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Mikrobielle Gemeinschaften an der Chemokline anoxischer Becken der zentralen Ostsee

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Inhalt

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Zusammenfassung

An oxisch-anoxischen Grenzschichten mariner Habitate finden vielfältige mikrobiell katalysierte Prozesse statt. Neben der Mineralisation von organischem Material durch heterotrophe Mikroorganismen stellen solche Grenzschichten auch Lebensräume für eine große Zahl von chemolithotrophen Mikroorganismen dar. In der Vergangenheit wurden viele Studien an oxisch-anoxischen Grenzschichten mariner Sedimente durchgeführt, jedoch gibt es bisher nur wenige umfassende Untersuchungen an den Übergangszonen der großen anoxischen Wasserkörper, wie dem Schwarzen Meer und den tiefen Becken der Ostsee. Ziel der vorliegenden Arbeit war es, Einblick in die chemischen Bedingungen und die Abundanz und Verteilung chemoorganotropher und chemolithotropher Mikroorganismen entlang der Gradienten an der Chemokline anoxischer Becken in der zentralen Ostsee zu gewinnen.

Zunächst wurde ein neues Verfahren zur Bestimmung mikrobieller Biomasse in Anreicherungen und Reinkulturen entwickelt, das auf Basis einer Floureszenzfärbung und anschließender Floureszenzmessung eine einfache und sensitive Detektion von Wachstum erlaubt. Mit Hilfe dieses Verfahrens war es möglich, eine große Zahl von selektiven Anreicherungen zur Bestimmung der Lebendzellzahl heterotropher und lithotropher Mikroorganismen anzulegen und empfindlich auf Wachstum zu untersuchen.

Die Lebendzellzahlbestimmungen ergaben, dass während stagnierender Bedingungen aerobe und anaerobe heterotrophe Mikroorganismen in vergleichbaren Zelldichten an der Chemokline vorkommen. Nach einer Durchmischung und Oxidation des anoxischen Tiefenwassers, wie sie im Sommer 2003 auftraten, verändern sich diese Verhältnisse gravierend. Vermutlich aufgrund des lateralen Eintrags von aeroben Mikroorganismen gemeinsam mit sauerstoffhaltigen Wassermassen stehen unter diesen Bedingungen erhöhte Zahlen aerober Mikroorganismen einer drastischen Abnahme fakultativ oder strikt anaerober Mikroorganismen an der neu gebildeten Chemokline gegenüber. Gemeinsam mit der gefundenen Schwefelabhängigkeit mikrobieller CO_2-Fixierung, legen die vergleichsweise hohen Lebendzellzahlen aerober und anaerober Schwefeloxidierer nahe, dass alternative Elektronenakzeptoren wie Nitrat und Manganoxid nach solchen Durchmischungs- und Oxidationsereignissen vorwiegend für mikrobielle oder chemische Sulfidoxidation zur Verfügung stehen und organisches Material hauptsächlich aerob mineralisiert werden könnte.

Summary

A variety of microbially catalysed processes occur at oxic-anoxic interfaces in the marine environment. Besides the mineralisation of organic matter by chemoorganotrophic microorganisms, such environments also provide a niche for a large number of chemolithotrophic microorganisms. In the past, several studies have investigated the oxic-anoxic interfaces of marine sediments. There are, however, only a few comprehensive studies available on oxic-anoxic interfaces of the large anoxic water bodies, like the Black Sea or the deep basins within the Baltic Sea. The goal of the present study was to gain insights into the chemical zonation, as well as the abundance and distribution of chemoorganotrophic and chemolithotrophic microorganisms along the gradients at the chemocline of anoxic basins within the Central Baltic Sea.

During the present study, a new method for the determination of microbial biomass in enrichments and pure cultures was developed. Based on fluorescent staining and subsequent measurement of fluorescence emission, this method allows a sensitive detection of microbial growth. Using this technique, it was possible to analyse a large number of selective enrichments to determine viable counts of chemoorganotrophic and chemolithotrophic microorganisms.

The results showed that aerobic and anaerobic heterotrophic microorganisms occur in similar numbers at the chemocline during stagnant conditions. This pattern changes significantly after mixing and oxidation of the anoxic bottom water, as observed during summer 2003. Presumably due to the lateral input of aerobic microorganisms together with oxygen-rich water masses, increased viable counts of aerobes and strongly reduced numbers of facultative or strict anaerobes were observed at the newly established chemocline.

High numbers of aerobic and anaerobic sulphur oxidisers in conjunction with the observed sulphur dependence of microbial *in situ* CO_2-fixation, suggested that nitrate and manganese oxide might preferentially serve as electron acceptors for microbial or chemical sulphur oxidation, whereas organic matter could be mineralized mainly aerobically.

Liste der Publikationen

Teile der vorliegenden Arbeit wurden bereits veröffentlicht, zur Veröffentlichung eingereicht oder zur Veröffentlichung vorbereitet:

Martens-Habbena, W., Sass, H. (2006). Sensitive determination of microbial growth by nucleic acid staining in aqueous suspension. *Appl. Environ. Microbiol.* 72: 87-95.

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Weitere in der vorliegenden Arbeit nicht enthaltene Veröffentlichungen:

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Abkürzungsverzeichnis

AO	Acridine Orange
AQDS	Anthrachinondisulfonat
Bp	Basenpaare
Chl*a*	Chlorophyll *a*
CTD	*Conductivity-temperature-density*
DAPI	4’,6-Diamidino-2-phenylindol-dihydrochlorid
DGGE	Denaturierende Gradienten Gelelektrophorese
dNTP	Desoxy-Nukleosidtriphosphat
DOC	*Dissolved Orcanic Carbon*
EDTA	Ethylendiamintetraessigsäure
FI	*Fluorescence intensity*
FISH	Fluoreszenz *in situ* Hybridisierung
HEPES	[4-(2-Hydroxyethyl)-piperazino]-ethansulfonsäure
HPLC	*High performance liquid chromatography*
ICP-OES	*Inductively coupled plasma-optical emission spectroscopy*
MPN	*Most Probable Number*
OD	*Optical density*
OTU	*Operational taxonomic unit*
p.a.	*Pro analysi*
PBS	*Phosphate buffered saline*
PCR	*Polymerase Chain Reaction*
PSU	*Practical Salinity Units*
RFU	*Relative Fluorescence Unit*
SD	*Standard deviation*
SRB	*Sulphate reducing bacteria*
TCC	*Total Cell Count*
Tris	Tris(hydroxymethyl)-aminomethan
v/v	*Volume per volume*
w/v	*Weight per volume*

1. Einleitung

1.1 Mikrobielle Kohlenstoffmineralisation in marinen Habitaten

Die biogeochemischen Kreisläufe der wichtigsten Elemente (C, H, O, N, P, S, Fe, Mn) sind geprägt durch die sauerstoffhaltige Erdatmosphäre. Die Quelle des atmosphärischen Sauerstoffs ist die Photosynthese. Phototrophe Organismen nutzen Lichtenergie zur Reduktion von CO_2 zu organischen Kohlenstoffverbindungen, aus denen sie zusammen mit anderen Nährstoffen ihre Biomasse aufbauen. Im marinen Milieu wird oxygene Photosynthese hauptsächlich von photosynthetischen Diatomeen, Grünalgen, Flagellaten und Cyanobakterien durchgeführt. Schätzungen zufolge bilden diese Organismen im Ozean jährlich etwa 45 Gigatonnen organischer Substanz (Falkowski *et al.*, 1998).

Der Großteil der photosynthetisch gebildeten organischen Substanz (50 bis 90 %) wird direkt in der euphotischen Zone der Wassersäule von heterotrophen Bakterien wieder mineralisiert und bildet die Grundlage für ein verzweigtes biologisches Nahrungsnetz (Azam *et al.*, 1983; Azam, 1998). Nur ein kleiner Teil der Primärproduktion sedimentiert aus der photischen Zone herab in tiefere Wasserschichten und weniger als 1 % erreicht im offenen Ozean die Sedimentoberfläche. An nährstoffreichen Kontinentalrändern und in Randmeeren mit hoher photosynthetischer Primärproduktion sind die Sedimentationsraten wesentlich höher. Hier werden zwischen 10 und 50 % der jährlichen Primärproduktion auf dem Sediment abgelagert und von Mikroorganismen mineralisiert. Nur ein sehr kleiner Teil der organischen Substanz wird nicht abgebaut und dauerhaft in marinen Sedimenten abgelagert (Hedges & Keil, 1995).

Die Mineralisation von organischer Substanz im Sediment unterscheidet sich deutlich von der Mineralisation in der Wassersäule. Während Mikroorganismen im Wasser organische Substanz ausschließlich durch aerobe Atmung abbauen, wird in Sedimenten mit hohem Eintrag von organischem Material der gelöste Sauerstoff innerhalb weniger Millimeter unterhalb der Sedimentoberfläche von Mikroorganismen vollständig verbraucht. Die aerobe Mineralisation im Sediment wird daher durch Nachlieferung von Sauerstoff über Bioturbation und Diffusion limitiert. Tiefere Sedimentschichten sind frei von Sauerstoff und der Abbau organischer Substanz erfolgt durch Gärung und anaerobe Atmungsprozesse (Denitrifikation, dissimilatorische Nitratammonifikation, Manganreduktion, Eisenreduktion, Sulfatreduktion, Methanogenese sowie Acetogenese) (Canfield *et al.*, 1993; Lovley, 1991).

Mit zunehmender Sedimenttiefe lässt sich oftmals eine vertikale Abfolge der Nutzung alternativer Elektronenakzeptoren feststellen. Diese Abfolge lässt sich anhand der Redoxpotentiale der verschiedenen Elektronenakzeptoren nachvollziehen. Die Oxidation von organischem Material oder dessen Gärprodukten (z. B. Formiat, Lactat, Acetat und Wasserstoff) setzt, abhängig vom Redoxpotential des verwendeten Elektronenakzeptors, unterschiedlich viel Energie frei (Abb. 1, Tab.1; Zehnder & Stumm, 1988).

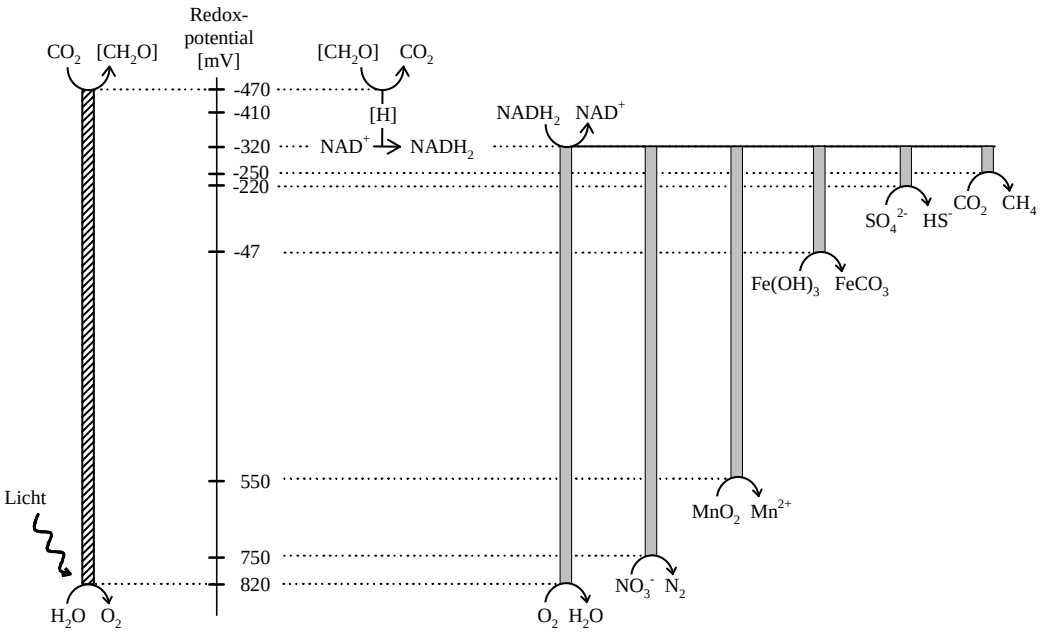

Abb. 1: Vereinfachte schematische Darstellung der Redoxpotentialdifferenzen, die bei der Bildung von organischer Substanz durch photosynthetische Organismen und beim Abbau organischer Substanz durch heterotrophe Organismen überbrückt werden. Mit Hilfe von Lichtenergie koppeln grüne Pflanzen und das Phytoplankton die Oxidation von Wasser an die Reduktion von CO_2 zu organischer Substanz $[CH_2O]$ (schraffierter Balken). Organotrophe Organismen koppeln die Oxidation organischer Substanz an die Reduktion von Sauerstoff oder anderer Elektronenakzeptoren. Der dabei frei werdende Energiebetrag hängt vom Redoxpotential des verwendeten Elektronenakzeptors ab (graue Balken).

Ein typisches Beispiel eines Gärproduktes, das von Mikroorganismen durch anaerobe Atmungsprozesse oxidiert werden kann, ist Acetat. Nach der aeroben Atmung liefern in absteigender Reihenfolge prinzipiell Denitrifikation, Mangan- und Eisenreduktion den größten Energiegewinn je oxidiertem Acetat-Molekül (Tab. 1). Nach dem Verbrauch von Sauerstoff an der Sedimentoberfläche werden demnach theoretisch Nitrat und Manganoxid als

erste Elektronenakzeptoren verwendet, da die betreffenden Mikroorganismen z. B. Eisenreduzierer und Sulfatreduzierer auskonkurrieren können.

