11). The peak height ratio of DPA/ChA normalized to concentration was about 4.5, the peak area ratio even 7.5 due to the narrower ChA peak. This is contrary to observations of Lamture et al. (1995), who compared the fluorescence intensities of Tb(III) complexes of a series of 4-substituted analogues of dipicolinic acid in TRIS buffer at pH 8. However, Lamture et al. (1995) added ligands in excess, favouring the formation of terbium trisdipicolinate complexes (Rosen & Niles, 2001). Furthermore, concentrations of dipicolinic acid derivates were extremely high, exceeding the measurement range of our approach by two orders of magnitude. In addition, the choice of buffer could have affected the fluorescence intensities of DPA and ChA complexes (Barela & Sherry, 1976).

3.4.6 Determination of DPA from endospores added to canned tuna

The post-column complexation HPLC approach turned out to be suitable for determination of endospores in samples with complex matrices. This was demonstrated by analysis of canned tuna spiked with 3.1×10^6 *B. subtilis* spores per gram. An aliquot of the tuna sample was previously tested to contain no detectable amounts of DPA (Fig. 12A). In general, the fluorescence chromatograms of the tuna extracts were characterised by several peaks forming a hump between 1 and 3 min retention time and three peaks between 10 and 13 min. In the spiked tuna sample (Fig. 12B), DPA was almost baseline-separated from the hump and detected after 3.8 min retention time.

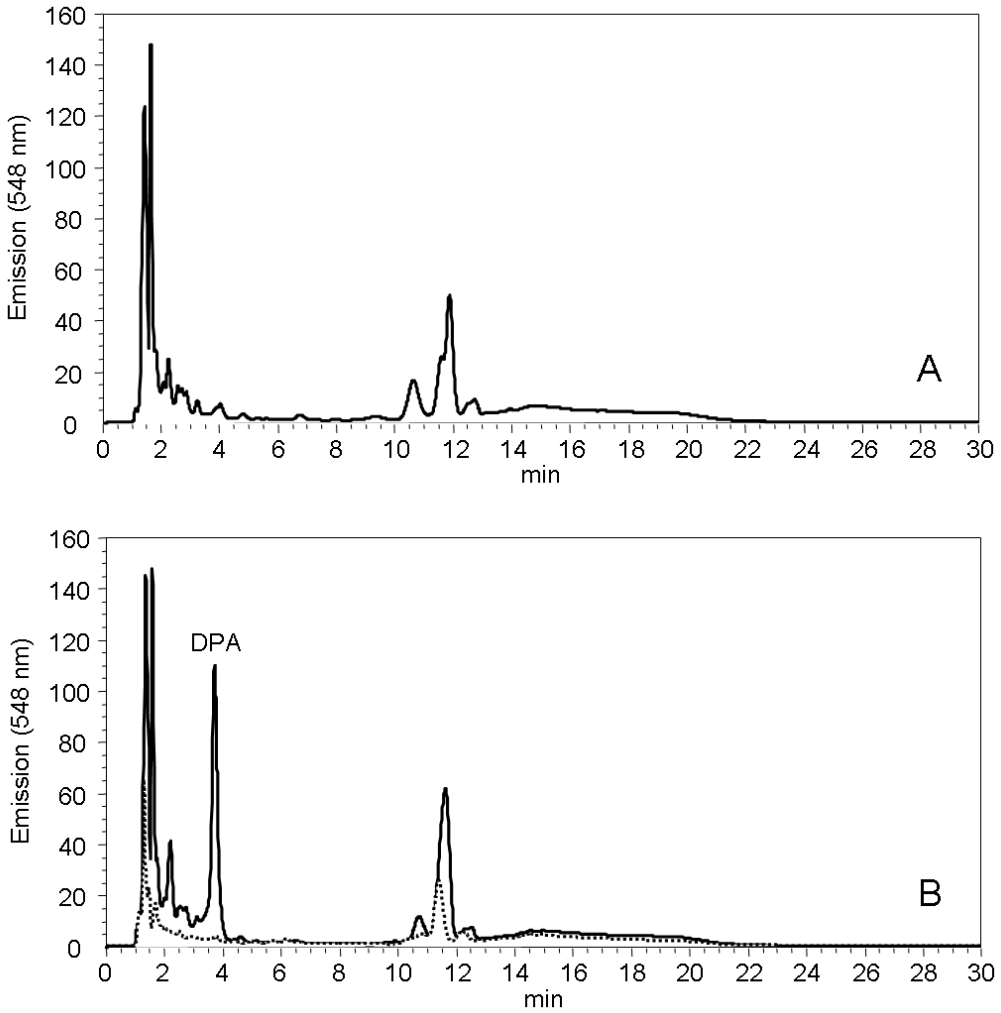

Fig. 12: Determination of DPA in spiked tuna. A) Fluorescence chromatogram of tuna extract after post-column addition of $TbCl_3$. B) Chromatogram of a tuna aliquot spiked with 3.1×10^6 *Bacillus subtilis* spores per gram. The dotted line shows the emission derived from the extract without addition of $TbCl_3$ (post-column addition of 0.5 ml min^{-1} H_2O), the solid line displays the emission after post-column complexation with $TbCl_3$. Via complexation with Tb^{3+} contamination with endospores was clearly detected by a large DPA peak occurring at 3.8 min.

For verification of spore contamination a second sample run was performed without post-column addition of terbium. For this purpose, the complexation solution was replaced with pure water and added at the same flow rate. The resulting fluorescence chromatogram did not show any peak occurring in the elution window of DPA, since DPA itself does not exhibit fluorescence. In contrast, the peak triplet between 10 and 13 min was visible also without addition of terbium. Differences in intensity can partially be attributed to changes in pH. With addition of sodium acetate solution (without terbium) instead of water, calculation of difference chromatograms may provide a possibility to quantitatively differentiate DPA and coeluting substances, permitting in certain cases a better quantification of non-baseline-separated DPA peaks.

3.4.7 Estimation of spore numbers in a tidal flat sediment rich in organic matter

Spore numbers in a tidal flat sediment sample were estimated via quantification of DPA. Determination of DPA in tidal flat sediments was previously hampered by high amounts of fluorescent organic matter interfering with terbium dipicolinate fluorescence. High fluorescence background in sediment extracts, but also strong quenching of terbium dipicolinate fluorescence was observed, requiring a protocol for selective extraction of DPA (Fichtel et al., 2007a).

By application of the newly developed HPLC method, this fluorescent organic matter background was almost completely separated from DPA as can be seen in the fluorescence chromatogram of the sediment extract (Fig. 13). The emission chromatogram showed a hump between 1 and 3 min retention time, which was also visible in the absorption chromatogram recorded at 270 nm wavelength (data not shown). DPA was detected as a distinct, baseline-separated peak at about 4 min retention time, but only in the fluorescence chromatogram.

External calibration was used for quantification and resulted in a DPA concentration of 274 nmol DPA l^{-1} extract, corresponding to 686 pmol DPA g^{-1} sediment (dry weight). By spiking sediment samples with 250 pmol DPA g^{-1}, recoveries of about 80 % were determined. This was done by suspending sediment aliquots in DPA standard solution as described in the methods section. It turned out that spiking concentrations were required

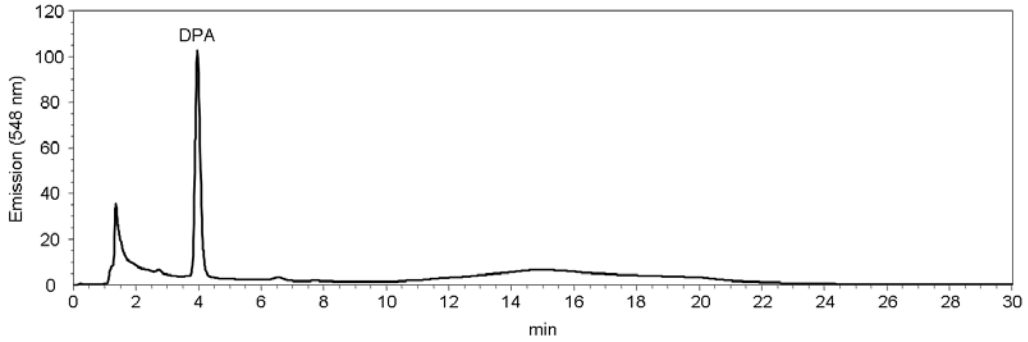

Fig. 13: Fluorescence chromatogram of the extract of a sediment sample taken from about 1 m depth in the backbarrier tidal flat of Spiekeroog Island in the southern North Sea. Via the post-column complexation HPLC approach, DPA was separated from the fluorescent sediment matrix and could be quantified without further sample preparation. Determination of DPA content was used to estimate endospore numbers in the sediment sample.

that amount to at least one quarter of the natural DPA concentration. Lower spiking concentrations often affected determination of recovery due to the inhomogeneity of the sediment samples. Completeness of extraction was determined by multiple extraction of the sediment samples. The additionally extracted DPA was calculated with respect of expected carryover effects, but concentrations were not significantly higher and did not result in an increase of recovery. Therefore, DPA concentrations determined after single extraction were expected to give a good basis for estimating endospore numbers in sediments. Nevertheless, determination of recovery is recommended in order to assess remaining possible interferences in different sediment matrices.

For conversion of sediment DPA contents into endospore numbers, an average DPA content of 2.24×10^{-16} mol per spore was assumed (Fichtel et al., 2007a). This factor was determined from spore DPA contents of several strains isolated from the investigated tidal flat. The spores of these strains showed only little variation in DPA content. Using this conversion factor, DPA content of the sediment resulted in 3×10^{6} spores g^{-1} sediment (dry weight), corresponding to about 1 % of total cell counts (Köpke et al., 2005).

3.5 Conclusions

The presented HPLC method succeeded in chromatographic separation of DPA from interfering compounds and utilised the entire sensitivity of terbium dipicolinate fluorescence for detection of bacterial endospores. It permitted the detection of less than 1 nmol DPA l^{-1} without the need of extensive and time-consuming sample preparation or derivatisation steps. Furthermore, the post-column complexation HPLC approach was very insensitive to interferences and allowed the quantification of spore-derived DPA in complex samples in the presence of high amounts of organic material. Therefore, potential fields of application range from detection of spores for quality control in food industry to quantification of endospores in environmental microbiology.

3.6 Acknowledgements

We thank Wolfgang Mayer (Waters GmbH, Eschborn, Germany) for discussion and suggestions concerning HPLC set-up. This work was supported by the Deutsche Forschungsgemeinschaft (DFG) through a grant for the Research Group on BioGeoChemistry of Tidal Flats (grant no. RU 458/24).

4 Assessment of spore contamination in pepper by determination of dipicolinic acid with a highly sensitive HPLC approach

This chapter is in press: Fichtel J.*, Sass H. and Rullkötter J., 2008. Assessment of spore contamination in pepper by determination of dipicolinic acid with a highly sensitive HPLC approach. Food Control, (doi:10.1016/j.foodcont.2007.09.006).

4.1 Abstract

A protocol for quantification of dipicolinic acid (DPA) in spices by high-performance liquid chromatography (HPLC) with post-column complexation and fluorescence detection was developed. DPA is a specific component of bacterial endospores and can be used for rapid assessment of endospore contamination without the need of cultivation. The highly sensitive approach was used to determine the DPA contents of whole black, white and green peppercorns. For conversion into endospore numbers, the DPA content of endospores of *Bacillus subtilis*, one of the most frequently detected species of spore-forming bacteria in pepper, was used. Estimated total spore numbers were highest for black pepper (1.6×10^8 spores g^{-1}), for white and green pepper lower endospore loads were determined (about 8×10^7 spores g^{-1} in each case).