Tab. 1: Einfluss von Elektronenakzeptoren auf den möglichen Energiegewinn durch aerobe und anaerobe Atmungsprozesse am Beispiel der Oxidation von Acetat. Angegeben ist die Änderung der freien Energie ($\Delta G^{0'}$) je Mol oxidierten Acetats.[1]

Aerobe Atmung:	$C_2H_3O_2^- + 2\ O_2 + H^+$	=>	$2\ CO_2 + 2\ H_2O$	-853.9 kJ/mol
Denitrifikation:	$5\ C_2H_3O_2^- + 8\ NO_3^- + 13\ H^+$	=>	$4\ N_2 + 14\ H_2O + 10\ CO_2$	-801.8 kJ/mol
Manganreduktion:	$C_2H_3O_2^- + 4\ MnO_2 + 9\ H^+$	=>	$2\ CO_2 + 4\ Mn^{2+} + 6\ H_2O$	-535.5 kJ/mol
Eisenreduktion:	$C_2H_3O_2^- + 8\ Fe(OH)_3 + 6\ HCO_3^- + 7\ H^+$	=>	$8\ FeCO_3 + 20\ H_2O$	-367.9 kJ/mol
Sulfatreduktion:	$C_2H_3O_2^- + SO_4^{2-} + 2\ H^+$	=>	$2\ CO_2 + HS^- + 2\ H_2O$	-57.4 kJ/mol
Methanogenese:	$C_2H_3O_2^- + H^+$	=>	$1\ CO_2 + CH_4$	-35.9 kJ/mol

[1] Thermodynamische Daten aus Thauer *et al.* (1977), Lide (1992), Stumm & Morgan (1981).

Den größten Anteil an der mikrobiellen Mineralisation in marinen Sedimenten macht dennoch oft die Sulfatreduktion aus (Jørgensen, 1982). Der Energiegewinn aus der Oxidation organischer Substrate mit Sulfat liegt zwar deutlich unter dem der anderen Elektronenakzeptoren, jedoch liefert das überlagernde Meerwasser an der Sediment-Wassergrenzschicht etwa 100fach mehr Sulfat nach als beispielsweise Sauerstoff. In Oberflächensedimenten ist die mikrobielle Sulfatreduktion daher meist nicht durch die Verfügbarkeit des Elektronenakzeptors limitiert.

Die Konzentrationen der anderen Elektronenakzeptoren sind im Meerwasser oder an der Sedimentoberfläche selten groß genug, um substantiell zur Kohlenstoffmineralisation beizutragen (Canfield *et al.*, 1993). Eine Ausnahme davon sind z. B. besonders manganreiche Sedimente, wo Manganoxid als Elektronenakzeptor einen wesentlichen Anteil der Mineralisation ausmachen kann (Canfield *et al.*, 1993; Thamdrup *et al.*, 2000). Methanogenese herrscht im marinen Milieu nur in tieferen Sedimentschichten vor, die kein Sulfat mehr enthalten.

1.2 Mikrobielle Oxidation von reduzierten anorganischen Verbindungen

Abhängig von der Intensität der mikrobiellen Remineralisierung organischen Materials werden durch anaerobe Atmungsprozesse große Mengen von Sulfid, reduziertem Mangan und

Eisen sowie Ammonium freigesetzt. Aufgrund des großen Anteils der Sulfatreduktion an der Mineralisation macht Sulfid den wesentlichen Teil dieser reduzierten Verbindungen aus. Gemessen an den Sulfatreduktionsraten akkumulieren jedoch nur etwa 10 % davon als Eisensulfid dauerhaft im Sediment (Jørgensen, 1977; Zopfi *et al.*, 2004). Der überwiegende Teil des Sulfids und der anderen reduzierten Verbindungen diffundiert an die Sedimentoberfläche und wird dort entweder chemisch oder von lithotrophen Mikroorganismen wieder oxidiert (Jørgensen, 1977; Jørgensen *et al.*, 1990; Yao & Millero, 1993; Yao & Millero, 1996; Zhang & Millero, 1993). An der oxisch-anoxischen Grenzschicht konkurrieren daher organotrophe und lithotrophe Mikroorganismen mit chemischen Redoxreaktionen um Elektronenakzeptoren.

Abgesehen vom Wasserstoff, der durch Gärungsprozesse freigesetzt wird und aufgrund seines niedrigen Redoxpotentials auch in tiefen Sedimentschichten von Sulfatreduzierern und Methanogenen genutzt werden kann, sind die anderen lithotrophen Prozesse eng an die Gegenwart von Sauerstoff und Nitrat gekoppelt und kommen folglich oft an oxisch-anoxischen Grenzschichten vor. Die von lithotrophen Organismen zur Energiegewinnung nutzbare Redoxpotentialdifferenz hängt daher im Wesentlichen von dem verwendeten Elektronendonor ab (Abb. 2). Die Oxidation von Mangan kann mikrobiell katalysiert werden, jedoch ist bisher unklar, ob diese Reaktion auch zur Energiekonservierung genutzt werden kann (Tebo *et al.*, 2005).

Auf Grundlage der Redoxpotentialdifferenzen sind noch viele weitere lithotrophe Prozesse denkbar, z. B. eine mikrobielle Kopplung von Sulfid-, Eisen- oder Ammoniumoxidation an Manganreduktion. Jedoch konnten nicht alle theoretisch möglichen Prozesse auch in der Umwelt nachgewiesen werden (z. B. Broda, 1977; Luther III *et al.*, 1997).

Lithotrophe Mikroorganismen müssen in der Regel mit chemischen Redoxreaktionen konkurrieren. Dies trifft insbesondere für die Oxidation von Sulfid, Eisen und Mangan zu. Während die Wasserstoff- und Ammoniumoxidation chemisch relativ langsam ablaufen und daher in marinem Milieu fast ausschließlich mikrobiell katalysiert werden, sind die chemischen Reaktionsgeschwindigkeiten der Sulfid- und Metalloxidationen vergleichsweise hoch (Thamdrup, 2000; Yao & Millero, 1993; Yao & Millero, 1996; Zhang & Millero, 1993). Entsprechend der Redoxpotentialdifferenzen kann Sulfid chemisch durch Sauerstoff, Mangan- und Eisenoxid oxidiert werden, während reduziertes Eisen durch Sauerstoff sowie Manganoxid und reduziertes Mangan nur durch Sauerstoff oxidiert werden kann (Abb. 2).

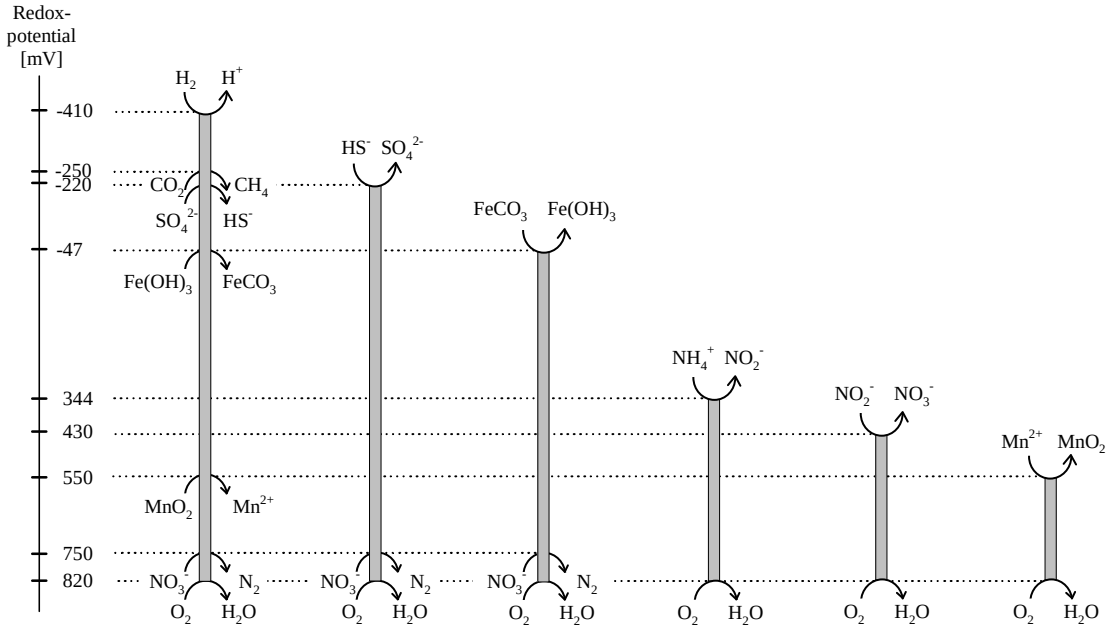

Abb. 2: Vereinfachte schematische Darstellung einiger Redoxpotentialdifferenzen, die von lithotrophen Mikroorganismen durch die aerobe oder anaerobe Oxidation inorganischer Verbindungen zur Energiegewinnung genutzt werden können.

An oxisch-anoxischen Grenzschichten mariner Sedimente laufen die verschiedenen Prozesse räumlich sehr eng nebeneinander ab. Die Schwefel- und Metallverbindungen können daher intensiven Kreisläufen unterliegen. Die genauen Mechanismen der Schwefeloxidation und der dabei auftretenden Intermediate (z. B. elementarer Schwefel, Thiosulfat, Sulfit) sowie die Metallkreisläufe und die Rolle von Mikroorganismen in diesen Prozessen sind bisher im Detail noch nicht verstanden (Thamdrup, 2000; Zopfi *et al.*, 2004).

In den vergangenen Jahren ist erkannt worden, dass auch Huminstoffe als Elektronendonoren und -akzeptoren für organotrophe und lithotrophe Organismen fungieren können (Benz *et al.*, 1998; Coates *et al.*, 2002). Die Redoxpotentiale natürlicher Huminstoffe lassen sich recht schwer bestimmen und sind stark abhängig von deren chemischer Struktur und Herkunft. Anhand des Redoxpotentials von Huminstoff-Präparationen aus Bodensee-Sediment (+84 mV, Benz *et al.*, 1998) lässt sich jedoch nachvollziehen, dass sie als Elektronendonoren und -akzeptoren von Bedeutung sein könnten (vgl. Abb. 1 und 2). Auch diese Prozesse sind bisher kaum untersucht.

1.3 Struktur geschichteter Randmeere am Beispiel der Ostsee

Gegenüber dem offenen Ozean sind Randmeere wie das Mittelmeer, das Schwarze Meer und die Ostsee durch Meeresschwellen von der globalen Zirkulation des Ozeans abgeschnitten. Während das Mittelmeer kontinuierlichen Wasseraustausch mit dem Atlantischen Ozean hat, ist die Wasserbilanz des Schwarzen Meeres und der Ostsee durch überwiegenden Einstrom von Süßwasser aus Flüssen bzw. Niederschlag geprägt. Der Abfluss von salzarmem Oberflächenwasser ins Mittelmeer bzw. in die Nordsee überwiegt daher deutlich gegenüber dem Einstrom von Meerwasser. Ins Schwarze Meer und in die Ostsee dringt Meerwasser nur in unregelmäßigen Abständen ein und unterschichtet das Oberflächenwasser aufgrund seiner höheren Dichte. Infolgedessen sind diese Randmeere durch eine permanente Dichtesprungschicht, eine sogenannte Halokline, gekennzeichnet. Diese Sprungschicht trennt das salzarme Oberflächenwasser vom salzreicheren Tiefenwasser und unterbindet die Durchmischung beider Wasserschichten. Nährstoffe und organisches Material können nur über Diffusion oder Partikelsedimentation durch die Halokline zwischen beiden Wasserkörpern ausgetauscht werden.

In der Ostsee nimmt der Süßwassereinfluss auf das Oberflächenwasser mit zunehmender Entfernung von der Nordsee zu. Die Salinität des Oberflächenwassers und die Wassertiefe, in der sich die Halokline befindet, sinken ab. In der zentralen Ostsee beträgt die mittlere Salinität etwa 8 ‰ im Oberflächenwasser und 12 ‰ im Tiefenwasser und die Halokline liegt zwischen 60 und 80 m Wassertiefe (Abb. 3). Das salzärmere Oberflächenwasser wird im Sommer zusätzlich durch eine Temperatursprungschicht, eine so genannte Thermokline, unterteilt. Der Wasserkörper ist daher durch eine thermo-haline Schichtung in drei Zonen geteilt: (I) eine warme Oberflächenzone geringer Salinität, (II) eine kalte Zwischenzone geringer Salinität und (III) eine stagnierende Tiefenwasserzone höherer Salinität (Abb. 3).

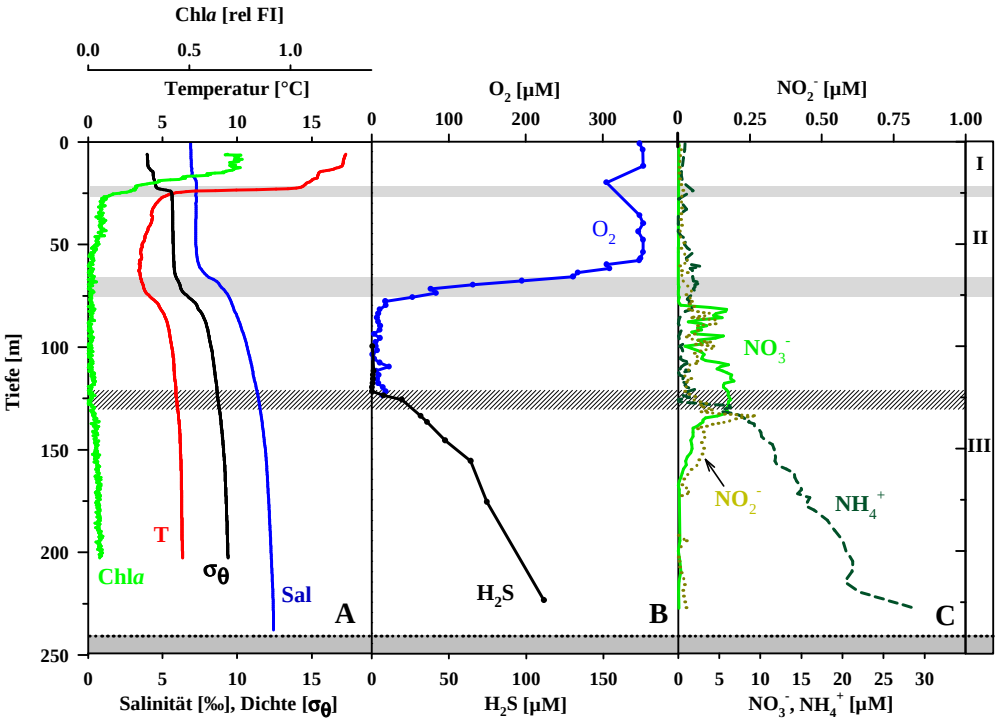

Abb. 3: Physikalische und chemische Zonierung des Gotland Beckens in der zentralen Ostsee im Juli 2002. A) Tiefenprofile von Temperatur (T), Salinität (Sal), Dichte (σ_θ) und Chlorophyll*a* Fluoreszenz (Chl*a*). B) und C) Tiefenprofile chemischer Parameter. Die warme Oberflächenschicht (I), die kalte Zwischenschicht (II) und das stagnierende Tiefenwasser (III) sind auf der rechten Seite markiert. Thermokline, Halokline und die Sedimentoberfläche sind grau, die Chemokline schraffiert hinterlegt.