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4.2 Introduction

Spices and herbs can contain high numbers of microorganisms (McKee, 1995). Particularly black pepper was reported to be heavily contaminated. Christensen et al. (1967), Schwab et al. (1982) and Pafumi (1986) determined more than 10^8 viable bacteria per gram of black pepper. Baxter & Holzapfel (1982) showed that the majority of the detected bacterial cells in spices and herbs had been present as endospores. After pasteurisation of the samples, aerobic spore formers were found to represent between 50 % and 95 % of total plate counts of bacteria. Total spore counts of more than 10^7 CFU per gram of black and white pepper were also reported by de Boer et al. (1985). High numbers of endospores in spices are a potential hazard even for cooked food since spores might be able to survive the cooking process, may germinate and result in food poisoning or spoilage of the spiced product. Generally, standard plate counts after pasteurisation of the samples are used in food industry for determination of spore counts to check processing parameters and for control of food quality. This technique is sensitive, but often requires several days of incubation and sometimes results in considerable variability.

In the present study, we describe a new cultivation-independent approach for sensitive detection of endospores in spices. The approach is based on the determination of dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid), a universal and specific component of bacterial endospores (Powell, 1953), which makes up 5–14 % of endospore dry weight (Murrell & Warth, 1965; Murrell, 1969). DPA was shown to be a useful analytical indicator for the presence of bacterial endospores and even permits to estimate total spore numbers, if spore DPA contents are known (Fichtel et al., 2007a). For quantification of endospore-derived DPA in pepper samples we used a sensitive high-performance liquid chromatographic method with indirect fluorescence detection of DPA, which is described in detail by Fichtel et al. (2007b). The method is based on detection of terbium dipicolinate fluorescence after chromatographic separation and post-column complexation of DPA. It permits the quantification of 0.5 nmol DPA l^{-1}, corresponding to less than 10^4 endospores per ml. This approach, in combination with a quantitative extraction protocol for DPA, allowed the assessment of spore contamination in black, white and green pepper without potential cultivation bias.

4.3 Materials and methods

4.3.1 Chemicals

Dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid) was purchased from Sigma (Taufkirchen, Germany) and sodium bisulphate monohydrate (Bio-Chemika Ultra, $\geq 98.0\,\%$) from Fluka (Taufkirchen, Germany). Methanol used for HPLC was gradient grade (VWR International, Darmstadt, Germany). For the post-column rise of pH, sodium acetate of luminescence grade with a purity of $\geq 99.5\,\%$ (Fluka) was used for buffer preparation. Terbium(III) chloride hexahydrate used for post-column complexation of DPA had a purity of 99.999 % (Aldrich, Taufkirchen, Germany).

4.3.2 Apparatus

Chromatography and detection of DPA were carried out on a Waters HPLC system (Eschborn, Germany) consisting of a gradient pump (model 600 E Multisolvent Delivery System), an HPLC pump (model 515) for adding the post-column complexation reagent, and an autosampler (model 717 plus) with 2000 µl sample loop and 2500 µl syringe. Mobile phases and complexation reagent were degassed by an in-line degasser (In-Line Degasser AF). Absorbance in the range from 210 nm to 400 nm was measured using a photodiode array (PDA) detector (model 996). A multi-wavelength fluorescence detector (model 2475) was set up to excite at 270 nm and measure the resulting emission at 548 nm. In order to eliminate second-order diffraction of elastically scattered light, a circular long pass filter N-WG320 (Schott, Mainz, Germany) with 7 mm diameter (1 mm thickness) was inserted between flow cell and emission monochromator of the fluorescence detector. The software Waters Empower 2 was used to control the operation of the system, for data collection and analysis.

4.3.3 Chromatography

A Gemini C18 110A HPLC column (5 µm particle diameter) with 150 mm length and 4.6 mm inner diameter (Phenomenex, Aschaffenburg, Germany) was used for separation. The analytical column was protected with a Phenomenex Gemini C18 guard column (4 mm $\times$ 3.0 mm). A column temperature of 20 °C was maintained by means of a column thermostat (BFO 04 svnl, W.O. Electronics, Langenzersdorf, Austria). The mobile phase consisted of

50 mmol l^{-1} sodium bisulphate. It was adjusted to pH 1.2 with sulphuric acid and passed through HPLC-certified GH Polypro membrane disc filters (Pall, Dreieich, Germany) with 0.45 µm pore size to remove particles. A multistep binary gradient programme with a methanol ratio of 20 % to 40 % was used at a flow rate of 2.0 ml min^{-1} (Table 3).

Table 3: HPLC gradient programme

Time (min)	Sodium sulphate buffer (%)	Methanol (%)
0	80	20
5	80	20
10	60	40
15	60	40
20	80	20
30	80	20

4.3.4 Post-column complexation

Sodium acetate solution (1 mol l^{-1}) with 50 µmol l^{-1} $TbCl_3$ was degassed by means of an in-line degasser and added to the eluent at a flow rate of 0.5 ml min^{-1}. To prevent pulsations in the fluorescence signal derived from the Waters 515 HPLC pump, a pulse damper (Model LP-21, SSI-LabAlliance, Pa., USA) was installed together with a backpressure regulator (part no. 39024, 0.7–10 MPa, Alltech, Worms, Germany) set to 7 MPa. Complexation reagent was added by means of a post-column reactor (Metrohm, Herisau, Switzerland) with a built-in pulsation-absorbing silicone capillary. By default, a PEEK capillary with smaller inner diameter (0.25 mm inner diameter, 3 m length) was installed in line with the pulsation absorbing silicone capillary to increase the backpressure and maximize the damping. Due to the high flow rate in our application, this PEEK capillary had to be shortened to about 10 cm length to prevent bursting of the silicone capillary.

4.3.5 Pepper samples

Whole black, white and green peppercorns (*Piper nigrum* L.) were supplied by Werner & Co. Gewürze GmbH (Gelsenkirchen, Germany).

4.3.6 Extraction of DPA

For determination of spore contamination in spices, about 10 g whole peppercorns were weighed into 50-ml polypropylene tubes with screw caps (Roth, Karlsruhe, Germany). For complete release of DPA from spores adhering to the peppercorns 15 ml of 0.5 M HCl were added, and the samples were autoclaved at 121 °C for 30 min (Pellegrino et al., 2002). After cooling, the samples were transferred into preextracted extraction tubes by rinsing with 0.5 M HCl and placed into a Soxhlet apparatus. The samples were extracted with 300 ml of 0.5 M HCl for 48 h to ensure quantitative extraction of the released DPA from the peppercorns. For purification via selective extraction of DPA with ethyl acetate, pH of the extract was readjusted to 0.5 by adding of concentrated NaOH solution (50–52 %). The aqueous phase was four times extracted with ethyl acetate (volume ratio in each extraction step 1:1). Ethyl acetate extracts were volume reduced, combined and evaporated. The residue was redissolved in 25 ml of $NaHCO_3$ buffer (10 mmol l^{-1}, pH 7) and the obtained extract transferred into a 40-ml centrifugation tube by rinsing with further 10 ml of $NaHCO_3$ buffer. Since buffer capacity was not sufficient, pH was readjusted by addition of NaOH. After transferring the extract into a separatory funnel by rinsing the tube with further 5 ml of $NaHCO_3$ buffer, the pH neutral extract was subsequently washed once with diethyl ether (40 ml) and twice with ethyl acetate (40 ml). For fluorimetric determination of DPA via HPLC, the aqueous extracts of the pepper samples were diluted at least 1:100 with sodium bisulphate buffer (50 mmol l^{-1}, pH 1.2) and filtered through cellulose acetate syringe filters (0.2 µm pore size) into polypropylene HPLC vials.

4.4 Results and Discussion

4.4.1 Quantitative extraction of DPA and purification of the extract

Soxhlet extraction turned out to be suitable for analysis of the pepper samples, since it allows an efficient extraction with small volumes of 0.5 M HCl. Purification was achieved by selective extraction of DPA from the aqueous phase. Since extraction efficiency is strongly influenced by pH, it was necessary to readjust the pH after Soxhlet extraction. At pH 0.5 the distribution coefficient of the two phase system was about 1, resulting in an extraction efficiency of $> 93\,\%$ after four times extraction with equal volumes of ethyl acetate. Purification by ethyl acetate extraction was observable with the naked eye, in particular for the black pepper sample. The DPA-containing ethyl acetate extracts were yellowish, whereas the discarded aqueous solution was coloured dark brown. Further purification was achieved by dissolving the residue of the ethyl acetate fractions in buffer and washing the obtained aqueous phase at pH 7 with diethyl ether and ethyl acetate. DPA was not removed by this washing step, since at pH 3.5 or higher the distribution coefficient is less than 10^{-2} (Warth, 1979). For the washing procedure a sodium carbonate buffer of low concentration with poor buffer capacity is recommended, since for chromatographic separation of DPA a pH of 1.2 was required, which was obtained by diluting the extract with sodium bisulphate buffer.

4.4.2 Quantification of DPA via HPLC

Via PDA detector there was no DPA response in the pepper samples, indicating DPA concentrations of less than 1 µmol l^{-1} in the final extracts. In contrast, quantification of terbium dipicolinate complexes using a fluorescence detector has an about three orders of magnitude higher sensitivity (Fichtel et al., 2007b) and allowed the detection of spore contaminations in all analysed pepper samples. Figure 14 shows the chromatogram of black pepper extract with DPA being detected (via emission at 548 nm) as a distinct, baseline separated peak at about 4 min retention time. External calibration based on peak area was used for quantification, resulting in DPA contents of 30, 16 and 15 nmol g^{-1} for black, white and green pepper, respectively.

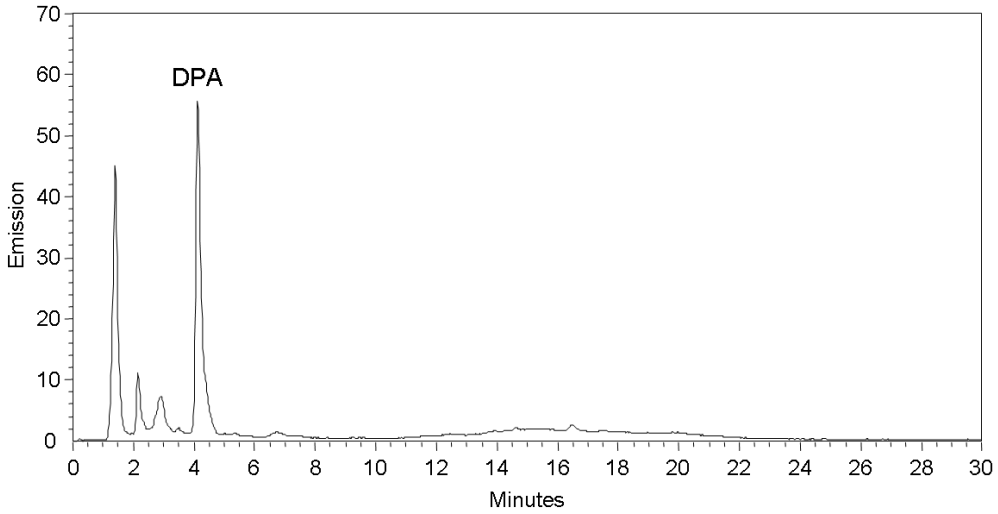

Fig. 14: HPLC chromatogram of black pepper extract recorded with the fluorescence detector at 548 nm wavelength (excitation at 270 nm) after post-column addition of $TbCl_3$ and complexation of DPA. Highly fluorescent terbium dipicolinate complexes were detected as a clear, baseline-separated peak at about 4 min retention time.

For differentiation between DPA-derived fluorescence and possible coeluting autofluorescent substances, the characteristic emission spectrum of terbium dipicolinate complexes can be utilised (Fig. 15). Since the emission maximum of terbium dipicolinate complexes at 548 nm is very narrow ($\pm$ 15 nm), DPA-derived fluorescence is very characteristic and can be distinguished from fluorescent coeluting substances with generally wider emission maxima. In the white pepper extract, for example, quantification of DPA was impaired by a coeluting substance overlaying the DPA peak at 4 min retention time (Fig. 16). Since it showed a wide emission maximum, this coeluting substance was clearly identified by simultaneous measurement at 548 nm and 525 nm, i. e. additionally at one of the terbium dipicolinate emission minima (Fig. 15). Furthermore, simultaneous measurement of emission at 548 nm and 525 nm can be used for calculation of difference chromatograms. In the case of the analysed pepper samples, direct recording of difference chromatograms with the Waters 2475 fluorescence detector provided the possibility to differentiate DPA from coeluting substances and to quantify DPA at the same time. Concentrations determined via difference chromatograms (emission at 548 nm minus emission at 525 nm) differed by less than 2 % from the quantification exclusively based on measurement of emission at 548 nm.