1.4 Entstehung oxisch-anoxischer Grenzschichten im Tiefenwasser der Ostsee

Die photosynthetische Primärproduktion an der Wasseroberfläche ist auch in der zentralen Ostsee die wesentliche Quelle des organischen Materials. In der südlichen und zentralen Ostsee kommt es in der Regel jährlich zu zwei Algenblüten, deren Länge und Intensität von Temperatur, Lichtintensität und Nährstoffverfügbarkeit bedingt werden. Die Frühjahrsblüte zehrt die über die Wintermonate aus den Flüssen eingetragenen Nährstoffe auf. Ihr Maximum liegt im April und Mai und ist in den nährstoffreichen Küstengebieten stärker ausgeprägt als in der zentralen Ostsee. Sie wird von den Diatomeenarten *Achnanthes taeniata* und *Skeletonema costatum* dominiert. Nach auftretender Stickstofflimitierung stirbt diese Algenblüte innerhalb weniger Wochen ab und es folgen gewöhnlich einige Wochen geringer Algendichte. Im Juli bis Oktober wächst dann eine zweite, hauptsächlich von *Microcystis* sp. und den stickstofffixierenden Arten *Aphanizomenon flos-aquae* und *Nodularia spumigena*

dominierte Cyanobakterien-Blüte heran. Beiden Algenblüten folgt leicht zeitversetzt eine Blüte von heterotrophem Bakterioplankton (Rheinheimer, 1995).

Die gesamte jährliche photosynthetische Primärproduktion liegt in der zentralen Ostsee mit ca. 6.5 mol C m^{-2} deutlich höher als im offenen Ozean (Schneider & Kuss, 2004). Ein großer Teil des fixierten Kohlenstoffs (ca 3.2 mol C m^{-2}) wird analog zum offenen Ozean direkt in der photischen Zone von organotrophen Mikroorganismen remineralisiert (Gast & Gocke, 1988; Rheinheimer *et al.*, 1989; Schneider & Kuss, 2004). Im Durchschnitt sedimentieren jährlich etwa 3.3 mol C m^{-2} als partikuläres organisches Material über die Halokline in das Tiefenwasser ab (Schneider *et al.*, 2002). Insbesondere nach dem Absterben von Algenblüten können jedoch große Ansammlungen von Aggregaten entstehen, die innerhalb weniger Tage bis Wochen auf das Sediment absinken (Rheinheimer, 1995).

Unterhalb der Halokline ist die Nachlieferung von sauerstoffhaltigem Wasser nur durch lateralen Transport innerhalb des Tiefenwassers möglich. Es kommt daher insbesondere in den abgeschnittenen tiefen Becken durch den mikrobiellen Abbau des an der Sedimentoberfläche akkumulierenden organischen Materials zum vollständigen Verbrauch von Sauerstoff, Nitrat, Mangan- und Eisenoxid. Die Mineralisation an der Sedimentoberfläche erfolgt demnach ausschließlich durch Fermentation, Sulfatreduktion und Methanogenese (Piker *et al.*, 1998). Der von Sulfatreduzierern produzierte Schwefelwasserstoff, im Sediment akkumuliertes reduziertes Mangan und Eisen sowie Ammonium können nicht wie im Ozean direkt an der Sedimentoberfläche wieder oxidiert werden. Sie diffundieren in die Wassersäule hinein bis an die oxisch-anoxische Grenzschicht, die sogenannte Chemokline (Abb. 3). Dort treffen diese potentiellen Elektronendonoren für lithotrophe Organismen auf Elektronenakzeptoren und werden entweder chemisch oder mikrobiell oxidiert (Brettar & Rheinheimer, 1991; Neretin *et al.*, 2003).

Die Stoffkreisläufe solcher stagnierenden anoxischen Becken unterscheiden sich deutlich von denen an einer oxischen Sedimentoberfläche. An der Chemokline im Wasserkörper akkumuliert kein absedimentierendes partikuläres organisches Material. An der Sedimentoberfläche wird organisches Material zudem vollständig anaerob abgebaut und die Reoxidationsprozesse laufen mehrere zehn bis hundert Meter oberhalb in der Wassersäule ab.

In der Vergangenheit sind umfassende Studien an oxisch-anoxischen Übergangszonen mariner Sedimente durchgeführt worden (z. B. Froelich *et al.*, 1979; Jørgensen, 1977; Jørgensen, 1982; Jørgensen *et al.*, 1990; Kostka *et al.*, 2002; Llobet-Brossa *et al.*, 1998; Llobet-Brossa *et al.*, 2002; Thamdrup & Canfield, 1996; Thamdrup *et al.*, 1998; Wieringa *et al.*, 2000). Im Gegensatz dazu gibt es abgesehen von einzelnen Studien an Chemoklinen

hypersaliner Becken und anoxischer Fjorde (Daffonchio *et al.*, 2006; Ramsing *et al.*, 1996; Sass *et al.*, 2001; Zopfi *et al.*, 2001) wenige vergleichbare Studien an der Wassersäule der großen anoxischen Wasserkörper, wie dem Schwarzen Meer und den tiefen Becken der zentralen Ostsee. Die vorhandenen Studien konzentrierten sich weitgehend auf einzelne physiologsiche Gruppen von Mikroorganismen, wie Denitrifizierer (Brettar & Rheinheimer, 1991; Brettar & Rheinheimer, 1992; Brettar *et al.*, 2002), Manganreduzierer (Nealson *et al.*, 1991), Sulfatreduzierer (Teske *et al.*, 1996), Schwefeloxidierer (Jannasch *et al.*, 1991; Sorokin, 2003) oder Manganoxidierer (Tebo, 1991). Bisher gibt es keine Studien, welche die verschiedenen physiologischen Gruppen von Mikroorganismen gemeinsam untersuchen, um deren Verteilung entlang der chemischen Gradienten an oxisch-anoxischen Grenzschichten in der Wassersäule aufzuklären.

1.5 Moderne Strategien zur direkten Untersuchung von Mikroorganismen in der Umwelt

Aufgrund der Entwicklung und Anwendung neuer Untersuchungsmethoden in der Mikrobiologie hat sich die Sicht auf Mikroorganismen in der Natur in der jüngeren Vergangenheit wesentlich verändert (Amann *et al.*, 1995; Handelsman, 2004). Im Folgenden sollen einzelne wichtige Beispiele und deren Einfluss auf die mikrobielle Ökologie kurz dargestellt werden.

Traditionelle lichtmikroskopische Verfahren zur Untersuchung von Mikroorganismen ermöglichen nur sehr selten eine genaue Bestimmung der gesamten Zellzahl, da die Zelldichten in natürlichen Proben meist zu gering sind. Die Einführung von epifluoreszenzmikroskopischen Methoden, in Verbindung mit der Filtration größerer Probenvolumina, haben dieses Hindernis weitgehend überwunden und werden heute standardmäßig zur Bestimmung von Zellzahlen eingesetzt (Hobbie *et al.*, 1977; Kepner & Pratt, 1994). Neben speziellen Verfahren zur Abschätzung der Zahl vitaler Zellen hat sich für die epifluoreszenzmikroskopische Bestimmung von Gesamtzellzahlen die Nutzung von Fluoreszenzfarbstoffen durchgesetzt, die selektiv an Nukleinsäuren binden (Kepner & Pratt, 1994; Noble & Fuhrman, 1998; Porter & Feig, 1980; Weinbauer *et al.*, 1998). Mit Hilfe dieser Methode wurde festgestellt, dass die Zahl von Mikroorganismen in den meisten Habitaten um Größenordnungen höher liegt, als bis dahin durch Kultivierungsverfahren detektiert werden konnte (Amann *et al.*, 1995; Kepner & Pratt, 1994). Man geht daher davon aus, dass bisher

weniger als 1 % der Mikroorganismen aus Wasserproben und noch deutlich weniger aus Sedimentproben mit Hilfe klassischer Verfahren kultiviert werden können (Amann *et al.*, 1995). Demzufolge ist vermutlich eine große Zahl von Organismen und möglicherweise auch eine Vielfalt von mikrobiell katalysierten Prozessen einer direkten mikrobiologischen Untersuchung unzugänglich (Handelsman, 2004; Leadbetter, 2003; Lorenz & Schleper, 2002; Tyson & Banfield, 2005).

Ein erster Schritt zur Untersuchung dieser bisher nicht kultivierten Organismen wurde durch die Entwicklung und Anwendung neuer molekularbiologischer Methoden ermöglicht. Hier ist insbesondere die Entwicklung der Polymerase-Kettenreaktion (PCR, Saiki *et al.*, 1988) von Bedeutung. Dieses Verfahren zur enzymatischen Vervielfältigung von Nukleinsäuresequenzen erlaubt heute die Analyse geringster Mengen von Nukleinsäuren. Die PCR ist daher Ausgangspunkt oder wichtiges Hilfsmittel fast aller molekularbiologischen Untersuchungsmethoden und wird unter anderem für die Bestimmung von Nukleotidsequenzen mittels DNA-Sequenzierung und der Untersuchung mikrobieller Diversität verwendet (Kowalchuk *et al.*, 2004).

Zur Untersuchung mikrobieller Diversität steht in der molekularen Ökologie bis heute die Analyse von einzelnen Markergenen im Vordergrund. Dieses sind insbesondere Gene, von denen angenommen wird, dass sie aufgrund ihrer Funktionen bei der DNA-Replikation oder der Proteinsynthese in jedem lebenden Organismus vorkommen. Das am häufigsten verwendete Markergen für die Untersuchung phylogenetischer Diversität natürlicher Mikroorganismengemeinschaften ist das Gen für die ribosomale 16S rRNA (Olsen *et al.*, 1986; Pace, 1997; Woese & Fox, 1977).

Durch die direkte Isolierung von DNA aus Umweltproben, anschließender PCR-Amplifikation, Auftrennung der unterschiedlichen Sequenztypen mittels Klonierung oder elektrophoretischer Methoden, wie der Denaturierenden Gradienten-Gelelektrophorese (DGGE, Muyzer *et al.*, 1993) oder *Single Stranded Conformation Polymorphism* (SSCP, Sunnucks *et al.*, 2000) und anschließender Sequenzierung, kann ein Überblick über die phylogenetische Zusammensetzung mikrobieller Gemeinschaften gewonnen werden. Auf diese Weise sind bisher mehr als 220.000 Sequenzen des 16S rRNA Gens von Reinkulturen und aus Umweltproben bestimmt und in öffentlichen Datenbanken hinterlegt worden (*The Ribosomal Database Project*, Cole *et al.*, 2005, http://rdp.cme.msu.edu/, abgerufen am 12.06.2006).

Anhand der vorhandenen 16S rRNA-Gensequenzen und der phänotypischen Unterschiede können heute drei Organismengruppen, sogenannte Domänen, abgegrenzt

werden: Bakterien, Archaeen und Eukaryonten (Hugenholtz *et al.*, 1998; Woese & Fox, 1977). Innerhalb der Domäne der Bakterien lassen sich aufgrund von Sequenzähnlichkeiten derzeit etwa 53 Großgruppen, sogenannte Phyla, unterteilen. Etwa die Hälfte dieser Phyla enthält keine kultivierten Vertreter und viele weitere Phyla umfassen 16S rRNA-Gensequenzen mit einem sehr geringen Anteil von bereits kultivierten Organismen (Rappé & Giovannoni, 2003).

Ein wichtiger Schritt zur direkten mikroskopischen Identifizierung von Mikroorganismen aus der Umwelt, und damit auch der Identifizierung bisher unbekannter Organismen, wurde durch die Entwicklung der Fluoreszenz-In-Situ-Hybridisierung (FISH) eingeleitet (Amann *et al.*, 1990; DeLong *et al.*, 1989). Anhand von fluoreszenzmarkierten Sonden, die komplementär zu Abschnitten der 16S rRNA sind, ermöglicht dieses epifluoreszenzmikroskopische Verfahren eine Zuordnung einzelner Zellen zu einzelnen phylogenetischen Gruppen.

Mit den genannten Methoden ist es gelungen, Einblicke in die Struktur natürlicher Bakteriengemeinschaften zu gewinnen. Jedoch lassen sich damit nur selten Einblicke in die Lebensweise der Organismen oder deren Rolle im Ökosystem gewinnen. In der Regel ist dies nur anhand des Vergleichs mit bereits kultivierten Stämmen möglich.

Jüngste methodische Entwicklungen zur Untersuchung natürlicher mikrobieller Gemeinschaften basieren daher in der Regel auf der Kombination verschiedener Untersuchungsmethoden, die sowohl Einblick in die Zusammensetzung mikrobieller Gemeinschaften, als auch in deren Aktivität erlauben. Beispielsweise wurde die FISH-Technik mit gleichzeitiger mikroautoradiographischer Analyse des Umsatzes von ^{14}C-markierten Substraten kombiniert (MAR-FISH, Daims *et al.*, 2001; Gray *et al.*, 2000; Nielsen *et al.*, 2003). Auf diese Weise konnten in den genannten Studien Organismengruppen identifiziert werden, die organische Substrate aufnehmen oder CO_2 fixieren. Ein weiteres Beispiel stellt die Kombination der FISH-Technik mit der Analyse stabiler Kohlenstoffisotopenverhältnisse in einzelnen Zellen dar (Orphan *et al.*, 2001). Enzymatische Umsetzungen sind mit der Diskriminierung von Kohlenstoffisotopen verbunden. Dieser Effekt tritt besonders bei Enzymen in Erscheinung, die kleine Moleküle wie CO_2 oder CH_4 umsetzen. Wenn Mikroorganismen diese Moleküle als Kohlenstoffquelle verwenden, spiegelt das Isotopenverhältnis ihres Zellkohlenstoffs diese Isotopenfraktionierung wieder. Werden komplexere Kohlenstoffverbindungen aufgenommen, so entspricht das Isotopenverhältnis der Biomasse in etwa derjenigen der Substratquelle. Die Analyse stabiler Isotopenverhältnisse

kann daher Aufschluss über die verwendeten Kohlenstoffquellen in natürlichen Habitaten geben (Manefield *et al.*, 2002; Radajewski *et al.*, 2000).