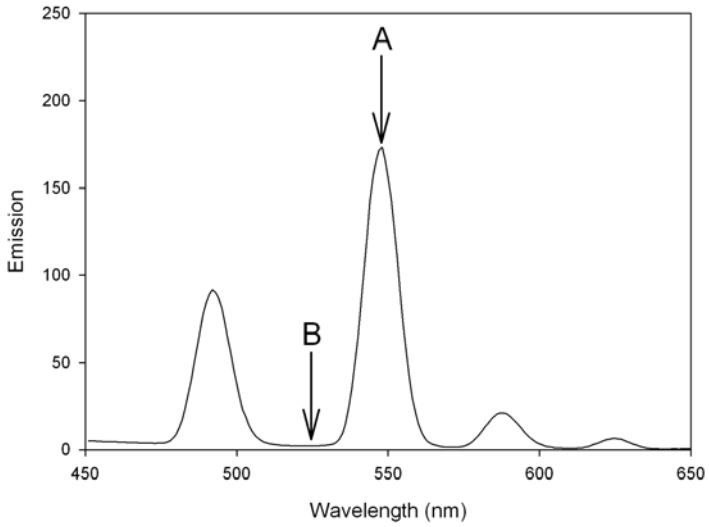

Fig. 15: Emission spectrum at 270 nm excitation wavelength of 1 µmol l^{-1} DPA in sodium acetate buffer (1 mol l^{-1}, pH 5.6) with 10 µmol l^{-1} $TbCl_3$ recorded with a Shimadzu RF-1501 Fluorescence Spectrophotometer. For quantification of terbium dipicolinate complexes via HPLC the strongest emission maximum at 548 nm was used (A). Since the emission maxima of terbium dipicolinate complexes are very narrow, DPA-derived fluorescence is very characteristic and can be distinguished from the fluorescence of coeluting substances by simultaneous measurement at one of the terbium dipicolinate emission minima, e. g. at 525 nm wavelength (B).

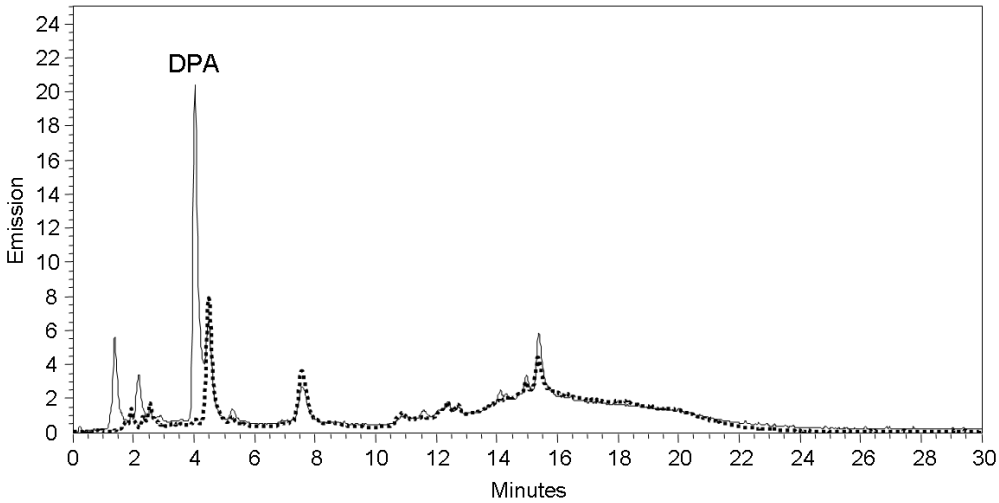

Fig. 16: Fluorescence chromatograms of white pepper extract recorded at 548 nm (solid line) and 525 nm (dotted line) wavelength. Since the coeluting substance at 4.5 min had a wide emission maximum, it also showed strong fluorescence at the terbium dipicolinate fluorescence emission minimum at 525 nm and could be clearly identified.

4.4.3 Conversion of DPA contents into endospore numbers

Spore contamination of the samples was assessed by conversion of pepper DPA contents into endospore numbers. One of the most frequently detected species of spore-forming bacteria in pepper is *Bacillus subtilis* (Palumbo et al., 1975; Seenappa & Kempton, 1981; Juri et al., 1986; Antai, 1988). Conversion based on the DPA content of endospores of *Bacillus subtilis* (Fichtel et al., 2007a) resulted in about 1.6×10^8 spores g^{-1} for black and 8×10^7 spores g^{-1} for white and green pepper. Determined endospore numbers are in a range expected from literature data (Baxter & Holzapfel, 1982; de Boer et al., 1985), keeping in mind that viable counts of spores generally underestimate true endospore numbers (e. g. Turnbull et al., 2007). Since our approach does neither discriminate between viable and nonviable spores nor between different physiological groups, we are confident that it provides a more realistic estimate of total endospore numbers than cultivation-dependent approaches.

4.5 Conclusions

The presented HPLC approach offers a sensitive and cultivation-independent possibility for assessment of spore contaminations in spices. If necessary, total analysis time can be reduced to a few hours by replacing Soxhlet extraction with repeated ultrasonic extraction or accelerated solvent extraction (ASE). Therefore, determination of DPA via HPLC provides a rapid and effective tool for an initial quality control of spices.

4.6 Acknowledgements

We thank Werner & Co. Gewürze GmbH (Gelsenkirchen, Germany) for providing the pepper samples. The development of the HPLC system used for quantification of DPA was supported by Deutsche Forschungsgemeinschaft (DFG) through a grant for the Research Group on BioGeoChemistry of Tidal Flats (grant no. RU 458/24).

5 High variations in endospore numbers within tidal flat sediments revealed by quantification of dipicolinic acid

This chapter was submitted: Fichtel J.*, Köster J., Rullkötter J. and Sass H., 2007. Dipicolinic acid contents reveal high variations in endospore numbers within tidal flat sediments. Geomicrobiology Journal.

5.1 Abstract

Bacterial endospores are resting stages without detectable metabolism that can remain viable for a long time and hence might accumulate in sediments during burial. They can be stained by the dyes commonly used for the determination of total cell counts, while cultivation-based approaches have suggested a significant contribution to sediment microbiota. However, the actual contribution of endospores to total cell counts in marine sediments is largely unknown, because of the lack of methods for reliable quantification. In the present study, dipicolinic acid (DPA), which is accumulated in the endospore core, was used to quantify endospores in up to 5.5 m long sediment cores collected from tidal flats off the German North Sea coast (Wadden Sea). Dipicolinic acid contents were determined fluorimetrically using a highly sensitive post-column complexation HPLC approach and ranged from 0.02 to 4.4 nmol DPA g^{-1} sediment dry weight. Dipicolinic acid contents of spores of pure cultures, that were isolated from the sampling area, were used for the conversion into endospore numbers. Estimated numbers ranged from 1×10^5 to 2×10^7 spores g^{-1} sediment dry weight. In the uppermost 50 cm of the sediment section endospore numbers represented less than 1 % of the total cell counts. However, in the layers beneath their contribution to total cell counts apparently increased with depth reaching up to 10 % of total cell counts. The endospore depth profile was irregular, but reflected the vertical changes in lithology. The highest endospore numbers were found in thin black mud layers, significantly lower numbers in sandy sediments.

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5.2 Introduction

Marine sediments are one of the largest habitats on Earth (Parkes et al., 1994; Whitman et al., 1998) extending hundreds of meters into Earth's interior and harboring the majority of global prokaryotic cells. However, there is uncertainty about the ratio of active versus inactive cells, particularly in deeper sediment layers that are characterized by low metabolic activities (D'Hondt et al., 2002). But even in surface-near sediments apparently not all cells are active. Luna et al. (2002) suggested, that even in surface sediments dead and inactive cells that can be stained might represent up to about 90 % of the total cell counts.

A special case of resting (inactive) cells are endospores that are formed by certain members of the bacterial phylum *Firmicutes*. Endospores can remain viable for thousands (Gest & Mandelstam, 1987) and probably millions of years (Cano & Borucki, 1995). Therefore, it can be expected that their relative contribution to sediment microbial communities increases with sediment depth and age. However, quantifying endospores in environmental samples has not attracted much attention so far. But since endospores can be stained with fluorescent dyes like acridine orange or DAPI (Cragg and Sass, unpublished), there is evidence to suggest that they are included in the determination of total cell counts.

Endospores in sediments are usually quantified by viable counts obtained after pasteurization (Isaksen et al., 1994; Rothfuss et al., 1997). Applied to marine sediments these methods have revealed the presence of up to 10^5 spores g^{-1} sediment (Isaksen et al., 1994; Sass et al., unpublished). Like total cell counts, absolute numbers of cultured endospores decreased with depth but also suggested an increase in their relative contribution to the microbial communities. Some tens of centimeters to a few meters deep in marine and freshwater sediments viable counts of spores even outnumbered those of vegetative cells (Köpke et al., 2005; Miskin et al., 1998; Rothfuss et al., 1997; Sass et al., 2003a). On the other hand, due to the high physiological diversity of endospore-forming bacteria, and the relatively low absolute spore numbers in most studies so far (e. g. Köpke et al., 2005), it seems obvious that cultivation-based methods underestimate the actual in situ numbers of endospores. In fact, during studies on pure cultures viable counts were generally lower than microscopic counts indicating that a proportion of spores in a population may not be stimulated to germinate, may be genuinely nonviable or may have become viable but not cultivable (Turnbull

et al., 2007). For environmental samples, determination of viable counts of spores is furthermore influenced by species-specific variations of the optimal amount of heat necessary to activate spores, by the species-specific response to concentrations of added germinants, and the suitability of the medium in which the spores are suspended.

To obtain a more realistic estimate of endospore numbers in sediments, Fichtel et al. (2007a) developed a cultivation-independent method targeting dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid), which is a major component of bacterial endospores (Powell, 1953). This method, based on the fluorescence of terbium dipicolinate, was very sensitive but was also prone to interference from the sediment matrix and hence required a specific extraction protocol. Furthermore, internal calibration by standard addition was necessary to correct for remaining quenching of terbium dipicolinate fluorescence and the specific background fluorescence of the samples. Application of this protocol to tidal flats of the southern North Sea revealed that endospores may account for at least 3 % of total cell counts, orders of magnitude higher than viable counts obtained after pasteurization (Köpke et al., 2005).

However, the time-consuming nature of this procedure only allowed processing of a limited number of samples, and some of the samples did not yield reliable data at all. This was attributed to the high background fluorescence and the almost complete adsorption of DPA to the sediment matrix in some samples. To circumvent these problems an HPLC approach based on post-column complexation and fluorimetric quantification of DPA (Fichtel et al., 2007b) was developed and proved less sensitive to interference. In the present study this HPLC approach was used to analyze the presence of endospores in up to 5.5 m long sediment cores from tidal flats in the backbarrier area of Spiekeroog Island (southern North Sea). Main focus of this investigation was to compare different lithological subunits for their DPA content to identify possible correlations between endospore content and type of lithology and to analyze layers that did not yield reliable data by fluorimetric detection after chemical extraction.

5.3 Materials and methods

5.3.1 Sediment samples

Sediment samples were taken in the backbarrier tidal flats of Spiekeroog Island, one of the East Frisian Islands along the German North Sea coast (Fig. 17). Sediment cores were taken close to the margins of a mixed flat (site Neuharlingersieler Nacken, NSN) and a sand flat (site Janssand, JS). Cores NSN5 and NSN7 were taken in October 2003 and February 2004 from the same position (53°43.270´N, 7°43.718´E), whereas core NSN10 (53°43.249´N, 7°43.713´E) was taken in April 2005 in about 40 m distance. Core JS11 (53°44.178'N, 7°41.974'E) was also taken in April 2005.