Trotz der methodischen Entwicklungen sind bis heute viele Prozesse und deren mikrobielle Beteiligung schwer nachzuweisen. Ausgehend von den geringen Kultivierungserfolgen und dem hohen biotechnologischen Potenzial, das in der genetischen Vielfalt natürlicher mikrobieller Gemeinschaften vermutet wird, wird daher zunehmend versucht, durch Sequenzierung großer DNA-Mengen aus Umweltproben direkt die genetische Information unkultivierter Mikroorganismen zu entschlüsseln bzw. ganze Genome unkultivierter Mikroorganismen zu rekonstruieren und deren Stoffwechselkapazitäten zu verstehen (Handelsman *et al.*, 1998; Lorenz & Schleper, 2002; Tyson *et al.*, 2004; Venter *et al.*, 2004).

Solche metagenomischen Studien haben ein großes Potential, neue Einsichten in die Biologie bisher nicht kultivierter Organismen zu vermitteln (Handelsman, 2004). So wurden beispielsweise durch metagenomische Studien Bakteriorhodopsine in bisher unkultivierten Bakterien der SAR86-Gruppe in Küstengewässern der USA gefunden. Dies hat einen völlig neuen Anhaltspunkt für den Energiestoffwechsel dieser bisher unkultivierten Organismen gegeben (Béjà *et al.*, 2000). Nach der hierdurch stimulierten gezielten Suche konnten diese Rhodopsine dann ebenfalls in einem Bakterienstamm der im Ozean sehr abundanten SAR11-Gruppe, Candidatus *Pelagibacter ubique*, nachgewiesen werden (Giovannoni *et al.*, 2005). Im Rahmen einer weiteren metagenomischen Studie wurde ein von nur fünf Organismen dominierter Biofilm aus sauren Minenwässern nahezu vollständig sequenziert. Dadurch konnten wesentliche Einblicke in den Energie-, Kohlenstoff- und Stickstoffmetabolismus der bis dahin unkultivierten Bakterien gewonnen werden (Tyson *et al.*, 2004). Die Erkenntnis, dass ein Organismus in diesem Biofilm die genetische Ausstattung zur Stickstofffixierung besitzt, wurde anschließend zur selektiven Anreicherung und Isolierung einer der beiden dominanten Mikroorganismen in diesem Biofilm genutzt (Tyson *et al.*, 2005).

Häufig sind natürliche Organismengemeinschaften jedoch zu vielfältig, um sie mittels konventioneller DNA-Sequenzierung ausreichend zu erfassen. Die Entwicklung neuer Sequenziertechniken, z. B. der sogenannten Polony Sequenzierung (Margulies *et al.*, 2005; Shendure *et al.*, 2005), wird in naher Zukunft die Sequenzierleistung vervielfachen und noch wesentlich detailliertere metagenomische Studien erlauben. Jedoch stößt auch dieser Ansatz methodisch an Grenzen, da sich vielen Genen keine eindeutige Funktion zuordnen lässt und vorhandene Gene nicht zwangsläufig auch genutzt werden. Ein einfaches Beispiel dafür ist die Tatsache, dass selbst im Genom von einem der bestuntersuchten Mikroorganismen,

E. coli, großen Teilen der vorausgesagten Gene keine Funktion zugeordnet werden kann und ein genaueres Verständnis des Genoms von *E. coli* gegenwärtig durch Intensivierung physiologischer Untersuchungen erzielt werden soll (Prieto *et al.*, 2004).

Anhand des gerade erst aufkommenden Wissens über Wachstum von Mikroorganismen und der Organisation bzw. Selbstorganisation zellulärer Prozesse kommen bereits Zweifel an der weit verbreiteten Konzeption auf, dass zelluläre Abläufe rein genetisch determiniert sind (Harold, 2005). Das genetische Material von Organismen ist nur ein Teilbestandteil dessen, was im Laufe der Evolution von Generation zu Generation weitergegeben wird. Eine intakte räumliche Organisation der Zelle, stereochemische Selektivität von enzymatischen Reaktionen und intakte Membranen werden ebenfalls weitergegeben und sind gleichermaßen Bedingung und organisatorische Basis für lebende Organismen und Gegenstand natürlicher Selektion (Cavalier-Smith, 2001). Es erscheint zweifelhaft, ob Phänomene, wie die Kontinuität der Membranen, Interaktion von Genprodukten, intrazelluläre physikalische Kräfte oder die strukturelle Integrität von Zellen allgemein sich überhaupt auf genetische Information beziehen bzw. durch sie zu erklären sind (Cavalier-Smith, 2001; Harold, 1990; Harold, 2005).

1.6 Gegenwärtige Ansätze zur Verbesserung von Kultivierungsverfahren

Das Fehlen von kultivierten Vertretern vieler phylogenetischer Gruppen von Mikroorganismen und die Diskrepanz zwischen Gesamt- und Lebendzellzahlen in Proben aus natürlichen Mikrobengemeinschaften hat die skizzierten methodischen Entwicklungen kultivierungsunabhängiger Verfahren wesentlich stimuliert (Amann *et al.*, 1995; Handelsman, 2004). Jedoch ist die Untersuchung kultivierter Mikroorganismen nach wie vor eine unverzichtbare Quelle von prinzipiellen Erkenntnissen über deren Aufbau und Lebensweise (Fry, 2000; Harold, 2005; Leadbetter, 2003) und dient darüber hinaus auch zum besseren Verständnis molekular-phylogenetischer und metagenomischer Analysen (Leadbetter, 2003; Tyson & Banfield, 2005).

In den vergangenen Jahren haben Erkenntnisse aus kultivierungsunabhängigen Untersuchungen wiederum nachhaltig zu neuen Kultivierungsuntersuchungen angeregt. Dabei sind wesentliche Fortschritte in der Kultivierung besonders abundanter phylogenetischer Gruppen sowie neuer physiologischer Typen von Mikroorganismen erzielt worden

(Leadbetter, 2003). Die gewählten Ansatzpunkte lassen sich generell in drei verschiedene Kategorien einteilen oder stellen Kombinationen dieser Kategorien dar.

Zum einen hat die Nutzung molekularbiologischer Untersuchungsmethoden wesentlich zur gezielten Anreicherung und Isolierung neuer phylogenetischer Gruppen beigetragen. Entgegen ihrer physiologischen Vielfalt sind Mikroorganismen morphologisch oft schwer zu differenzieren. PCR- oder FISH-basierte Analysen von Anreicherungen haben ermöglicht, einzelne phylogenetische Gruppen zu identifizieren und zu selektieren. Der erste erfolgreiche Bericht über die Nutzung einer solchen Strategie war die Isolierung neuer hyperthermophiler Archaeen, die zuvor durch 16S rRNA-Gen Analysen identifiziert wurden (Huber *et al.*, 1995). Auf ähnliche Weise wurde später auch das schon erwähnte Isolat aus der SAR11-Gruppe der Gammaproteobakterien gewonnen (Rappé *et al.*, 2002). Weitere Beispiele für die erfolgreiche Anwendung dieser Strategie sind die Isolierung eines Archaeons aus der Gruppe der marinen *Crenarcheota* (Könneke *et al.*, 2005) und neuer bisher unkultivierter Bodenbakterien aus den bakteriellen Phyla *Acidobacteria* und *Verrumicrobia* (Chin *et al.*, 1999; Janssen *et al.*, 2002; Stevenson *et al.*, 2004) sowie die stabile Anreicherung von Organismen aus dem bisher unkultivierten bakteriellen Phylum TM7 (Hugenholtz *et al.*, 2001).

Ein zweiter Ansatzpunkt zur Kultivierung neuer Mikroorganismen ist die gezielte Suche nach neuen mikrobiell katalysierten Prozessen, Energiestoffwechselwegen und physiologischen Eigenschaften, die entweder durch chemische Messungen in der Umwelt, durch thermodynamische Berechnungen oder andere Beobachtungen postuliert werden können. Dieser Ansatz ist unabhängig von der ersten Strategie und mit sehr unterschiedlichen Versuchen zur selektiven Anreicherung von neuen Organismen verbunden, wird aber häufig mit phylogenetischen Analysen kombiniert. Beispiele für diese Herangehensweise sind viele in den vergangenen Jahren neu entdeckte mikrobielle Energiestoffwechsel, wie die mikrobielle Reduktion von Metallen, z. B. Eisen (Lovley & Phillips, 1988), Mangan (Lovley & Phillips, 1988; Myers & Nealson, 1988), Vanadium (Ortiz-Bernad *et al.*, 2004) und Uran (Lovley, 1993), die Reduktion von halogenierten Verbindungen (Coates *et al.*, 1999) und Trinitrotoluol (TNT, Esteve-Núñez *et al.*, 2000) sowie die anaerobe nitrat- oder nitritabhängige Oxidation von Methan (Raghoebarsing *et al.*, 2006), Ammonium (Van de Graaf *et al.*, 1995), Eisen (Straub *et al.*, 1996), Phosphonat (Schink & Friedrich, 2000), Arsenit (Santini *et al.*, 2000), Huminstoffen (Coates *et al.*, 2002) und Benzol (Coates *et al.*, 2001).

Ein dritter Ansatzpunkt besteht in der Verbesserung oder Neuentwicklung von Kultivierungsmethoden, um den Kultivierungserfolg prinzipiell zu verbessern. Derartige

Studien versuchen ein differenziertes Verständnis der Wachstumsphysiologie von Mikroorganismen und der natürlichen Bedingungen in der Umwelt zu nutzen, um besser an die natürlichen Bedingungen angepasste Kultivierungsbedingungen zu entwickeln und mit der phylogenetischen Analyse von Anreicherungen zu kombinieren.

Neue methodische Entwicklungen in diesem Sinne sind beispielsweise die physikalische Separation mariner Bakterien durch Einbettung in Agar-Mikrokapseln, die anschließend im Durchfluss von natürlichem Meerwasser inkubiert und mittels Durchfluss-Zytometrie isoliert werden (Zengler *et al.*, 2002). Auf diese Weise konnte ein breites Spektrum an bisher unkultivierten Bakterien der Phyla *Proteobacteria, Planctomycetes* und *Bacteroidetes* zum Wachstum *in vitro* stimuliert werden. Vergleichbare Beispiele sind die Weichagar-Einbettung von Mikroorganismen aus Wattsedimenten in Diffusionskammern, die nach Inkubation mit Ursprungssediment in Laboraquarien vereinzelt und isoliert werden können, sowie die Agar-Einbettung intakter Sedimentstücke in Substratgradienten. Durch beide Methoden konnte die Diversität neuer Isolate deutlich erhöht werden (Kaeberlein *et al.*, 2002; Köpke *et al.*, 2005).

Andere erfolgreiche Studien basieren auf der sensitiven Detektion von geringem Wachstum in Flüssigkultur durch Fluoreszenzmikroskopie oder Durchfluss-Zytometrie (Connon & Giovannoni, 2002; Rappé *et al.*, 2002; Könneke *et al.*, 2005; Schut *et al.*, 1993) bzw. auf Agarplatten durch sensitive mikroskopische Untersuchung von Anreicherungen nach langen Inkubationszeiten (Chin *et al.*, 1999; Stevenson *et al.*, 2004). Darüber hinaus spielt die Variation der Zusammensetzung synthetischer Medien eine wesentliche Rolle. In diesem Zusammenhang sind z. B. die Verbesserung der Kultivierungserfolge von natürlichem Bakterioplankton durch Herabsetzung der Substratkonzentrationen (Connon & Giovannoni, 2002; Köpke *et al.*, 2005; Schut *et al.*, 1993; Süß *et al.*, 2004), Zusatz von mikrobiellen Signalstoffen wie zyklisches Adenosinmonophosphat (cAMP) oder Homoserinlaktone (HSL) (Bruns *et al.*, 2002; Bruns *et al.*, 2003; Stevenson *et al.*, 2004), oder die Anhebung des CO_2-Partialdrucks in der Atmosphäre als Beispiele zu nennen (Chin *et al.*, 1999; Stevenson *et al.*, 2004).

1.7 Ziele der vorliegenden Arbeit

Ziel der vorliegenden Arbeit war es, einen Einblick in die Abundanz und Verteilung verschiedener physiologischer Gruppen von organotrophen und lithotrophen Mikroorganismen in der Wassersäule und insbesondere an der Chemokline anoxischer Becken in der zentralen Ostsee zu gewinnen. Von besonderem Interesse war dabei die Frage, welche Bedeutung die verschiedenen Elektronenakzeptoren an der Chemokline für die mikrobielle Mineralisation von organischem Material und der Oxidation anorganischer Verbindungen haben.

Bisher erlauben weder Aktivitätsmessungen noch molekular-phylogenetische Analysen oder mikroskopische Verfahren eine direkte Identifizierung und gleichzeitige Analyse mehrerer verschiedener physiologischer Gruppen von Mikroorganismen. Metagenomische Studien, mit deren Hilfe die Rekonstruktion der notwendigen genetischen Kapazitäten möglich wäre, sind extrem aufwendig und lassen sich nur an einzelnen Proben realisieren. Es war daher wesentliches Ziel der vorliegenden Arbeit, kultivierungsbasierte Verfahren so zu modifizieren und an die natürlichen Bedingungen zu adaptieren, dass sie zum einen eine selektive Anreicherung einzelner physiologischer Gruppen von Mikroorganismen erlauben und zum anderen einen größtmöglichen Anteil der mikrobiellen Gemeinschaft zum Wachstum *in vitro* stimulieren können. Durch eine anschließende chemische und molekularbiologische Analyse der Anreicherungen sollte dann die spezifische Aktivität nachgewiesen und die phylogenetische Zugehörigkeit der angereicherten Organismen festgestellt werden. Chemische Gradienten, Lebendzellzahlen und phylogenetische Analysen sollten schließlich zu einer ersten Einschätzung der Bedeutung der einzelnen physiologischen Gruppen für die verschiedenen Umsetzungsprozesse an der Chemokline genutzt werden.

Teilprojekt 1:

In den vergangenen Jahren haben verschiedene Untersuchungen gezeigt, dass neue Isolate von im Meerwasser häufig vorkommenden Phylotypen nicht zu hohen Zelldichten heranwachsen (Könneke *et al.*, 2005; Rappé *et al.*, 2002; Schut *et al.*, 1993). Zur Identifizierung von Wachstum sind daher empfindliche Methoden nötig. Bisher war dies nur durch aufwendige mikroskopische Verfahren möglich (z. B. Connon & Giovannoni, 2002). Das wesentliche Ziel dieses Teilprojektes bestand daher darin, ein einfaches Verfahren zu entwickeln, dass einen sensitiven Nachweis von mikrobiellem Wachstum nicht nur in partikelarmen oxischen Proben, sondern auch in schwer filtrierbaren eisensulfid- oder

manganoxidhaltigen Anreicherungen anaerober Bakterien ermöglicht. Dieses Verfahren sollte die Grundlage für die nachfolgenden Analysen an einer großen Zahl von selektiven Anreicherungen darstellen.

Teilprojekt 2:

Ziel dieses Teilprojektes war es, einen Einblick in die Verteilung aerober und anaerober Mikroorganismen entlang der chemischen Gradienten an der Chemokline des Gotland Beckens und des Fårö Tiefs zu gewinnen. Durch die Oxidation des sulfidischen Tiefenwassers im Gotland Becken und der nachfolgenden Neubildung der Chemokline im Jahr 2003 ergab sich zudem die Möglichkeit, die mikrobiellen Gemeinschaften vor und nach der Reoxidation des Tiefenwassers zu vergleichen. Dazu wurden die chemischen Gradienten an der oxisch-anoxischen Grenzschicht beider Becken untersucht und Wassertiefen für die Analyse der mikrobiellen Gemeinschaften ausgewählt. Mit Hilfe des *Most Probable Number* (MPN)-Verfahrens wurden Lebendzellzahlen von Mikroorganismen aus den unterschiedlichen Wasserschichten und der Chemokline mit verschiedenen Elektronenakzeptoren bestimmt. Anhand der Ergebnisse wurde die Verteilung von aeroben und anaeroben Mikroorganismen in beiden Becken sowie der Einfluss von Durchmischung und Oxidation des sulfidischen Tiefenwassers auf Mikroorganismen an der Chemokline beurteilt.

Teilprojekt 3:

Basierend auf den in Teilprojekt 2 festgestellten chemischen Gradienten, Lebendzellzahlen und CO_2-Fixierung lithotropher Mikroorganismen an der Chemokline des Gotland Beckens, ergab sich die Frage, welche Energiestoffwechselwege von diesen Mikroorganismen genutzt werden. Ziel dieses Teilprojektes war es daher, die *in situ* CO_2-Fixierungsaktivität und die Verteilung von potentiellen Elektronendonatoren (Schwefelwasserstoff und Ammonium), Elektronenakzeptoren (Sauerstoff, Nitrat, Manganoxid) sowie von intermediären Schwefelverbindungen (Elementarschwefel, Thiosulfat und Sulfit) hochauflösend zu untersuchen. Durch Stimulationsexperimente sollten darüber hinaus Anhaltspunkte über die limitierenden Substrate lithoautotropher Mikroorganismen gewonnen werden. Basierend auf diesen Analysen sollten diffusive Flüsse von Elektronendonatoren und -akzeptoren berechnet und potentielle Energiestoffwechselwege abgeleitet werden.

Teilprojekt 4:

Ziel dieses Teilprojektes war es, parallel zu den Analysen in Teilprojekt 3, organotrophe und lithotrophe Mikroorganismen mit Hilfe des MPN-Verfahrens anzureichern. Dabei wurde das Spektrum der Anreicherungen erweitert und ein Schwerpunkt auf die Anreicherung aerober und anaerober Schwefeloxidierer gelegt. Mit Hilfe chemischer und molekularbiologischer Analysen wurden die Aktivität und die phylogenetische Zugehörigkeit einzelner Anreicherungen untersucht. Die Ergebnisse wurden abschließend zu einer Einschätzung der Rolle verschiedener physiologischer Mikroorganismentypen für die Stoffkreisläufe an der Chemokline in der Wassersäule des Gotland Beckens genutzt.

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2. Publikationen

2.1 Sensitive Determination of Microbial Growth by Nucleic Acid Staining in Aqueous Suspension

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Sensitive Determination of Microbial Growth by Nucleic Acid Staining in Aqueous Suspension

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The determination of cell numbers or biomass in laboratory cultures or environmental samples is usually based on turbidity measurements, viable counts, biochemical determinations (e.g., protein and lipid measurements), microscopic counting, or recently, flow cytometric analysis. In the present study, we developed a novel procedure for the sensitive quantification of microbial cells in cultures and most-probable-number series. The assay combines fluorescent nucleic acid staining and subsequent fluorescence measurement in suspension. Six different fluorescent dyes (acridine orange, DAPI [4′,6′-diamidino-2-phenylindole], ethidium bromide, PicoGreen, and SYBR green I and II) were evaluated. SYBR green I was found to be the most sensitive dye and allowed the quantification of 50,000 to up to 1.5×10^8 *Escherichia coli* cells per ml sample. The rapid staining procedure was robust against interference from rRNA, sample fixation by the addition of glutaric dialdehyde, and reducing agents such as sodium dithionite, sodium sulfide, and ferrous sulfide. It worked well with phylogenetically distant bacterial and archaeal strains. Excellent agreement with optical density measurements of cell increases was achieved during growth experiments performed with aerobic and sulfate-reducing bacteria. The assay offers a time-saving, more sensitive alternative to epifluorescence microscopy analysis of most-probable-number dilution series. This method simplifies the quantification of microbial cells in pure cultures as well as enrichments and is particularly suited for low cell densities.

The quantification of microbial cells in pure cultures, enrichments, and environmental samples is a key measurement in microbiology, ecology, and biotechnology. Several methods are currently applied to obtain direct (light and epifluorescence microscopy and flow cytometry) or indirect (turbidimetry, nephelometry, and biochemical determinations [17]) measures of cell density or biomass. These methods vary considerably regarding their sensitivities and time requirements. While biomass determination by turbidimetric measurement is fast and easy to perform, and therefore still frequently used, its sensitivity is rather low, and it is susceptible to interference, for example, by precipitates or cell aggregate formation. Microscopic measures, in turn, are more sensitive, but particularly in the case of epifluorescence microscopy, are more time-consuming, require some experience, and may be influenced by biases between individuals. The application of flow cytometry is an emerging technique with a high sensitivity that simplifies the counting procedure, but it needs careful setup, and the equipment is not generally available (16, 18).

Cultivation-based estimates such as plate counts and most probable numbers (MPN) are widely used, since they offer the opportunity to target particular physiological groups (e.g., sulfate reducers) and can serve as a source for the isolation of pure cultures (12, 28, 30). However, some bacterial groups exhibit little growth, such as chemolithoautotrophic ammonia oxidizers (5) and oligocarbophilic marine bacteria (11), and thus are hardly analyzable by simple turbidimetric measurements. Therefore, following the growth of these organisms requires the use of fluorescence microscopy, flow cytometry, or chemical or activity measurements (1, 5, 12, 27).

During balanced growth of microbial cultures, every cell component is supposed to change at the same rate (23). Accordingly, the culture biomass and its particular constituents, such as proteins and nucleic acids, will occur at constant ratios, although for a single cell the biomass and nucleic acid content may vary significantly during the cell cycle (6, 23). Therefore, the increase in cellular constituents such as proteins or nucleic acids is supposed to serve as a reliable marker of biomass increase during balanced microbial growth. In recent years, the determination of cellular nucleic acid contents by fluorescent staining and flow cytometry has been successfully applied (10, 16). Hence, the determination of bulk nucleic acid content in microbial cultures is likely to provide a reliable and sensitive tool for the determination of microbial growth. However, little research has focused in this direction, although fluorescence measurements have already been applied for determinations of microbial cell numbers in samples from soils, sediments (34), and pelagic environments (14, 31).

The aim of the present study was to develop a sensitive method for the quantification of microbial cells in pure and enrichment cultures that is particularly suitable for low cell densities. A simple protocol combining nucleic acid staining of fixed or untreated cultures or enrichments and subsequent measurement of fluorescence emission was developed and applied to growth experiments and most-probable-number dilution series.

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MATERIALS AND METHODS

Nucleic acid dyes and staining procedures. Stock solutions (10 mg ml^{-1}) of acridine orange (3,6-bis[dimethylamino]acridine hydrochloride) and DAPI (4′,6′-diamidino-2-phenylindole dihydrochloride) were freshly prepared from

TABLE 1. Bacterial and archaeal strains used for the present study

Domain	Phylum	Strain	Cultivation conditions and substrate			Fluorescence/OD ratio[d]	
			Medium (oxygen status)	Temp (°C)	Substrate (concn)	Fresh cells	Fixed cells
Bacteria	*Alphaproteobacteria*	*Ruegeria algicola* ATCC 51440^T	Marine (oxic)	20	Glucose (10 mM)	8.06 ± 3.52	5.09 ± 1.55
	Gammaproteobacteria	*Shewanella baltica* DSM 9439^T	Brackish (oxic)	20	Monomer mix[a]	3.40 ± 1.34	3.39 ± 1.20
		Oceanospirillum sp. strain GM1[b]	Brackish (oxic)	20	Acetate (100 μM)	ND[c]	3.43 ± 1.15
	Deltaproteobacteria	*Desulfovibrio acrylicus* DSM 10141^T	Marine (anoxic/FeS)	30	Lactate (20 mM)	4.29 ± 0.40	NA[c]
	Epsilonproteobacteria	*Arcobacter* sp. strain Na105[b]	Marine (oxic)	20	Monomer mix[a]	3.02 ± 1.35	1.49 ± 0.41
	"*Firmicutes*"	*Desulfosporosinus orientis* DSM 765^T	Freshwater (anoxic/dithionite)	28	Lactate (10 mM)	5.23 ± 1.20	8.16 ± 2.14
	"*Bacteroidetes*"	*Muricauda ruestringensis* DSM 13258^T	Marine (oxic)	20	Mannose (10 mM)	6.93 ± 0.82	11.6 ± 3.79
Archaea	*Euryarchaeota*	*Methanospirillum hungatei* DSM 864^T	Freshwater (anoxic/sulfide)	30	Acetate (10 mM), H_2	8.31	9.66
		Methanosarcina barkeri DSM 800^T	Freshwater (anoxic/sulfide)	30	Acetate (10 mM), H_2	7.94	9.22

[a] According to the work of Süß et al. (30).
[b] Sass and Martens-Habbena (unpublished).
[c] NA, was not fixed because of a possible reaction of aldehydes with sulfide. ND, not done.
[d] Data are means ± standard deviations.

crystalline powder by dissolving the powder in double-distilled and sterilely filtered (0.2 μm) water and were stored at 4°C. Stock solutions of ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide; 10 mg ml^{-1}) were prepared in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and subsequently sterilely filtered and stored at 4°C. Stock solutions of SYBR green I and II as well as of PicoGreen were purchased from Molecular Probes (Eugene, OR), subdivided into small aliquots upon receipt, and stored in 1.5-ml reaction tubes at −20°C. All other dyes were obtained from Sigma (Deisenhofen, Germany).

Working solutions of dyes were freshly prepared each day at five times the desired final assay concentrations in sterile plastic petri dishes. Working solutions of DAPI were prepared in sterilely filtered phosphate-buffered saline (PBS) (8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per liter; pH 7.2). All other working solutions were prepared in sterilely filtered TE buffer concentrate (200 mM Tris-HCl, 50 mM sodium EDTA, pH 8.0). Unless otherwise stated, staining experiments were performed with 200 μl of sample plus 50 μl of dye working solution in black untreated 96-well microplates (Nunc 237108; VWR International, Darmstadt, Germany).

Comparison of nucleic acid dyes. The applicability of nucleic acid dyes was tested with suspended *Escherichia coli* cells. Therefore, *E. coli* K-12 was cultured aerobically in liquid Luria-Bertani broth (17) at 37°C on a rotary shaker. Early-stationary-phase cultures were harvested by centrifugation for 20 min at 10,000 × *g* (Beckman J2-HS centrifuge with a JA20 rotor). Cells were washed, resuspended in sterilely filtered (0.2-μm) vitamin-free freshwater medium (see below) to a final cell density of approximately 2×10^9 cells per ml, and stored at 4°C. The cell suspension or cell-free medium was dispensed into black microplates, stained by the addition of dye working solution, incubated at room temperature in the dark, and subsequently analyzed by fluorescence measurement (see below). For dilution series of *E. coli*, the cell numbers were determined exactly by epifluorescence microscopy after SYBR green I staining and checked again after fluorescence measurements. For pH optimization of each dye, the respective dye working solution was adjusted to the desired pH by the addition of HCl or NaOH prior to use. Because of the small sample volume, the final pHs in the assays were checked with narrow-range pH paper (CS series; Whatman, Maidstone, United Kingdom), and they deviated no more than 0.1 pH units.

Nucleic acid quantification. For DNA quantification, a λ phage DNA standard (100 μg ml^{-1}; Molecular Probes, Eugene, OR) was diluted 100-fold in sterilely filtered TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). Aliquots were dispensed into black microplates and brought up with TE buffer to a final volume of 200 μl. Staining was performed by the addition of 50 μl SYBR green I working solution (2,000-fold dilution of the stock solution). After 5 minutes of incubation in the dark, the samples were analyzed on a fluorescence microplate reader (see below).

The quantification of rRNAs in the presence of DNA was performed with a λ phage DNA standard and *E. coli* rRNAs (16S and 23S rRNAs at 4 mg ml^{-1}; Roche, Mannheim, Germany). Briefly, RNase-free TE buffer was prepared by incubation in the presence of 0.1% diethyl pyrocarbonate at 37°C overnight and subsequent autoclaving. The DNA standard (200 ng ml^{-1}) and rRNA standard (0 to 1,600 ng ml^{-1}) were prepared with RNase-free TE buffer. One-hundred-microliter aliquots of DNA standard were mixed with 100 μl diluted rRNA standard and 50 μl SYBR green I working solution (2,000-fold dilution of the stock solution in RNase-free concentrated TE buffer) in sterile and RNase-free microplates (Costar 3596; Corning Inc., NY). Fluorescence measurements were performed after 5 minutes of incubation in the dark.