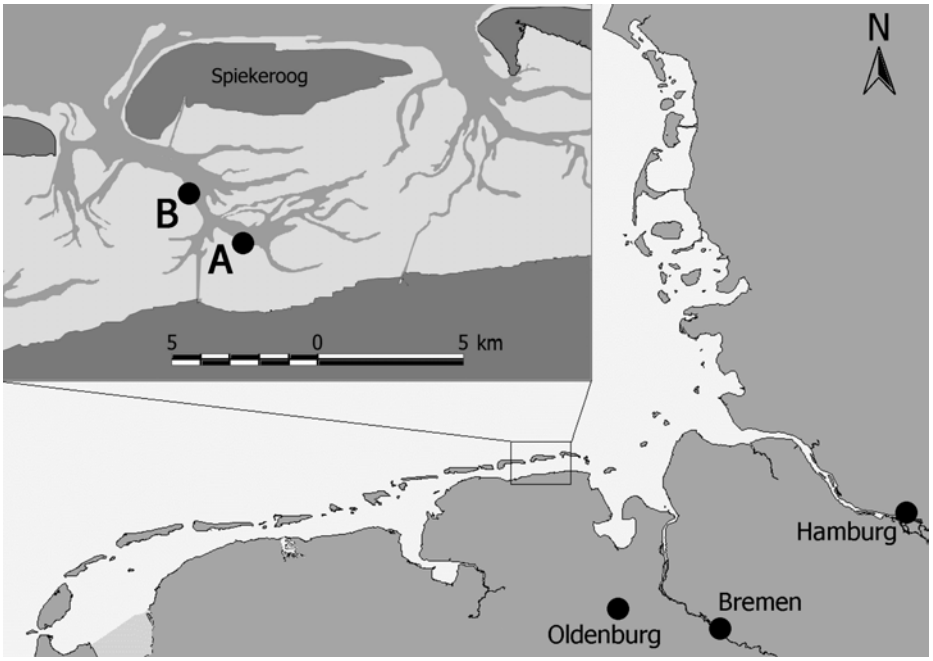

Fig. 17: Sampling sites in the backbarrier tidal flat area of Spiekeroog Island, one of the East Frisian Islands along the coast of Germany in the southern North Sea. Sediment cores were collected from the tidal flats Neuharlingersieler Nacken (A, cores NSN5, 7 and 10) and Janssand (B, core JS11).

Aluminum tubes (8 cm diameter) were driven into the sediment using a vibrating unit. The cores were recovered using a tripod with a hoist. The cores were cut longitudinally for subsampling. The sedimentary structure and lithology of the cores was determined by visual inspection. Sediment samples for DPA analysis were taken from the innermost part of the cores to avoid cross-contamination due to smear along the aluminum tubes. Cores NSN5 and NSN7 were subsampled at a vertical resolution of about 20 cm.

Cores NSN10 and JS11 were analyzed at higher vertical resolution (about 10 cm increments) allowing to separate layers dominated by different sediment types. Sediment samples were freeze-dried and stored at −20 °C until processing.

5.3.2 Sediment bulk parameters

Total organic carbon (TOC) contents of the sediment samples were determined as the difference between total carbon determined by combustion in a CS-444 instrument (Leco Instruments GmbH, Mönchengladbach, Germany) and inorganic carbon measured with a CM5012 CO_2 coulometer coupled to a CM5130 acidification module (UIC Inc., Joliet, IL).

Total cell counts were obtained after staining with 4,6-diamidino-2-phenylindole (DAPI) as described by Süß et al. (2004). Counts for core NSN5 were determined by Köpke et al. (2005), those for core JS11 by A. Gittel (personal commun.).

5.3.3 Reagents for HPLC analysis

Dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid) was purchased from Sigma (Taufkirchen, Germany) and sodium bisulfate monohydrate (BioChemika Ultra, $\geq 98.0\,\%$) from Fluka (Taufkirchen, Germany). Methanol used for HPLC was gradient grade (VWR International, Darmstadt, Germany). For the post-column rise of pH, sodium acetate of luminescence grade with a purity of $\geq 99.5\,\%$ (Fluka) was used. Terbium(III) chloride hexahydrate added for post-column complexation of DPA had a purity of 99.999 % (Aldrich, Taufkirchen, Germany).

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Chromatography and detection of DPA were carried out using a Waters HPLC system (Eschborn, Germany) consisting of a gradient pump (model 600 E Multisolvent Delivery System), an HPLC pump (model 515) for adding the post-column complexation reagent, and an autosampler (model 717 plus) with 2 ml sample loop and 2.5 ml syringe. Mobile phases and complexation reagent were degassed (In-Line Degasser AF). A fluorescence detector (model 2475) was set up to excite at 270 nm and measure the resulting emission at 548 nm wavelength. In order to eliminate second-order diffraction of elas-

tically scattered light, a circular long pass filter (N-WG320, Schott, Mainz, Germany) with 7 mm diameter and 1 mm thickness was inserted between flow cell and emission monochromator. The software Waters Empower 2 was used to control the operation of the system for data collection and analysis.

5.3.5 Chromatography

A Phenomenex Gemini C18 110A HPLC column (5 µm particle diameter) with 150 mm length and 4.6 mm inner diameter (Phenomenex, Aschaffenburg, Germany) was used for separation. The analytical column was protected with a Phenomenex Gemini C18 guard column (4 mm × 3.0 mm). A column temperature of 20 °C was maintained by means of a column thermostat (BFO-04 svnl, W.O. Electronics, Langenzersdorf, Austria). The mobile phase consisted of an aqueous solution of 50 mmol l^{-1} sodium bisulfate. It was adjusted to pH 1.2 with sulfuric acid and passed through HPLC-certified GH Polypro membrane disc filters (Pall, Dreieich, Germany) with 0.45 µm pore size to remove particles. A multistep binary gradient programme with a methanol ratio of 20 % to 40 % was used at a flow rate of 2.0 ml min^{-1} (Fichtel et al., 2007b).

5.3.6 Post-column complexation

Sodium acetate solution (1 mol l^{-1}) with 50 µmol l^{-1} $TbCl_3$ was degassed by means of an in-line degasser and added to the eluent at a flow rate of 0.5 ml min^{-1}. To prevent pulsations in the fluorescence signal caused by the Waters 515 HPLC pump used for addition of the complexation agent, a pulse damper (Model LP-21, SSI-LabAlliance, Pa., USA) was installed together with a backpressure regulator (part no. 39024, 100–1500 psi, Alltech, Worms, Germany) set to 1000 psi. Complexation reagent was added by means of a post-column reactor (Metrohm, Herisau, Switzerland) with a built-in pulsation-absorbing silicone capillary (Fichtel et al., 2007b).

5.3.7 Determination of DPA in sediment samples

For determination of DPA depth profiles, 0.5 to 2.0 g freeze-dried sediment were weighed into autoclavable 15-ml polypropylene tubes with screw caps (Sarstedt, Nümbrecht, Germany). Duplicates were prepared for each sample to determine recovery: One of the sediment aliquots was suspended in 5 ml

sodium bisulfate buffer (50 mmol l^{-1}, pH 1.2), the other one was spiked by adding buffer with 100 or 200 nmol DPA l^{-1}. Both duplicates were autoclaved to completely release DPA from the endospores within the sediment. After cooling, the samples were centrifuged (4000 *g*, 5 min, 15 °C), and the supernatants were filtered through cellulose acetate syringe filters (0.2 µm pore size, Nalgene Nunc International, Rochester, NY) into polypropylene vials. For calculation of recovery, the difference in DPA concentrations of the duplicates was determined and divided by the concentration of the standard solution used for spiking (Fichtel et al., 2007b).

5.3.8 Determination of DPA in pore water samples

Pore water samples were obtained at site Janssand (53°44.183´ N, 7°41.904´ E) with a permanently installed in situ pore water sampler as described by Beck et al. (2007). Pore water samples were prefiltered through 50 µm nylon mesh at the sampling ports. In order to discriminate dissolved pore water DPA (e. g. released during spore germination) from particulate (spore-bound) DPA, pore water aliquots were sterile-filtered (0.2 µm pore size). For release of DPA from endospores in the unfiltered pore water aliquots, all samples were transferred into 15-ml polypropylene tubes with screw caps and autoclaved at 121 °C for 30 min. Previously unfiltered pore water aliquots were filtered after autoclaving. For determination of DPA via HPLC 2 ml pore water were diluted with 1 ml threefold concentrated mobile phase (150 mmol l^{-1} sodium bisulfate, adjusted to pH 1.2 with sulfuric acid).

5.3.9 Estimation of endospore numbers from DPA contents

For conversion of sediment DPA contents into endospore numbers an average DPA content of 2.24×10^{-16} mol per spore (SD: 0.63×10^{-16}) was assumed. This factor was determined from spore DPA contents of six strains isolated from the respective area. The spores of these strains showed only little variation in volume and DPA content. Therefore, calculation of endospore numbers was based on the assumption that the cultivated and investigated spores adequately represented the DPA contents of the in situ spore community (Fichtel et al., 2007a). The relative contribution of endospores to the sedimentary microbial community was estimated on the basis of total cell counts determined with DAPI that were available for cores NSN5 and JS11.

5.4 Results and discussion

5.4.1 Characterization of the sediments at the sampling sites

The backbarrier area of Spiekeroog Island (Fig. 17) comprises sandy and mixed intertidal flats in an upper mesotidal regime with an average tidal range of 2.8 m. Surface sediments show a typical general grain size pattern with increasing mud content towards the mainland (Flemming & Ziegler, 1995). At site NSN the sediment column was characterized by three main sedimentary units. In the lowermost part, the cores comprised gray mud-rich sediments that were deposited in a salt marsh environment (Chang et al., 2006, Figs. 18C and 19C). These were erosively overlain by shell layers, which were dated to be around 600 years old (D. Ziehe, personal commun.). The sediments in the upper part were sand-dominated with thin intercalations of black mud and deposited in a sand flat to mixed muddy sand flat environment (Chang et al., 2006). The general lithological compositions of cores NSN5, 7 and 10 were similar to each other. However, small-scale differences occurred over short distances. Site JS11, located closer to Spiekeroog Island, was dominated by sand in the upper 50 cm. More deeply buried sediments consisted of intercalations of sand and mud showing sedimentary features typical for mixed intertidal flats. Only one thin shell layer was found at about 1 m depth (Fig. 20C).

Organic matter contents strongly varied with lithology and grain size (Figs. 18, 19, and 20) reflecting the general relationship between mineral surface area and TOC content in hemipelagic (Mayer, 1994) and coastal sediments (e. g. DeFlaun & Mayer, 1983; Volkman et al., 2000). Higher TOC contents found in fine-grained sediments were explained by the sorptive preservation of organic matter on mineral surfaces (Keil et al., 1994; Volkman et al., 2000). In our study TOC contents of sand dominated sediment layers were usually below 0.5 % (Fig. 21), whereas the thin intercalations of black mud were characterized by high TOC contents (1.2 % to 2.2 %), and for mixed sediment layers intermediate TOC contents were determined. In the gray mud section in the lower part of the cores from site NSN, TOC contents varied between 0.5 % and 1.9 % due to the presence or absence of remnants of higher plants, e. g. roots. With increasing depth δ^{13}C values of organic matter decreased (data not shown) as previously published for a core from site NSN by Webster et al. (2007). This general depth trend can be explained by the preferential microbial degradation of labile, mostly marine organic

matter relative to a more resistant fraction of TOC with a terrestrial carbon isotope signature (Böttcher et al., 1998; Volkman et al., 2000).