Cultivation of pure cultures. Pure cultures were grown in artificial seawater, brackish water, or freshwater. Artificial seawater contained (in g $liter^{-1}$) NaCl (24.3), $MgCl_2 \cdot 6H_2O$ (10.0), $CaCl_2 \cdot 2H_2O$ (1.5), KCl (0.66), and Na_2SO_4 (4.0). One milliliter each of stock solutions of KBr (0.84 M), H_3BO_3 (0.4 M), $SrCl_2$ (0.15 M), NH_4Cl (0.4 M), KH_2PO_4 (0.04 M), and NaF (0.07 M) was added per liter of medium. Brackish water contained (in g $liter^{-1}$) NaCl (5.5), $MgCl_2 \cdot 6H_2O$ (2.3), $CaCl_2 \cdot 2H_2O$ (0.34), KCl (0.15), and Na_2SO_4 (0.91). A 0.23-ml volume each of stock solutions of KBr (0.84 M), H_3BO_3 (0.4 M), $SrCl_2$ (0.15 M), NH_4Cl (0.4 M), KH_2PO_4 (0.04 M), and NaF (0.07 M) was added per liter of medium. Freshwater medium contained (in g $liter^{-1}$) NaCl (1.0), NH_4Cl (0.3), $MgSO_4 \cdot 7H_2O$ (0.025), $CaCl_2 \cdot 2H_2O$ (0.1), $MgCl_2 \cdot 6H_2O$ (0.4), and KH_2PO_4 (0.6). For cultures of methanogens, a slightly modified freshwater medium was used, which contained (in g $liter^{-1}$) KH_2PO_4 (0.1), NH_4Cl (0.1), NaCl (0.25), KCl (0.1), $MgCl_2 \cdot 6H_2O$ (0.31), $CaCl_2 \cdot 2H_2O$ (0.1), and resazurine (0.5 mg $liter^{-1}$). All oxic media were buffered by the addition of HEPES (2.38 g $liter^{-1}$) and by adjusting the pH to 7.2 with 1 M NaOH. Anoxic media were cooled under a N_2/CO_2 atmosphere (80/20 [vol/vol]) after being autoclaved and were supplemented with 30 ml of a sodium bicarbonate solution (1 M). The pH was adjusted to pH 7.2 with sterile 1 M HCl or NaOH, if necessary. All media received 10 ml of a sterilely filtered vitamin solution (4), 1 ml trace element solution SL10 (36) (for methanogens, SL9 was used [32]), and 0.2 ml of selenite tungstate solution (35) per liter. Anoxic media were reduced by adding separately 1.2 ml of 1 M Na_2S and 0.6 ml 1 M $FeSO_4$ per liter of medium (FeS-reduced medium) or by the addition of a few crystals of sodium dithionite to medium containing resazurine as a redox indicator (dithionite-reduced medium). The pure cultures used in the present study and the respective cultivation conditions are given in Table 1.

Growth experiments. Growth experiments were performed with selected bacterial strains (Table 2). All oxic growth experiments were performed in 250-ml Erlenmeyer flasks on a rotary shaker at 90 rpm. Anoxic growth experiments were performed in 100-ml serum bottles or 100-ml screw-cap bottles. Subsamples (2 ml) were taken aseptically from the growth vessels, and the optical density at 436 nm (OD_{436}) was immediately determined on a spectrophotometer (Shimadzu RF-1501) relative to distilled water. Samples were diluted with growth medium if the optical density exceeded 0.3. Samples were then stored in 2-ml reaction tubes on ice until fluorescence analysis. Parallel samples were fixed by the addition of glutaric dialdehyde (2% final concentration).

Fluorescence analyses of all culture samples were performed in duplicate by the addition of 50 μl SYBR green I working solution (1,000-fold dilution of

TABLE 2. Comparison of growth rates of selected strains

Phylum	Strain	Growth rate μ (d^{-1})[a]		
		OD_{436}	FH	FX
Alphaproteobacteria	*Ruegeria algicola*^{T}	0.15	0.17	0.17
Gammaproteobacteria	*Shewanella baltica*^{T}	0.18	0.22	0.18
	Oceanospirillum sp. strain GM1	0.09	n.d.	0.09
Deltaproteobacteria	*Desulfovibrio acrylicus*^{T}	0.16	0.16	n.d.
Epsilonproteobacteria	*Arcobacter* sp. strain NA105	0.13	0.18	0.16
"*Firmicutes*"	*Desulfosporosinus orientis*^{T}	0.13	0.16	0.15
"*Bacteroidetes*"	*Muricauda ruestringensis*^{T}	0.05	0.05	0.05

[a] Growth rates were calculated from optical density (436 nm) and fluorescence data obtained upon SYBR green I staining of fresh (FH) and glutaric dialdehyde-fixed cells (FX).

stock), incubation in the dark for at least 2 hours, and subsequent analysis on a microplate reader (see below).

Environmental samples and total cell counts (TCC). Water was collected at Buzzards Bay (Woods Hole, MA) in July 2003, approximately 1 km offshore. About 20 liters of water from a 50-cm water depth was used to fill a clean plastic container that had been thoroughly flushed five times with deionized water and once with sample water before being filled. Immediately after return to the laboratory, a 50-ml subsample was transferred aseptically into a 50-ml Falcon tube, fixed with 3 ml 37% formaldehyde, and stored at 4°C in the dark until further processing.

Total cell counts were performed according to the protocol of Noble and Fuhrman (25). Briefly, 1 ml fixed sample was mixed with 100 μl staining solution (SYBR green I; 400-fold dilution of the stock solution) and incubated for 10 min in the dark. A 100-μl aliquot of this mixture was filtered through a black polycarbonate filter (0.2-μm pore size; Millipore, Eschborn, Germany), rinsed with particle-free (0.2-μm sterilely filtered) PBS, air dried, and fixed on a microscopic slide by the addition of 10 μl mounting solution (50% glycerol, 50% PBS, 0.1% *p*-phenylenediamine [Sigma]). Samples were counted using a Zeiss Axioscope microscope equipped with a 100-W mercury vapor lamp and filter set 09 (BP450-490, FT510, and LP515). At least 20 fields and 400 cells were counted.

Most-probable-number dilution series. Most-probable-number dilution series were inoculated with the Buzzards Bay sample immediately after return to the laboratory, using three different media based on oxic artificial seawater (described above) with reduced vitamin contents (0.02 ml $liter^{-1}$ instead of 2 ml $liter^{-1}$). Medium A was supplemented with 70 mg $liter^{-1}$ Bacto peptone, 14 mg $liter^{-1}$ Bacto yeast extract (both from BD Biosciences, San Diego, CA), 1.4 mg $liter^{-1}$ ferric citrate, and 1 ml $liter^{-1}$ substrate mix (glucose, lactose, cellobiose, fructose, glucosamine, sodium salts of acetate, malate, succinate, tartrate, and pyruvate, and amino acids [cysteine, methionine, leucine, proline, glycine, alanine, and phenylalanine] [1 mM each], as well as 0.05% chitin). Medium B received only 1 ml $liter^{-1}$ substrate mix. Medium C received no organic substrates at all.

MPN series were prepared in 2-ml 96-well polypropylene deep-well plates. First, 800 μl of medium was dispensed into each well of the plates, and then 200-μl samples were added as the inoculum to seven wells of the first row. One row of wells served as a control without inoculum. Subsequently, the contents of the first row of wells were diluted fivefold into consecutive wells, creating 12 dilutions. The MPN series were incubated in total for 141 days at 15°C. After 3, 17, and 141 days, 200-μl subsamples from the wells were transferred to black microplates. Fifty microliters of SYBR green I working solution was added to each well. Fluorescence was measured after 2 h of incubation in the dark. Fluorescence measurements of MPN samples after 3 and 17 days of incubation were conducted in a Tecan SPECTRAFluor Plus microplate reader (Tecan GmbH, Gröding, Austria) (excitation, 485 nm; emission, 540 nm). After 141 days, fluorescence measurements were done as described below. The mean fluorescence emission of uninoculated controls was 45.7 relative fluorescence units (RFU), and the standard deviation was 6.1. Growth was scored as positive if the fluorescence emission in the inoculated wells was at least 80 RFU. This value exceeds the average of the controls by at least five times the standard deviation. The rationale for this was that it would detect significant but low growth, as can be expected for assays with little or no substrate addition.

Bias-corrected most probable numbers with approximate Cornish and Fisher confidence limits were calculated using the MPN calculator software described by Klee (22).

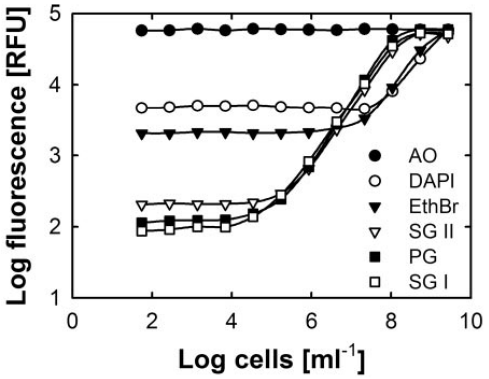

FIG. 1. Comparison of six nucleic acid dyes for the detection of bacterial cells in a microplate assay. Each data point represents the mean of four (PicoGreen) or eight replicates. Standard deviations were omitted for clarity. AO, acridine orange; EthBr, ethidium bromide; PG, PicoGreen; SG II, SYBR green II; SG I, SYBR green I.

Fluorescence measurements. Fluorescence intensities were determined in a microplate reader (Fluostar Optima; BMG Labtechnologies, Offenburg, Germany) at the following excitation/emission wavelengths: 485/520 nm (acridine orange), 360/460 nm (DAPI), 540/590 nm (ethidium bromide), and 485/520 nm (SYBR green dyes and PicoGreen). All measurements were carried out in three reading cycles, with integration of 20 flashes, a 0.5-s delay between plate movement and reading, and 10 s of shaking before each cycle. During comparisons of the different dyes, the gain of the photomultiplier was adjusted for each dye separately so that the fluorescence intensity (FI) of the highest *E. coli* cell density tested (ca. 2×10^9 cells per ml) equaled approximately 60,000 RFU. All subsequent measurements of SYBR green I-stained samples were carried out with a constant detector gain of 1,300 arbitrary units. Unless otherwise stated, stained samples were incubated overnight before fluorescence measurement (see Results).

However, the time dependence of SYBR green I fluorescence emission was analyzed by transferring the microplates to the reader directly after the addition of the SYBR green I working solution. Fluorescence data were acquired over 50 to 200 reading cycles, with integration of 10 flashes. Data points represent the averages of two parallels.

No data manipulation was applied to the raw data, except for subtraction of a blank when noted.

RESULTS

Comparison of nucleic acid dyes. For staining of *E. coli* cells, the optimal final dye concentrations were found to be 2 μg ml^{-1} (ethidium bromide and DAPI), 1:5,000 to 1:10,000 (SYBR green I and II), and 1:400 (PicoGreen). Acridine orange turned out to be unsuitable, since it displayed very high background fluorescence even at very low concentrations (Fig. 1) and did not allow discrimination between a cell-free control and 10^9 *E. coli* cells ml^{-1}. While DAPI worked best in PBS (pH 7.2), the best signal-to-noise ratios for all other dyes were found at pH 8.0 in TE buffer (data not shown). In particular, SYBR green I and II, PicoGreen, and ethidium bromide were found to be pH sensitive. Therefore, working solutions of these dyes (5× final concentration) were prepared at elevated buffer concentrations (200 mM Tris, 50 mM EDTA, pH 8.0) to ensure stable pH conditions even during the analysis of very acid or alkaline samples.

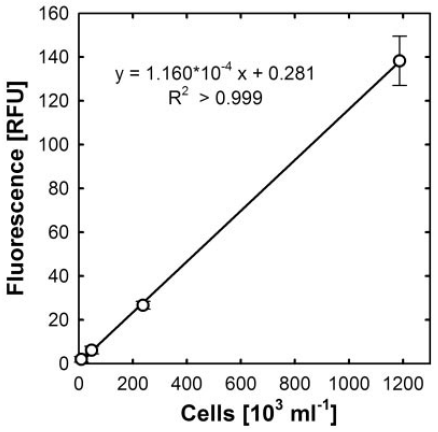

FIG. 2. Quantification of washed *E. coli* cells at low cell densities by SYBR green I staining. Error bars represent standard deviations for eight replicates.

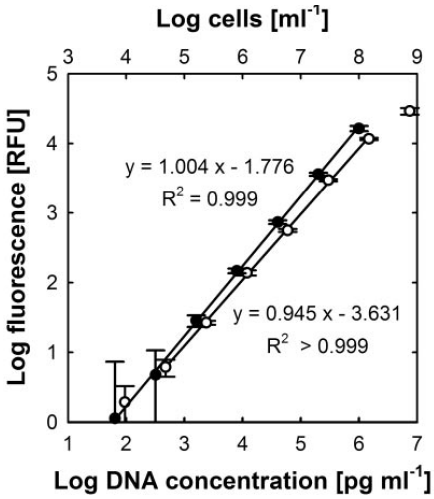

FIG. 3. Quantification of washed *E. coli* cells (open circles) and lambda phage DNA (closed circles) in a microplate assay. Samples were serially diluted, and fluorescence intensities were measured after staining with SYBR green I. Linear regression was calculated from log-transformed data. Error bars represent standard deviations for eight replicates.

The fluorescence yields and background fluorescence differed remarkably among the different dyes. With SYBR green I (1:10,000) or PicoGreen (1:400) in a sample volume of 200 μl, even as few as 10,000 *E. coli* cells per well (equivalent to 50,000 cells ml^{-1}) were unambiguously detected (Fig. 1), while SYBR green II (1:10,000) allowed the detection of 1.7×10^5 *E. coli* cells ml^{-1}. Ethidium bromide and DAPI were far less sensitive and required cell densities of at least 2×10^7 and 10^8 cells ml^{-1}, respectively (Fig. 1). However, further dilution to final concentrations of 1:20,000 (SYBR green dyes) and 1:800 (PicoGreen) did not result in the expected decrease in background fluorescence (data not shown). For further experiments, SYBR green I was chosen, because it is less prone to interference with various chemical compounds than SYBR green II or PicoGreen (20, 37).