In core NSN5 total cell counts decreased with depth from 8.1×10^8 cells g^{-1} sediment at the sediment surface to about 2×10^7 cells g^{-1} sediment at 3 m depth (Fig. 18E). In the gray mud layers beneath no apparent decrease in cell numbers was found. This depth profile reflects well previous reports for nearby tidal flat sediments (Köpke et al., 2005) but also for hemipelagic marine sediments (Parkes et al., 1994). In contrast, at site JS11 total cell numbers did not show an apparent depth trend (Fig. 20E). Cell numbers were constantly high from the surface down to 4 m depth, varying from 6.3×10^8 (surface) to 2.7×10^8 cells g^{-1} sediment (2 m depth).

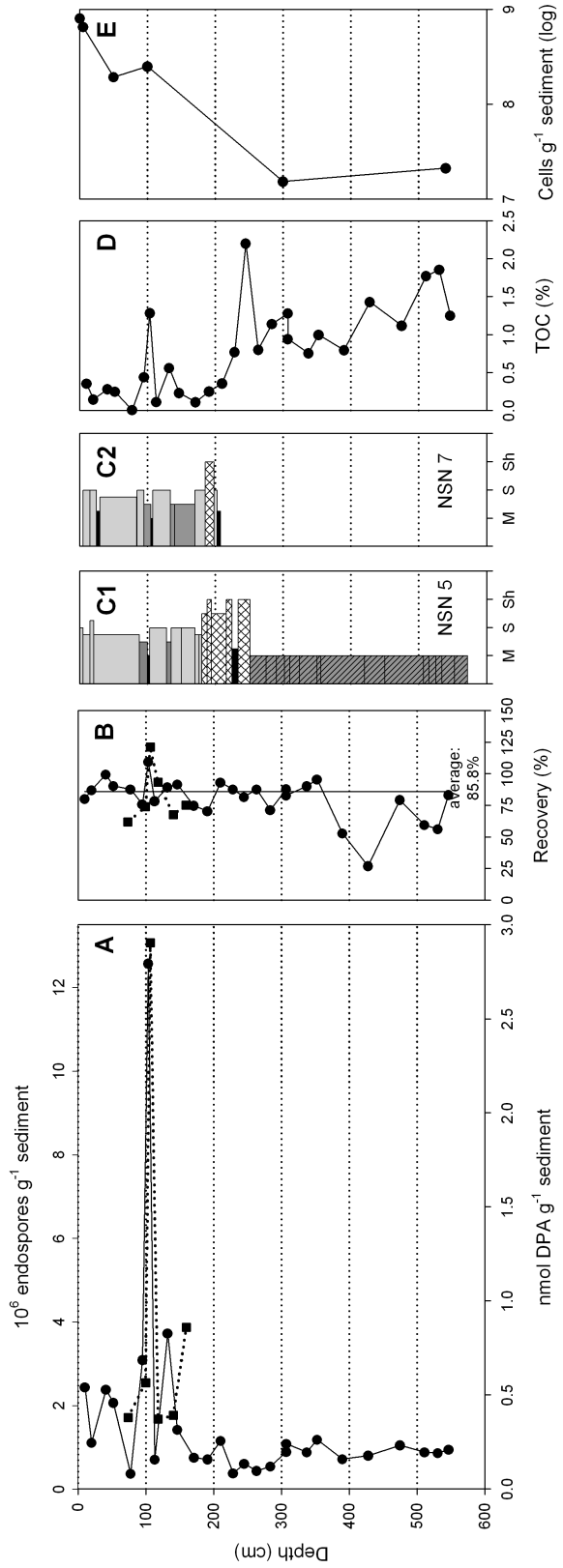

Fig. 18: Depth profiles of microbiological and sedimentological parameters in two cores taken from sites NSN5 and NSN7. A) Endospore numbers estimated from sediment DPA contents determined at sites NSN5 (circles) and NSN7 (squares). For conversion an average of 2.24×10^{-16} mol DPA per spore was assumed. B) Recovery determined by spiking of sediment aliquots with DPA (NSN5: circles, NSN7: squares). C1) and C2) Lithological profiles of the cores NSN5 and NSN7. The width of the bars represents the grain size (M: mud , S: sand, Sh: shells); black mud (black), mud/sand mixed sediments (dark gray), sand (light gray), shells (cross-hatched) and gray mud containing abundant plant remains (dark gray, hatched). D) Total organic carbon (TOC) content determined at site NSN5. E) Total cell counts for NSN5 determined by Köpke et al. (2005).

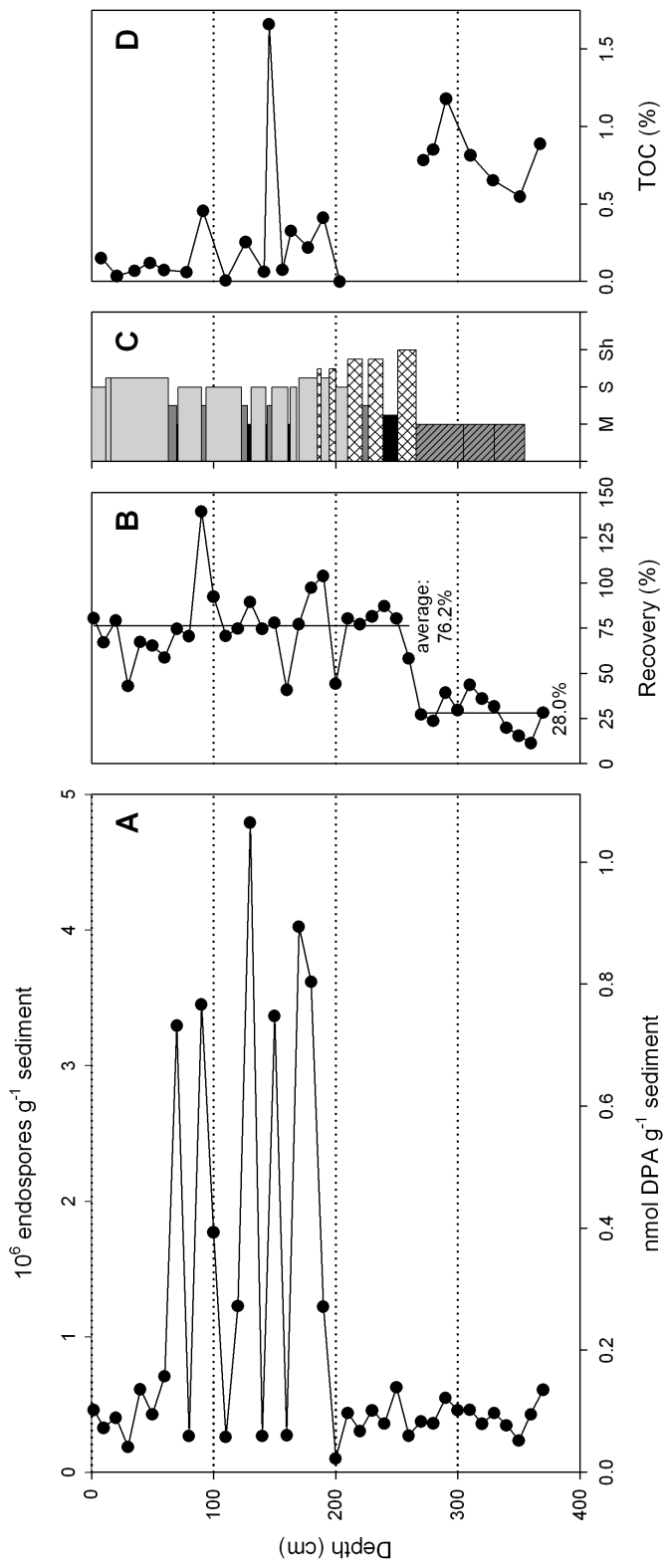

Fig. 19: High-resolution depth profiles of microbiological and sedimentological parameters in a core taken from site NSN10. A) Endospore numbers estimated from sediment DPA contents. For conversion an average of 2.24×10^{-16} mol DPA per spore was assumed. B) Recovery determined by spiking of sediment aliquots with DPA. C) Lithological profile of the core. The width of the bars represents the grain size (M: mud , S: sand, Sh: shells); black mud (black), mud/sand mixed sediments (dark gray), sand (light gray), shells (cross-hatched) and gray mud containing abundant plant remains (dark gray, hatched). D) Total organic carbon (TOC) content.

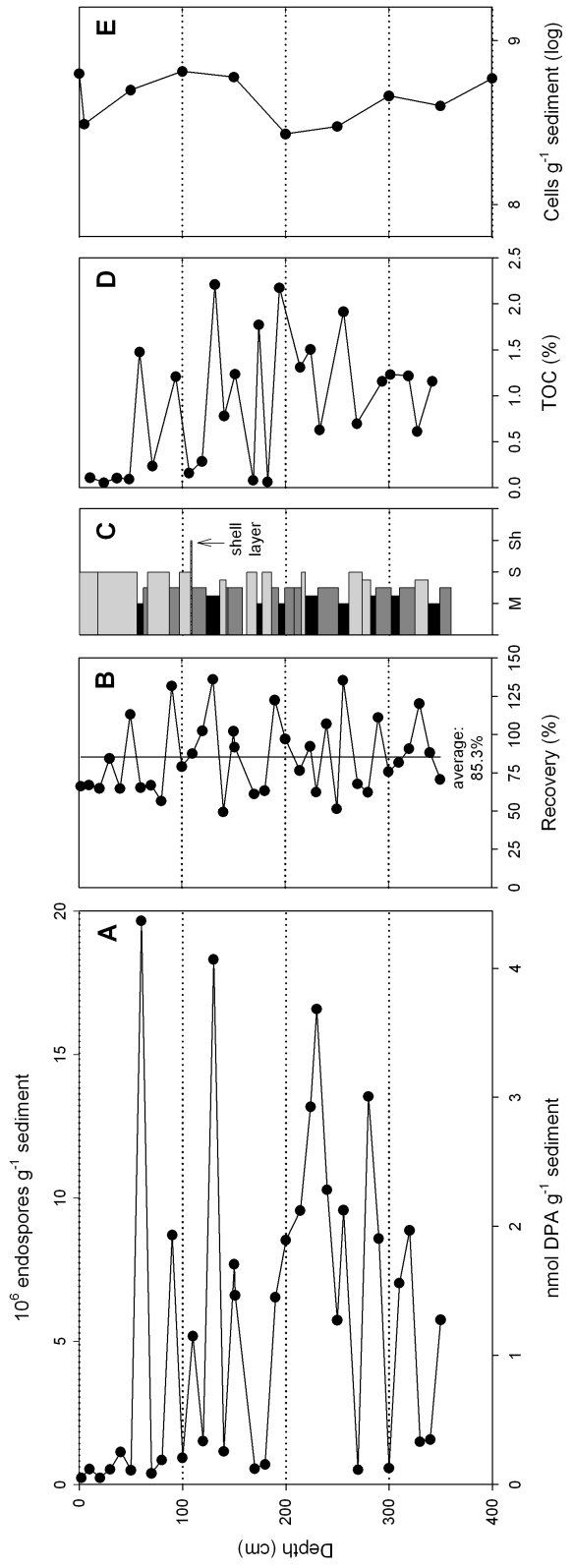

Fig. 20: High-resolution depth profiles of microbiological and sedimentological parameters in a core taken from site JS11. A) Endospore numbers estimated from sediment DPA contents. For conversion an average of 2.24×10^{-16} mol DPA per spore was assumed. B) Recovery determined by spiking of sediment aliquots with DPA. C) Lithological profile of the core. The width of the bars represents the grain size (M: mud , S: sand, Sh: shells); black mud (black), mud/sand mixed sediments (dark gray), sand (light gray) and shells (cross-hatched). D) Total organic carbon (TOC) content. E) Total cell counts determined by A. Gittel (personal commun.).