Characteristics of quantitative SYBR green I cell staining. Linear and log-transformed calibration curves obtained with washed *E. coli* cells are shown in Fig. 2 and 3. A double logarithmic plot of fluorescence intensities versus cell densities showed a linear correlation ($R^2 > 0.999$) over 4 orders of magnitude, from 50,000 to 2×10^8 cells ml^{-1}, following the equation $FI = (\text{cells ml}^{-1})^{0.945} \times 10^{-3.631}$, indicating that over a broad range of cell numbers the calibration curve follows a power law function. However, at low cell densities, when scattering and quenching of emitted fluorescence can be neglected, a direct linear correlation between cell numbers and fluorescence intensity was observed.

SYBR green I quantification of DNA. Quantification experiments with purified double-stranded DNA were performed to confirm the results of the cell staining experiments described above but also to investigate potential interference by RNA and fixation agents. The increase in fluorescence was linearly correlated ($R^2 > 0.999$) with increases in the DNA concentration from 10^2 to 10^6 pg ml^{-1} DNA, following the equation (Fig. 3) $FI = [\text{DNA}]^{1.004} \times 10^{-1.776}$, for DNA concentrations in pg ml^{-1}.

In contrast to the case for SYBR green I-stained cells, for purified DNA the slope of the regression curve was nearly 1.0 over almost the whole range of DNA concentrations tested. However, the quantification of very low DNA concentrations was limited by the intrinsic background fluorescence, and at DNA concentrations above 10^6 pg ml^{-1}, the amount of SYBR green I in the assays became limiting. This could be circumvented by the use of a dye working solution with a higher SYBR green I concentration. For the experiments presented here, a 1:10,000 dilution of the dye was chosen, since it worked well with the desired range of cell counts per ml. Higher cell numbers were avoided, since fluorescence emission would be negatively influenced by scattering and quenching.

From the plots of fluorescence versus DNA concentration or cell number (lower parts of the curves), it can be estimated that 1 RFU corresponds to approximately 9,250 cells ml^{-1} or 55.5 pg DNA ml^{-1} (Fig. 2 and 3). From these data, a DNA content of 6.0×10^{-3} pg per cell can be inferred. Considering a genome size of 4.75×10^6 bp for *E. coli* K-12 (3) and an average molar weight of 618 g for nucleotides (6), it can be calculated that the *E. coli* K-12 cells used in the present study contained, on average, 1.23 genomes per cell.

The addition of rRNA to DNA-containing assays led to a noticeable increase in fluorescence (Fig. 4). As expected, the increase in fluorescence correlated linearly with the amount of rRNA added. However, RNA yielded much less fluorescence than DNA. Even at the highest rRNA-to-DNA ratio tested in this study (eightfold), the increase in fluorescence compared to that with DNA alone was only by a factor of 0.8.

Influence of sample fixation on quantification of dissolved DNA. At concentrations commonly used for sample fixation, ethanol strongly inhibited fluorescence upon SYBR green I staining. After staining of glutaric dialdehyde- or formalde-

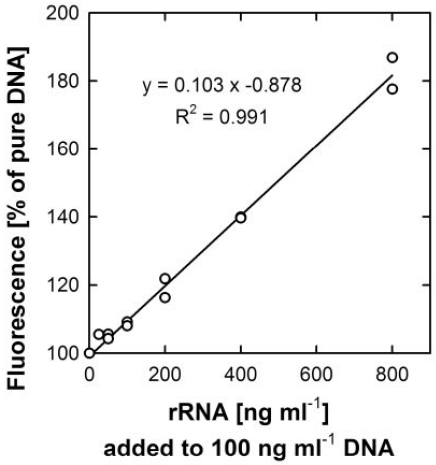

FIG. 4. Interference of rRNA with SYBR green I staining of double-stranded DNA. Increasing amounts of *E. coli* rRNA were added to 100 ng ml^{-1} lambda phage DNA and stained with SYBR green I. The fluorescence was measured and is displayed as a relative increase compared to that of pure DNA (defined as 100%). Linear regression was calculated from two parallel experiments.

hyde-fixed samples, a fixative concentration-dependent decrease in fluorescence emission was also observed but was much weaker than that caused by ethanol. This effect seemed to be independent of the actual DNA concentration (Fig. 5). Up to 2% glutaric dialdehyde did have a minor effect on fluorescence. However, at higher concentrations, the fluorescence decrease seemed to be linearly correlated with the glutaric dialdehyde concentration. With 5% fixative, an approximately 30% reduction in fluorescence was observed (Fig. 5). Overall, formaldehyde had a significantly stronger effect than glutaric dialdehyde. While 0.25 or 0.5% (vol/vol) formaldehyde had no effect on fluorescence, concentrations of 1% and 2% already resulted in 20% and 80% reductions in fluorescence, respectively. However, it was critical to measure the fluorescence of formaldehyde- or glutaric dialdehyde-fixed DNA samples within 10 minutes of dye addition, since a longer incubation regularly caused a further decrease in the fluorescence signal. After several hours of incubation, glutaric dialdehyde-fixed DNA samples stained with SYBR green I developed a visible yellowish color.

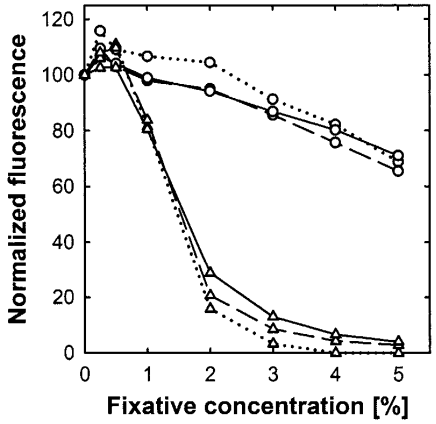

FIG. 5. Effects of different glutaric dialdehyde (circles) and formaldehyde (triangles) concentrations on fluorescence emission of SYBR green I-stained DNA. Different DNA concentrations (dotted lines, 10 ng ml^{-1}; dashed lines, 100 ng ml^{-1}; solid lines, 1,000 ng ml^{-1}) were incubated with fixative for 1 hour prior to staining. The resulting fluorescence was recorded and is displayed as a relative decrease compared to that of pure DNA (defined as 100%). Each data point represents the mean of three replicates. Standard deviations were negligible and were omitted for clarity.

SYBR green I staining of viable and glutaric dialdehyde-fixed cells. Fresh as well as glutaric dialdehyde-fixed cultures could be stained with SYBR green I, with similar fluorescence intensities, but they had different response times. Fresh (stained directly after sampling or stored on ice for some hours) cells needed several hours to reach stable fluorescence signals after the addition of dye, and it was advisable to incubate samples at least overnight or for approximately 20 h. Fixed samples attained stable signals after a minimum of 4 hours of incubation, while fluorescence could already be measured with sufficient accuracy after 2 hours. In contrast to the case with fresh samples, fluorescence in fixed samples should be measured within a maximum of 4 to 5 hours after the addition of dye, since glutaric dialdehyde-fixed cell suspensions developed a visible yellowish color after continued incubation, accompanied by an increasingly fluorescent background. However, in contrast to the case with glutaric dialdehyde-treated DNA samples, fluorescence levels in fixed cell suspensions did not decrease and remained constant for at least 6 hours. Controls without the addition of DNA or cells, however, also turned slightly yellowish with time, indicating an interaction of dye, buffer, and glutaric dialdehyde.

Growth experiments. Growth experiments were performed with several bacterial strains representing different physiological types, including aerobes and sulfate reducers. As an example, growth curves of aerobic bacteria growing in rich (panel A) and substrate-poor (panel C) media as well as of sulfate-reducing bacteria in ferrous sulfide-reduced medium (panel B) are shown in Fig. 6. Growth was monitored by both optical density and fluorescence measurements after SYBR green I staining. The latter was performed for comparison with glutaric dialdehyde-fixed cells and cells that were stored on ice between sampling and staining (fresh cells) but yielded no significant differences. Generally, both approaches (optical density and fluorescence determinations) showed similar time courses (Fig. 6) and consequently resulted in almost the same growth rates (Table 2). Minor deviations were observed only during the late lag and stationary phases. During late lag phase, some cultures, such as *Oceanospirillum* sp. strain GM1, already exhibited increased fluorescence while the turbidity remained stagnant, whereas in some stationary-phase cultures (Fig. 6C) the fluorescence started to decline earlier than the optical density.

The ratio of fluorescence to optical density was calculated separately for each strain. For all cultures (Table 1), this ratio remained almost constant, even during the different growth

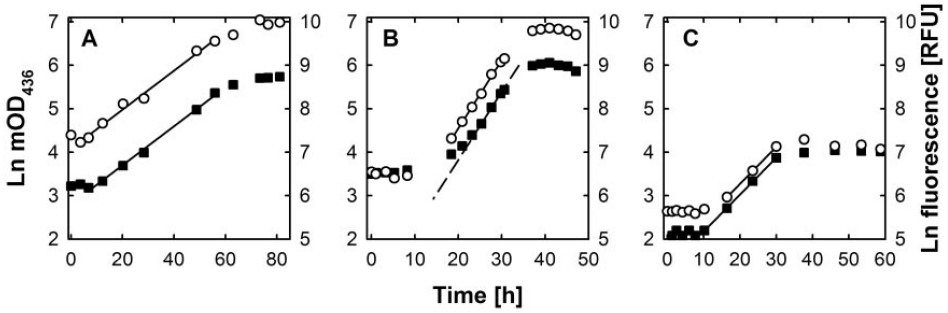

FIG. 6. Growth curves of *Muricauda ruestringensis* (A), *Desulfovibrio acrylicus* (B), and *Oceanospirillum* sp. strain GM1 (C) obtained by determining the optical density at 436 nm (filled squares) and by fluorescence measurement after SYBR green I staining (circles). The resulting growth rates of the strains in panels A to C and the other strains tested are given in Table 2.

phases. However, pronounced differences were found between the different strains, ranging from 1.5×10^4 (*Arcobacter* sp. strain NA105) to 11.6×10^4 (*Muricauda ruestringensis*^{T}) RFU OD^{-1}, and in some cases, between fresh and glutaric dialdehyde-fixed samples of a single strain (Table 1).

Most-probable-number dilution series. MPN series were monitored for growth after 3, 17, and 141 days (Fig. 7). Among the different series, pronounced differences were observed that could be easily linked to nutrient concentrations. Medium A, with the highest nutrient additions, stimulated the fastest growth, already reaching maximum cell numbers, as indicated by fluorescence, after 17 days. Growth on medium B commenced later and resulted in lower cell numbers than that on medium A. Surprisingly strong growth was obtained even without substrate addition (medium C). According to the DNA calibration curves (Fig. 3), the maximum fluorescence levels of 10,000, 1,000, and 700 RFU achieved with the different media correspond to DNA concentrations of approximately 500, 50, and 38 ng ml^{-1}, respectively. Assuming a DNA content of 5×10^{-3} pg DNA per cell (6), this would represent 10^8, 10^7, and 7.5×10^6 cells ml^{-1}, respectively. With a total cell count of 9.8×10^5 cells ml^{-1} in the original sample, an inoculum size of approximately 200,000 cells in the first dilution can be estimated, which would amount to a fluorescence of about 20 RFU. The values observed after 3 days even with the substrate-free series were clearly higher, demonstrating cell reproduction.

For evaluation of the fluorescence data, single wells of the MPN plates were checked by microscopy as well. Cells were never found in wells shown to be negative by SYBR green I staining. Because of their high sensitivity, fluorescence measurements revealed positive scores in cases when direct microscopic observation remained questionable and would require filtering of a larger volume of sample. The MPN count obtained with medium A was 42,000 cells ml^{-1} (4.3% of the TCC), while those with medium B and C reached 610 cells ml^{-1} (0.06% of the TCC) and 153 cells ml^{-1} (0.016% of the TCC), respectively.

DISCUSSION

A novel assay for the detection and quantification of microbial cells in aqueous suspension was developed. The described assay is simple, sensitive, and fast to perform, allowing a reliable quantification of cells in pure culture experiments as well as estimations of microbial biomass in enrichment procedures or MPN series. In combination with molecular phylogenetic analysis of successful enrichments, the selection of promising enrichments for subsequent isolation procedures is made easier. In addition, this assay can facilitate physiological investigations of strains which do not exhibit high growth yields, e.g., oligocarbophilic or strictly lithotrophic microorganisms.

Comparison of nucleic acid dyes. For the detection of microbial cells in suspension, fluorescent nucleic acid dyes were chosen because they stain any type of microbial cell (14, 25, 26, 34), irrespective of phylogeny or physiological capacities. However, the different dyes were found to vary remarkably in terms of sensitivity and the range of cell numbers they can detect. Cyanine dyes (PicoGreen and SYBR green) that are commonly employed for the quantification of dissolved DNA (20, 33, 37) were the most sensitive and allowed reliable quantification of microbial cells. Consistent with this, PicoGreen has previously been used for the determination of microbial biomass in environmental samples (14, 31). However, it is sensitive to interference from a broad range of organic compounds and salt concentrations present in seawater (20). Similarly, cations can impair the SYBR green I fluorescence yield. However, this effect can be minimized by maintaining a clear molar dye surplus. Zipper et al. (37) estimated an approximate concentration of 10 mM in the bulk stock solution of SYBR green I and discovered that at molar-dye-to-base-pair ratios higher than 10:1 (mol:mol nucleotide), this effect was negligible. Thus, with a 1:10,000 dilution of the SYBR green I stock, as used in the present study, DNA concentrations of up to 100 nM base pairs, corresponding to 1.2×10^7 *E. coli* cells per ml, can be expected to be determined free of interference. However, using freshwater media, even up to 1.5×10^8 *E. coli* cells per ml could reliably be determined (Fig. 3). For marine media or higher cell densities, a narrower working range is expected, but for these applications dye working solutions with a higher SYBR green I concentration can be prepared.