5.4.2 Variations in sediment DPA contents

DPA contents in the different sediment cores ranged from 0.02 to 4.4 nmol DPA g^{-1} sediment dry weight (Figs. 18A, 19A, and 20A). Determination of DPA down the cores did not reveal a continuous depth profile, instead DPA contents seemed to depend strongly on lithology (Figs. 18–20). DPA contents were highest in black mud layers and generally decreased with increasing proportions of sand. Whereas sand-dominated sediment layers were characterized by low DPA contents (generally less than 0.5 nmol DPA g^{-1} sediment dry weight), for mixed layers intermediate DPA contents were determined. Likewise, DPA contents changed with TOC contents (Fig. 21). However, correlation between DPA and TOC content was low (r^2=0.28) when all samples were included in the analysis, but improved significantly (r^2=0.56) when gray mud was excluded.

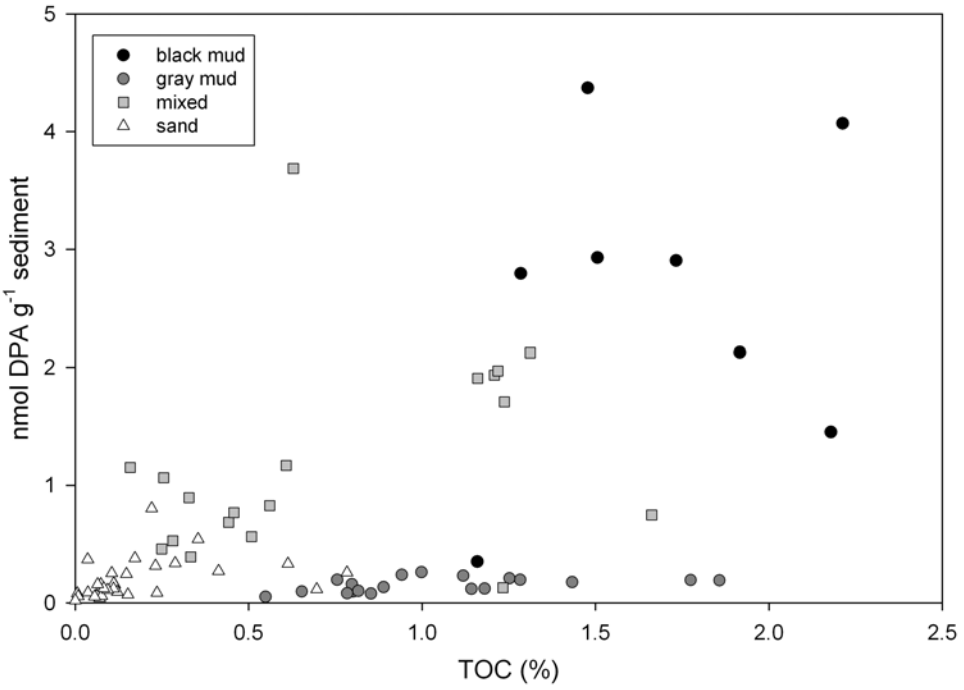

Fig. 21: Plot of DPA content versus total organic carbon (TOC) content of the samples analyzed in this study. Samples are labeled according to their sediment type: black mud (black circles), gray mud (dark gray circles), mixed sediments (light gray squares) and sand (white triangles).

Variations of up to one order of magnitude were found within a few centimeters depth between adjacent black mud and sand layers. In core NSN5, the highest DPA content was determined for a sample taken from a conspicuous, approximately 3 cm thin black mud layer at about 1 m depth. With 2.8 nmol DPA g^{-1} sediment dry weight, this sample showed an about one order of magnitude higher DPA content than the surrounding mixed to sandy sediments. These high variations were confirmed by analyzing the corresponding part of core NSN7, which yielded similar DPA concentrations (Fig. 18A). However, from cores NSN5 and 7 samples were taken every 20 cm and each sample usually covered a 10 cm depth interval. Therefore, DPA contents have to be regarded as an average of a possible mixture comprising different lithological subunits. In comparison to cores NSN10 and JS11, this may have resulted in a smoother DPA depth profile (see below).

To address the small scale variations of DPA content with lithology observed in cores NSN5 and 7, it was decided to analyze cores NSN10 and JS11 at a higher vertical resolution (about 10 cm increments) and to specifically address different sediment types. In core NSN10 relatively low and almost constant DPA contents were detected along the upper 50 cm of the sediment column and beneath a depth of 2 m (Fig. 19A), confirming the results obtained for core NSN5. However, in contrast to core NSN5, the depth interval from 70 cm to 180 cm was characterized by a highly irregular DPA depth profile. DPA contents in this section varied from 0.06 to 1.1 nmol DPA g^{-1} sediment (Fig. 19A), but did not reach the high values determined for the thin black mud layer in cores NSN5 and 7 (Fig. 18A).

The gray mud layers in the lowermost part of the cores from site NSN showed invariably low DPA contents (< 0.27 nmol DPA g^{-1} sediment). However, low recoveries of DPA derived from spiked sediment aliquots point out an interfering effect of the sediment matrix of these salt marsh deposits on quantification of DPA (see below).

The core from site JS did not contain sections of gray mud. Like the cores taken at site NSN, core JS11 was characterized by low DPA contents along the sand-dominated uppermost 50 cm, but showed a very irregular depth profile in the layers beneath (Fig. 20A). Maximum DPA contents were detected at 60, 130, 230 and 280 cm depth, generally in layers dominated by black mud. In these layers DPA contents exceeded even those of the thin black mud layer in cores NSN5 and 7 (Fig. 18A).

5.4.3 Determination of recovery and influence of lithology on extraction of DPA

In order to verify a reliable determination of the DPA content in the sediment samples, the degree of recovery was determined. An aliquot of each sample was spiked with a defined amount of DPA and analyzed like the untreated samples. Generally, sediment aliquots were spiked by suspending 2 g sediment in 100 nmol DPA l^{-1} standard solution. However, because of the influence the inhomogeneity of the sediment samples had on the determination of recovery, it turned out that spiking concentrations had to be set to a level corresponding to at least one quarter of the in situ value (data not shown). Therefore, in the case of black mud sediments with generally higher DPA contents, only 0.5 g sediment but a 200 nmol DPA l^{-1} standard solution was used instead.

Average recoveries ranged from 76 % (core NSN10, Fig. 19B) to 85–86 % (cores NSN5 and JS11, Figs. 18B and 20B). Significantly lower recoveries were found for the gray mud layers at the bottom of core NSN10 (Fig. 19B). Because of the extremely high mud fraction (85–99 %) these layers provided a large specific surface area for adsorption and it has been suggested that DPA was retained in this type of sediment and therefore resisted extraction (Fichtel et al., 2007a).

DPA recoveries differed significantly, often between adjacent samples. These variations showed no apparent correlation with lithology, except for the gray mud layers in core NSN10. Likewise, no correlation between TOC content and recovery was found (Fig. 22). One possible explanation for this finding might be small-scale patchiness within single samples. For spiking, each sample was subdivided and it can be imagined that the variation in recovery reflects small-scale inhomogeneities between the two sample aliquots. This might also explain recoveries exceeding 100 % found for a few samples (Fig. 22).

Determination of recovery was made possible by using HPLC for analysis (Fichtel et al., 2007b). In a previous study based on chemical extraction (Fichtel et al., 2007a), quenching effects and the background fluorescence of the samples did not allow external calibration. Internal calibration by standard addition, however, does not permit determination of recovery, since this requires a quantification of DPA in spiked samples in relation to external standards.

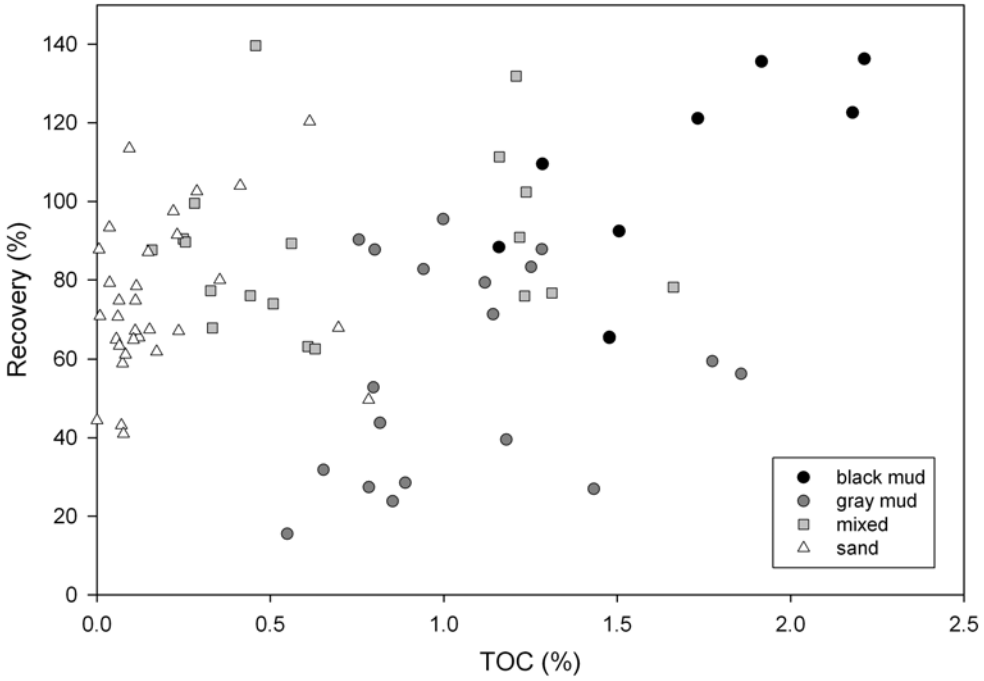

Fig. 22: Plot of recovery determined by analysis of spiked sediment aliquots versus total organic carbon (TOC) contents of the samples analyzed in this study. Samples are labeled according to their sediment type: black mud (black circles), gray mud (dark gray circles), mixed sediments (light gray squares) and sand (white triangles). Due to the inhomogeneity of the sediment samples, recoveries of up to 140 % were determined. The lowest recoveries were obtained for the gray mud samples at >2.5 m depth at site NSN.

5.4.4 Pore water DPA concentrations

Besides determination of recovery, discrimination of dissolved (e. g. released via germination of spores) and spore-bound DPA is necessary for an accurate estimation of endospore numbers. High concentrations of dissolved DPA may lead to an overestimation of spore numbers. Dissolved DPA concentrations in samples taken with a permanently installed pore water sampler at site JS (Beck et al., 2007) ranged from 0.6 to 6 nmol DPA l^{-1}. A local maximum was found at the sediment surface, lowest concentrations between 50 and 150 cm depth. Beneath, pore water DPA concentrations continuously increased with depth (Fig. 23). Pore water DPA was predominantly dissolved, only a small fraction was spore-bound (especially at about 2 m depth). Dissolved DPA made up less than 1 % of total sediment DPA contents at all depths and hence had not to be taken into consideration for conversion of sediment DPA contents into endospore numbers.

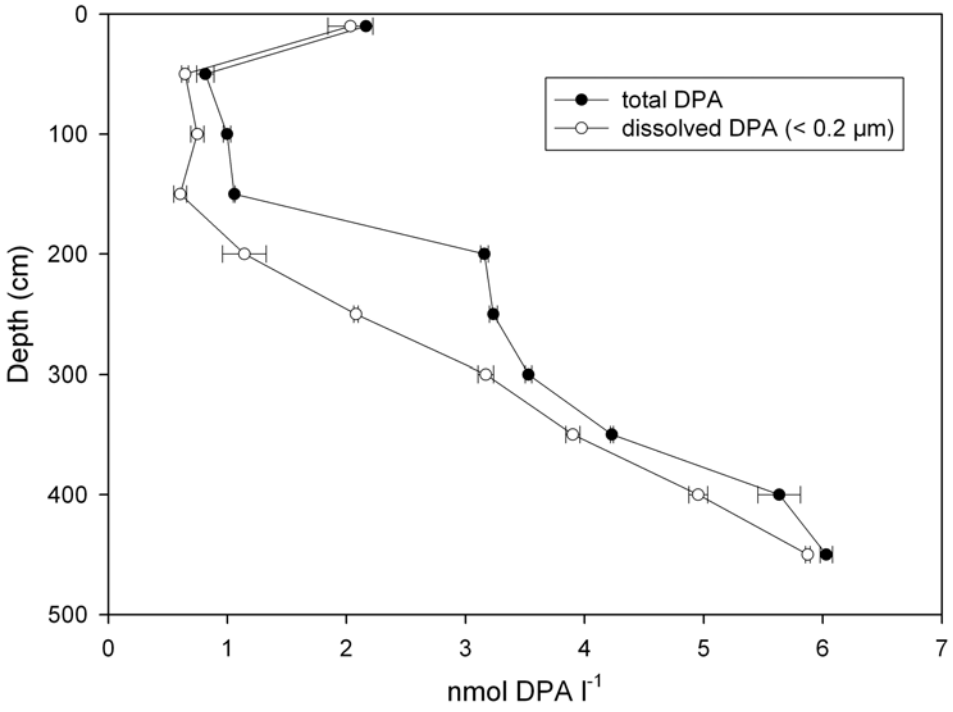

Fig. 23: Pore water depth profile of DPA concentrations determined at site JS with an *in situ* pore water sampler as described by Beck et al. (2007). Closed circles: total DPA concentrations (determined after autoclaving the unfiltered pore water samples). Open circles: concentrations of dissolved DPA (determined by filtration of the samples prior to autoclaving).

The dissolved DPA depth profile roughly reflected the depth profile of dissolved organic carbon (DOC) at site JS (Beck et al., 2007). Therefore, both dissolved DPA and DOC in the pore water are expected to be influenced by deep pore water circulation. Billerbeck et al. (2006) suggested that pore water in the sand flat has residence times in the order of years to tens of years and appears to act as a buffered nutrient source to the ecosystem.

5.4.5 Conversion of sediment DPA contents into endospore numbers

Generally, along the upper 50 cm estimated endospore numbers were low, contributing less than 1 % to total cell counts. Highest DPA contents in cores NSN5 and 7 were detected in the black mud layer at about 1 m depth. With 1.3×10^7 endospores g^{-1} sediment (Fig. 18A) estimated endospore numbers corresponded to approximately 5 % of the total cell counts. In the layers above and beneath, estimated numbers rarely exceeded 2×10^6 spores g^{-1} sediment. Except for the peak at approximately 1 m depth, endospore numbers remained more or less constant with depth whereas total cell counts decreased almost by two orders of magnitude. Hence, in the gray mud layers at the bottom of core NSN5 endospore numbers reached up to 10 % of total cell counts. However, since quantification of DPA in some of the gray mud samples was affected by low recoveries of only about 30 %, this is still a conservative estimate and DPA contents measured could underestimate actual endospore numbers. In core NSN10, estimated endospore numbers were lower than in the other two NSN cores with values ranging from 0.1×10^6 to 4.8×10^6 spores g^{-1} sediment (Fig. 19A). Highest endospore numbers were determined for site JS11 with maximum values of up to 2.0×10^7 spores g^{-1} sediment in black mud layers. However, these high endospore numbers coincided with higher total cell counts than those in the NSN cores. In addition, in core JS11 total cell counts did not decrease with depth, but remained relatively constant along the whole core. Therefore, endospores were estimated to account for less than 5 % of total cell counts at this site, even though at some depths endospore numbers exceeded 10^7 spores g^{-1} sediment.

5.4.6 Influence of sediment lithology on the accumulation of endospores

In the analyzed cores taken at sites NSN and JS the distribution of endospores corresponded with changes in lithology. The elevated endospore num-

bers in mud layers with higher TOC contents could be the result of higher initial total cell counts. Llobet-Brossa et al. (1998) found higher total cell numbers in the surface layers of muddy compared to sandy tidal flats. Assuming that endospore-forming bacteria represented the same fraction of the total population in surface layers of both sediment types, this may explain elevated endospore numbers in the mud layers. On the other hand, muddy sediments are generally characterized by small pore space and exhibit a lower hydraulic conductivity compared to sandy sediments (Chapelle & Lovley, 1990). This does not only limit diffusion and thereby hamper microbial activity and enhance spore formation; it may also facilitate capturing and fixing endospores that have been introduced by river runoff or wind. In permeable sandy sediments the situation is more complex. On the one hand particulate matter like phytoplankton cells can be easily transported a few centimeters deep into the sediment (Huettel et al., 1996). This interfacial water flow is driven by pressure gradients generated by bottom water currents deflected by small surface structures (mounds, ripples). On the other hand, on the downstream side of these sediment structures pore water is drawn out of the sediment, and potentially carrying endospores with it. Although sandy sediments may therefore not necessarily act as a sink for endospores, these pore water exchange processes can provide nutrients and enhance microbial activity even in deeper layers. In agreement with this, it was shown that permeable sandy sediments are characterized by higher microbial activities than muddy sediments (Rusch et al., 2006), rendering endospore formation less likely. However, whether the majority of endospores within the sediment was formed by the autochthonous community or introduced from external sources cannot be discriminated by the methods used here and remains a subject for future investigations.

5.5 Conclusions

The use of DPA as spore-specific biomarker and its quantification with a highly sensitive post-column complexation HPLC approach permitted a cultivation-independent determination of endospore numbers in marine sediments. Our data revealed high variations in endospore numbers with lithology. Although endospores represented only a minor fraction of total cell counts in the surface layers of the sediment, their relative contribution to the microbial community apparently increased with depth reaching a few percent of the total cell counts at several meters depth. The increase relative to

vegetative cells can be explained by the extreme longevity of endospores of at least thousands (Gest & Mandelstam, 1987) to millions of years (Cano & Borucki, 1995), whereas numbers of vegetative cells are expected to decrease more rapidly due to starvation. This is also reflected by the results of Batzke et al. (2007) who found spore-forming *Firmicutes* increasingly dominating culture collections with depth in deep sediments of the Pacific Ocean. Considering the vast vertical extension of the marine subsurface (Parkes et al., 2000), it can be expected that endospores contribute substantially to the deep biosphere. This in turn could help explain the discrepancy between the energy available for maintaining life and the extent of microbial communities in many deep subsurface environments (D'Hondt et al., 2002; Sass et al., 2003b).

5.6 Acknowledgements

We thank Melanie Beck for providing the pore water samples and Jan Tebben for his assistance in preparing the sediment samples. This work was supported by the Deutsche Forschungsgemeinschaft (DFG) through a grant for the Research Group BioGeoChemistry of Tidal Flats (grant no. RU 458/24).

6 Gesamtbetrachtung und Ausblick

6.1 Verwendung von Dipicolinsäure zur Bestimmung von Endosporenzahlen

6.1.1 Quantifizierung von Dipicolinsäure in Sedimenten

DPA ist ein Hauptbestandteil von Endosporen und kann als Biomarker für deren Nachweis und für eine Abschätzung von Endosporenabundanzen verwendet werden. Die Quantifizierung natürlicher Sporengehalte in Sedimenten erfordert jedoch sehr empfindliche Methoden, die einen Nachweis von einigen pmol bis wenigen nmol DPA pro Gramm Sediment ermöglichen. Eine ausreichend hohe Empfindlichkeit bietet der fluorimetrische Nachweis von DPA nach Komplexierung mit Tb^{3+}-Ionen (Rosen et al., 1997; Hindle & Hall, 1999). Dieses Verfahren stellt jedoch hohe Anforderungen an die Probenaufarbeitung, da in Sedimentextrakten eine fast vollständige Auslöschung des Fluoreszenzsignals beobachtet wurde. Auch die Eigenfluoreszenz des im Sediment enthaltenen organischen Materials erschwert den fluorimetrischen Nachweis von DPA. Zur Abtrennung von störenden quenchenden und fluoreszierenden Substanzen wurden im Rahmen dieser Arbeit zwei verschiedene analytische Verfahren entwickelt.

Mit der Flüssig-Flüssig-Extraktionsmethode (siehe Kapitel 2; Fichtel et al., 2007a) und der empfindlichen HPLC-Methode (siehe Kapitel 3; Fichtel et al., 2007b) stehen zwei geeignete Methoden zur Bestimmung von DPA in Sedimenten zur Verfügung. Über die Flüssig-Flüssig-Extraktion von DPA mit Essigsäureethylester wurde jedoch keine vollständige Abtrennung störender Substanzen erreicht. Für die Quantifizierung von DPA in den aufgereinigten Sedimentextrakten war deshalb ein sehr arbeits- und zeitintensives Standardadditionsverfahren notwendig, um eine Unterschätzung des DPA-Gehalts aufgrund eines durch *Quenching* verringerten Fluoreszenzsignals auszuschließen. Eine höhere Empfindlichkeit und bessere Reproduzierbarkeit bei gleichzeitig deutlich geringerem Aufwand für die Probenaufarbeitung wurde durch die Entwicklung einer speziellen HPLC-Methode erreicht. Das entwickelte HPLC-System kombiniert die chromatographische Trennung von Sedimentextrakten mit einer *Post-Column*-Komplexierung von DPA mit Tb^{3+}-Ionen zur anschließenden fluorimetrischen Detektion der gebildeten Komplexe. Die relativ kurze Analysenzeit und die Quantifizierung

über externe Kalibrierung erlaubten bei diesem Verfahren einen höheren Probendurchsatz. Auf der Grundlage der externen Kalibrierung und mit Hilfe von dotierten Sedimentproben war über die HPLC-Methode auch eine Bestimmung der Wiederfindung von DPA möglich. Im Gegensatz zum Flüssig-Flüssig-Extraktionsverfahren konnte somit die Vollständigkeit der Extraktion von DPA überprüft werden. Das HPLC-System erlaubt auch im Fall von Koelutionen eine zuverlässige Quantifizierung von DPA, da die gebildeten Terbiumdipicolinatkomplexe aufgrund ihrer charakteristischen Fluoreszenz über ein spezielles Detektionsverfahren zuverlässig bestimmt werden können. Dieses Prinzip wurde anhand der Analyse von Pfefferproben demonstriert, wodurch gleichzeitig eine mögliche Anwendung der HPLC-Methode im Bereich der Lebensmittelindustrie vorgestellt wurde (siehe Kapitel 4; Fichtel et al., 2008).

6.1.2 Umrechnung von Dipicolinsäuregehalten in Endosporenzahlen

Die Umrechnung der DPA-Gehalte von Sedimentproben in Endosporenzahlen erfolgte auf der Grundlage der DPA-Gehalte von Endosporen verschiedener Wattstämme (siehe Kapitel 2; Fichtel et al., 2007a). Diese Stämme wurden von Köpke et al. (2005) aus den Sedimenten im Rückseitenwatt der Insel Spiekeroog isoliert. Es stellte sich heraus, dass die Endosporen dieser Stämme keine großen Unterschiede in ihren DPA-Gehalten aufwiesen. Auf der Basis des für diese Stämme bestimmten mittleren DPA-Gehalts von $2{,}24\times10^{-16}$ mol pro Spore wurden die Endosporenzahlen in den untersuchten Wattsedimenten abgeschätzt. Der ermittelte Umrechnungsfaktor beruht auf den DPA-Gehalten von sechs Wattstämmen. Von Köpke et al. (2005) wurde zwar eine größere Anzahl von Sporenbildnern isoliert, die meisten dieser Stämme zeigten aber nur einen sehr geringen Sporenertrag in Kultur. Aufgrund der zu geringen Anzahl an Sporen konnte in diesen Fällen keine Bestimmung des DPA-Gehalt erfolgen, obwohl über Dichtegradientenzentrifugation eine Möglichkeit zur Abtrennung von Sporen und vegetativen Zellen zur Verfügung stand. Neue Isolate von Sporenbildnern sollten jedoch routinemäßig auf den DPA-Gehalt ihrer Sporen hin untersucht werden, um die Datenbasis des Umrechnungsfaktors stetig zu verbessern.

Zusätzlich zu den Wattstämmen analysierte Endosporen zeigten zum Teil große Unterschiede in ihren DPA-Gehalten. Eine Übertragung des Umrechnungsfaktors auf andere Standorte scheint deshalb nur eingeschränkt möglich zu sein. In ihrer Konzentration an DPA im Sporenprotoplasten unterschie-

den sich die analysierten Sporen jedoch kaum. Der DPA-Gehalt korreliert demnach offensichtlich mit dem Volumen von Endosporen (Fichtel et al., 2007a; Huang et al., 2007). Inwieweit die Kultivierungsbedingungen den DPA-Gehalt und das Volumen von Endosporen beinflussen, ist weitestgehend unklar. Im Gegensatz zu einer älteren Veröffentlichung (Hitchins et al., 1972) wurde in aktuellen Studien keine Beeinflussung des DPA-Gehalts und des Volumens von Sporen nach Variation der Kultivierungsbedingungen festgestellt (Melly et al., 2002; Carrera et al., 2007).

6.2 Anteil von Endosporen an der mikrobiellen Gemeinschaft in Sedimenten

6.2.1 Quantitative Bedeutung von Endosporen in Wattsedimenten

Über die entwickelte HPLC-Methode erfolgte eine lithologieabhängige, hochaufgelöste Untersuchung von bis zu 5,5 m langen Sedimentkernen auf ihren DPA-Gehalt. Die Sedimentkerne stammten von einem Sand- und einen Mischwattbereich aus dem Rückseitenwatt der Insel Spiekeroog. Bei den Analysen wurden DPA-Gehalte zwischen 0,02 und 4,4 nmol DPA g^{-1} Sediment ermittelt. Entsprechend dem oben genannten Umrechnungsfaktor ergaben diese Gehalte eine Anzahl von 1×10^5 bis 2×10^7 Endosporen g^{-1} Sediment (jeweils bezogen auf Trockengewicht). Es wurde keine generelle Abnahme der Endosporenzahl mit der Tiefe festgestellt. Die DPA-Gehalte spiegelten vielmehr die Lithologieänderungen in den Kernen wider. Die höchsten Sporenabundanzen wurden in dünnen schwarzen Schlickschichten bestimmt. Mit zunehmendem Sandanteil nahm der Sporengehalt gewöhnlich ab. In Sandlagen wurde meist ein DPA-Gehalt von weniger als 0,5 nmol DPA g^{-1} Sediment bzw. Endosporengehalte von weniger als 2×10^6 Endosporen g^{-1} Sediment bestimmt. Unsicherheiten gibt es noch bei der Quantifizierung von Endosporen in den schlickigen Salzwiesenablagerungen, die ausschließlich am Standort Neuharlingersieler Nacken in mehr als 2 m Tiefe gefunden wurden. Bei der Analyse von Sedimentproben dieses Typs wurden zum Teil sehr geringe Wiederfindungen bestimmt. Die geringen Wiederfindungen sind höchstwahrscheinlich auf eine Adsorption von DPA an die Sedimentmatrix zurückzuführen. Es ist deshalb zu vermuten, dass die Anzahl an Endosporen in diesem Bereich des Kerns noch unterschätzt wird.

Der relative Anteil von Endosporen an der mikrobiellen Gemeinschaft wurde auf der Grundlage der Gesamtzellzahlen abgeschätzt. Die Gesamtzellzahlen wurden von Köpke et al. (2005) und A. Gittel (persönliche Mitteilung) über Anfärben mit DAPI bestimmt und schließen auch Endosporen ein. In den oberen 50 cm des Sediments wurde an allen untersuchten Standorten ein Sporenanteil von weniger als 1 % der Gesamtzellzahl ermittelt. Am Neuharlingersieler Nacken wurde der höchste DPA-Gehalt in einer dünnen schwarzen Schlickschicht in etwa 1 m Tiefe bestimmt. Dieser Gehalt entspricht $1{,}3\times10^7$ Endosporen g^{-1} Sediment bzw. einem Sporenanteil von bis zu 5 % an der Gesamtzellzahl. Der Rest des Kerns wies deutlich geringere DPA-Gehalte auf. In den meisten Schichten lag der Sporengehalt bei weniger als 2×10^6 Endosporen g^{-1} Sediment. Während die Endosporenzahlen jedoch mit der Tiefe annähernd konstant blieben, nahmen die Gesamtzellzahlen um fast zwei Größenordnungen ab. Entsprechend wurde am Standort Neuharlingersieler Nacken ein mit der Tiefe zunehmender Endosporenanteil von bis zu 10 % der Gesamtzellzahl festgestellt. Am Standort Janssand wurden deutlich höhere Sporenzahlen bestimmt. In mehreren dünnen schwarzen Schlickschichten wurden über 10^7 Endosporen g^{-1} Sediment nachgewiesen. Der höheren Anzahl an Endosporen standen jedoch auch deutlich höhere Gesamtzellzahlen gegenüber. Des Weiteren wurde keine signifikante Abnahme der Gesamtzellzahl mit der Tiefe festgestellt. Aus diesem Grund lag am Standort Janssand der Sporenanteil bei weniger als 5 % an der Gesamtzellzahl (siehe Kapitel 5).

6.2.2 Bestimmung der Anzahl keimfähiger Endosporen

Die über den DPA-Gehalt bestimmten Endosporenzahlen liegen um mindestens drei Größenordnungen über den Werten, die von Köpke et al. (2005) über das MPN-Verfahren ermittelt wurden. Die MPN-Methode unterschätzt damit deutlich die Gesamtzahl an Endosporen in den untersuchten Sedimentproben (siehe auch Kapitel 2; Fichtel et al., 2007a). Es ist jedoch zu beachten, dass über den DPA-Gehalt alle Endosporen erfasst werden, während über das MPN-Verfahren nur der unter den gegebenen Bedingungen keimfähige und kultivierbare Anteil detektiert wird.

In derzeitigen Arbeiten wird versucht, die Anzahl keimfähiger Endosporen kultivierungsunabhängig über die Freisetzung von DPA nach induzierter Germination zu bestimmen. Über ein entsprechendes Verfahren haben Shafaat & Ponce (2006) etwa 300 keimfähige Endosporen ml^{-1} in Grönland-

eiskernen bestimmt. Hierfür haben sie die im Eis eingeschlossenen Endosporen über Filtration mehr als 150-fach aufkonzentriert. Das in Kapitel 3 vorgestellte HPLC-Verfahren (Fichtel et al., 2007b) sollte die Möglichkeit bieten, den Anteil keimfähiger Endosporen im Sediment, d.h. in einer deutlich komplexeren Matrix, zu bestimmen.

6.2.3 Bestimmung des Endsporenbildungspotentials

Derzeit werden zwei etwa 20 m lange Sedimentkerne analysiert, die im November 2007 im Rückseitenwatt der Insel Spiekeroog gewonnen wurden. Ziel dieser Analysen ist unter anderem eine Abschätzung des Anteils von *Firmicutes* bzw. von Endosporenbildnern an der mikrobiellen Gemeinschaft. Entsprechende Daten sollen mit Hilfe der quantitativen PCR-Methode und über CARD-FISH ermittelt werden, um das Endosporenbildungspotential im Sediment abzuschätzen. Außerdem soll ein möglicher Eintrag thermophiler Sporenbildner über selektive Kultivierung überprüft werden. Endosporen thermophiler Bakterien wurden in hoher Anzahl sowohl in marinen (Isaksen et al., 1994) als auch in limnischen Sedimenten (z.B. Parduhn & Watterson, 1985; Renberg & Nilsson, 1992) nachgewiesen. In diesen Sedimenten wurde ein Wachstum thermophiler Bakterien aufgrund der niedrigen Temperaturen verhindert. Es wird deshalb angenommen, dass die thermophilen Bakterien in Form von Endosporen in das Sediment eingetragen wurden.

6.2.4 Signifikanter Anteil von Endosporen an der tiefen Biosphäre?

In den untersuchten Wattsedimenten wurde keine generelle Abnahme der Endosporenzahlen mit der Tiefe beobachtet. Im Gegensatz dazu nahmen die Gesamtzellzahlen am Standort Neuharlingersieler Nacken innerhalb weniger Meter Tiefe um nahezu zwei Größenordnungen ab. Eine derartige Abnahme entspricht in etwa der von Parkes et al. (2000) ermittelten Tiefenverteilung von Prokaryonten in der tiefen Biosphäre (siehe auch Abb. 24). Während Endosporen in den oberen 50 cm der Wattsedimente nur einen geringen Anteil an der Gesamtzellzahl ausmachten, wurde bereits in wenigen Metern Tiefe ein Endosporenanteil im einstelligen Prozentbereich erreicht (siehe Kapitel 5). Diese relative Zunahme kann auf die enorme Überlebensdauer (siehe Kapitel 1.2.6) und die hohe Resistenz (siehe Kapitel 1.2.5) von Endosporen zurückgeführt werden, während die Anzahl vegetativer Zellen vermutlich infolge von eintretendem Nährstoffmangel deutlich abnimmt.

Aus diesem Grund wird vermutet, dass Endosporen signifikant zur tiefen Biosphäre beitragen und einen mit der Tiefe zunehmenden Anteil an den von Parkes et al. (2000) bestimmten Gesamtzellzahlen ausmachen. In Abb. 24 sind die in dieser Arbeit bestimmten Endosporen-Tiefenprofile zusammengefasst. Zum Vergleich ist die von Parkes et al. (2000) ermittelte Tiefenverteilung von Prokaryonten in der marinen tiefen Biosphäre dargestellt. Diese Daten lassen natürlich keine Abschätzung des Sporenanteils an der Gesamtzellzahl in der tiefen Biosphäre zu. Die Grafik verdeutlicht jedoch, dass die Anzahl an Endosporen in den oberen Metern der Sedimente nicht signifikant abnimmt. Ob der Endosporenanteil an der Gesamtzellzahl tatsächlich mit zunehmender Sedimenttiefe ansteigt, sollte in zukünftigen Arbeiten überprüft werden. Endosporenbildner stellen jedenfalls einen Großteil der bisherigen Isolate aus der tiefen Biosphäre dar (D'Hondt et al., 2004), insbesondere gewinnen sie mit zunehmender Sedimenttiefe an Bedeutung (Batzke et al., 2007). Aus 400 m tiefen Sedimentschichten des Pazifischen Ozeans mit einem geschätzten Alter von 16,5 Millionen Jahren haben Batzke et al. (2007) ausschließlich Bakterien des Phylums *Firmicutes* isoliert.

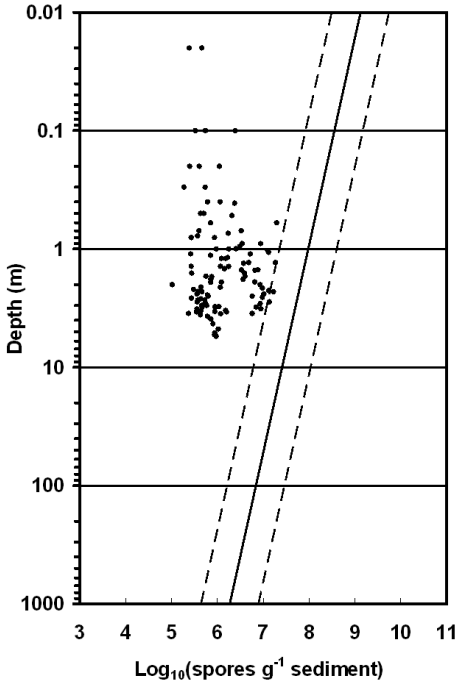

Abb. 24: Tiefenverteilung von Endosporen (Datenpunkte) an den untersuchten Standorten im Rückseitenwatt der Insel Spiekeroog. Dazu im Vergleich die von Parkes et al. (2000) ermittelte Tiefenverteilung von Prokaryonten in der marinen tiefen Biosphäre (in $\log_{10}$[Zellen cm^{-3} Sediment], gestrichelte Linie: 95 % Prognoseintervall)

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Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Zusätzlich erkläre ich, dass diese Dissertation weder in ihrer Gesamtheit noch in Teilen einer anderen wissenschaftlichen Hochschule zur Begutachtung in einem Promotionsverfahren vorliegt oder vorgelegen hat.

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