In addition, SYBR green I has been reported to exhibit some inherent limitations that are commonly found with cyanine dyes, as its binding behavior to double-stranded DNA depends on the GC content, fragment size, and conformation

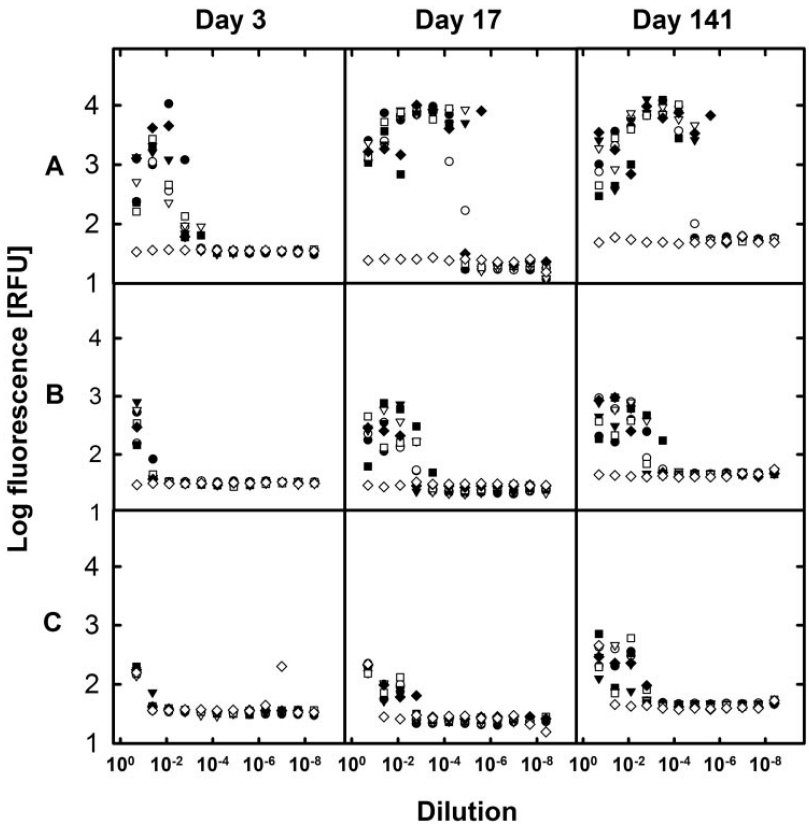

FIG. 7. Analysis of most-probable-number dilution series inoculated with water from Buzzards Bay. MPN series were set up in 96-well plates using three different media with different substrate additions. To 800 μl medium in the first dilution, 200 μl of sample was added and consecutively diluted into the following 11 wells. Subsamples were taken after 3, 17, and 141 days of incubation and were analyzed by SYBR green I staining and fluorescence determination. Different symbols represent seven parallel dilutions, and open diamonds represent uninoculated controls (by accident, the least diluted control of series C was inoculated but not further diluted). Medium A (top) contained peptone, yeast extract, and a substrate mix, while medium B (middle) received only the substrate mix and medium C (bottom) received no organic substrates.

of DNA (33, 37). These effects were not apparent, however, in the present study and seemed not to impair the applicability of SYBR green I for sensitive biomass determinations. A much more important effect was the sensitivity of SYBR green I stock solution to multiple freeze-thaw cycles, which was avoided by subdivision into small aliquots upon receipt (20, 24).

Possible constraints by fluorescent compounds. The use of nucleic acid stains for the measurement of fluorescence in aqueous cell suspensions is possible only if concentrations of interfering compounds emitting autofluorescence upon excitation are negligible. This effect can easily be recognized by fluorescence measurement prior to nucleic acid staining. Examples of fluorescent compounds are aromatics that may be present in considerable concentrations in culture media (e.g., amino acids or vitamins) but also compounds present in the inoculum, such as humic acids (34, 38). The latter, however, would probably be diluted out during the MPN procedure but still might interfere if only low cell growth occurs or even result in false-positive results for the low dilutions. Interference by autofluorescence of the medium, however, can be circumvented by separating cells from the medium by filtration prior to analysis. No loss of accuracy was found if samples were filtered onto black polycarbonate membranes and subjected to fluorescence measurements (data not shown).

Interference with ribosome content and sample fixation. rRNA is the most abundant nucleic acid in bacterial cells and in *E. coli* can surpass the DNA content by a factor of 11.5 (6). It is therefore likely to interfere with the quantification of microbial cells by nucleic acid staining. However, the fluorescence yield of rRNA was only 10% that of DNA (Fig. 4), confirming the results obtained for single-stranded DNA by Zipper et al. (37) and indicating that even in extremely fast-growing *E. coli* cells, rRNA might only double the fluorescence yield. Nevertheless, for growth rate determinations the fluorescence of rRNA can be neglected, since during exponential growth rRNA contents are supposed not to change dramatically (6, 9). A slight influence might be seen only during the lag

phase, when ribosome contents are built up, and during stationary phase, when cells reduce their cellular rRNA contents, like the case for *Oceanospirillum* sp. strain GM1 in Fig. 6. However, this effect is too weak to impede even the determination of cell numbers from fluorescence data. The present approach was particularly intended for the quantification of environmental isolates or chemolithoautotrophs that are slow growing and reach only low cell densities. These organisms are generally characterized by lower rRNA-to-DNA ratios, and therefore RNA interference should be of minor importance.

In many cases, an immediate analysis of cultures after sampling is not possible, and therefore sample fixation is required. Of the typical preservation protocols involving formaldehyde, glutaric dialdehyde, or ethanol, glutaric dialdehyde was most recommendable and could be used at final concentrations of up to 2%. Formaldehyde can be used as an alternative to glutaric dialdehyde, for example, for fluorescence in situ hybridization or flow cytometry (10), but must not be present at final concentrations exceeding 0.5%. Ethanol was found to be completely incompatible with SYBR green I staining. Since ethanol leads to a quantitative secession of SYBR green I from the DNA (20), it can be expected that it in turn also inhibits binding of the dye to DNA.

Alternatively, storing samples on ice until processing, such as during growth experiments, may be considered, since it avoids side reactions, but it requires several hours of incubation (for example, overnight) to achieve stable fluorescence signals. An advantage of this method, however, is that samples can serve subsequently for other purposes, e.g., nucleic acid extraction.

Biomass determination during growth experiments. Classical growth experiments typically rely on measurements of optical density, or in some cases, on microscopic counting. Counting is time-consuming and often associated with large standard deviations. Optical density, however, was found to be in good agreement with culture biomass for single bacterial types (9, 23), but there might be variations depending on the growth phase (6). Optical densities obtained for different organisms can hardly be compared, since cell size and shape vary between different species and strongly influence the optical density (19). This may be reflected by the variation in the FI/OD ratios found among the different strains in the present study (Table 1). DNA concentrations in prokaryotic cultures are directly dependent on cell numbers and are related to the growth phase (6). Each cell contains at least one and normally not more than two genomes, but genome sizes may vary from approximately 1 to 12 Mbp per cell among different prokaryotic taxa (15). However, the majority of bacterial and archaeal species seem to contain in the range of 1.5 to 5 Mbp (15). Similarly, little variation in DNA contents per cell (approximately 2.5 fg per cell) was found for aquatic prokaryotes and appeared to be independent of the phylogenetic position of the single cells (10, 29). Therefore, it seems that apart from exceptions with very large genomes, DNA fluorescence upon SYBR green I staining allows a better comparison of different strains than optical density.

Measuring the optical density is also hampered by cells forming chains, filaments, clumps, or aggregates or by the presence of particles (e.g., FeS in cultures of sulfate-reducing bacteria). It is also reliable over only 1 to 2 orders of magnitude (OD, ~0.01 to 0.3). At lower densities, no reliable signal is obtained, while at higher densities the OD deviates from linearity and cultures have to be diluted. Similarly, SYBR green I fluorescence may be scattered and quenched at very high cell densities. However, it offers a lower detection limit (by 1 to 2 orders of magnitude) and reliable signals over 4 to 5 orders of magnitude (Fig. 1 and 3). It therefore allows growth detection even in substrate-limited cultures (Fig. 6C) that are hardly or not analyzable by simple optical density determination.

Particles seem to affect fluorescence determinations less than they affect optical density measurements. During growth experiments with the sulfate-reducing bacterium *Desulfovibrio acrylicus*, OD measurements resulted in a rather sigmoid growth curve (Fig. 6B). A reliable determination of the growth rate was possible only several hours after exponential growth commenced, as could be seen by comparison with the plot of fluorescence data. Similarly, it is to be expected that fluorescence delivers more reliable data for filamentous cultures, clumping cultures, etc. OD measurements rely on the relative surface area of the cell and a homogenous distribution of cells in the sample. Fluorescence determination, in contrast, depends on the DNA (and RNA) content of the cells, and since normally the whole sample is scanned and integrated, the allocation of the cells within the sample is of minor importance.

Most-probable-number enrichments. Similar to dilution-to-extinction methods (11, 13), which are generally applied to aquatic habitats, during MPN analysis samples are serially diluted. Therefore, MPN series offer the opportunity to enrich potentially abundant cells from the highest positive dilutions (12, 21, 28, 30). For analyses of MPN series, dilution-to-extinction cultures, or dilution culturing assays (2), generally a large number of samples needs to be screened for growth. This is often achieved by visual inspection of color changes related to growth, turbidity measurement, or microscopic observation. During recent years, the use of low-substrate media helped to improve cultivation by yielding higher viable counts and more diversity (8, 13, 27, 30). However, the addition of small amounts of substrate allows only low cell densities to develop and makes growth detection more difficult. Microscopy is reliable, but screening of a single MPN series can take considerable time (several hours). Furthermore, an approximate detection limit of only 5×10^6 cells ml^{-1} has to be considered. Detection could be improved by filtration through membranes, but this cannot be applied to novel approaches with reduced culture volumes (13, 27, 30). The high sensitivity of the fluorescence assay, however, allowed growth detection in MPN series, even with low or no substrate additions allowing cell growth that was hardly detectable by light microscopy.

The fluorescence microplate assay is particularly advantageous if deep well plates are used for preparing MPN series (30), since multichannel pipettes can be used for the transfer of cultures samples to fluorescence assay plates. Therefore, the method presented in this study is faster, as it requires about 30 min of work for analysis of a 96-well plate plus the incubation of the dye, and is comparable in terms of sensitivity to flow cytometry (13) or microgrowth assays (7). Since it requires only small sample volumes, the remaining sample volume can be used for subculturing or chemical or molecular analysis.

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2.2 Spacial and temporal distribution of cultivated microbial communities in the Central Baltic Sea

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(in Bearbeitung)

Spacial and temporal distribution of cultivated microbial communities in the Central Baltic Sea

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Keywords

Chemocline, oxic-anoxic interface, chemocline, bacteria, heterotrophy, lithotrophy, denitrification, manganese, cultivation

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Abstract

The vertical distribution of aerobic, nitrate-reducing, manganese-reducing, and fermenting microorganisms along the chemical gradients of two adjacent anoxic basins in the Central Baltic Sea was investigated by means of a MPN dilution series approach. In both basins, the Gotland Basin, and the Fårö Deep, similar viable counts were obtained and varied between < 10 and 240.000 cells per ml. After mixing and oxidation of the sulphidic deep water in the Gotland Basin during 2003, viable counts of aerobes were strongly elevated whereas anaerobes were significantly reduced at the newly established oxic-anoxic interface. A distinct change from denitrification activity to incomplete nitrate reduction to nitrite was found in dilution series across the interface. Additional dilution series targeting manganese oxidisers, as well as aerobic and anaerobic ammonia oxidisers showed significant viable counts but no specific manganese- or ammonia oxidation activity. The results of the present study indicate that microbial populations in the Central Baltic Sea are tightly linked to the chemical gradients of the respective electron acceptors. Denitrifying and manganese reducing populations might significantly contribute to organic matter decomposition during long-term stagnant conditions, whereas their small populations might be of minor importance during successive reestablishment and upward movement of the oxic-anoxic interface.

Introduction

Mineralisation processes in sediments show a vertical sequence that follows the decreasing redox potentials of the electron acceptors available. In many sediments the most favourable electron acceptors like oxygen, nitrate and Mn(IV) are already consumed within the surface layers and strictly reducing conditions are established within a few centimeters (Llobet-Brossa *et al*., 2002). These resulting steep chemical gradients in turn indicate that different microbial respiration processes occur in close proximity. This is not the case in pelagic environments where, apart from a few exceptionally steep chemoclines, e.g. at the hypersaline Urania Basin in the Eastern Mediterranean (Sass *et al*., 2001; Daffonchio *et al*., 2006), the transition from oxygenated to sulfidic waters can span over several meters. These comparatively shallow gradients offer the opportunity to separately investigate layers defined by specific chemical settings and hence can be expected to be populated by specific microbial communities. Most pelagic chemoclines differ from their sedimentary counterparts also by their relatively low availability of organic carbon (Brettar & Rheinheimer, 1992). Hence,

lithotrophic processes may not only be important for recycling of potential electron acceptors but also by providing alternative electron donors for respiratory processes (Brettar & Rheinheimer, 1991; Labrenz *et al*., 2005; Brettar *et al*., 2006).

The Black Sea and the deep basins of the Central Baltic Sea are among the largest stratified water bodies, containing such pelagic oxic-anoxic interfaces. Whereas the Black Sea exhibited relatively stable chemical gradients during the last 7000 years, the Baltic Sea basins are characterized by more unsettled physicochemical conditions. The deep basins of the Central Baltic Sea are characterized by one or two pycnoclines, a summer thermocline at about 30 to 40 m and a permanent halocline at 60 to 80 m water depth. In contrast to the thermocline that disappears in autumn until re-establishing in late spring, the halocline prevents advective mixing processes leading to a permanent separation of the bottom waters (Rheinheimer, 1996). Microbial processes lead to a consumption of oxygen and other potential electron acceptors within the bottom waters establishing a chemocline that moves upwards from the sediment and can eventually reach the halocline. However, sporadically (e.g. 1993, Piker *et al*., 1998) major inflows of oxygenated water from the North Sea reach the central Gotland Basin and due to its higher density sinks to the bottom of the basin. This results in a partial or complete oxidation of the bottom waters and starts a new sequence of progressive oxygen consumption, establishment of a pelagic oxic-anoxic interface and it's upward movement. It was previously shown that the reoxidation of the bottom water had only a minor effect on the anaerobic microbial communities within the sediment and that sulfate reduction at the sediment surface was even slightly stimulated (Piker *et al*., 1998). However, it is still widely unknown how pelagic microbial communities, in particular if anaerobic, are affected by reoxidation events.

In the present work, the interrelation between geochemical settings and microbial communities at the Gotland Basin and the Fårö Deep in the Central Baltic Sea was investigated. An unexpected inflow of oxygen-rich North Sea water into the Gotland Basin additionally offered the opportunity to compare the depth distribution of targeted physiological groups before and after the reoxidation event.

A miniaturized cultivation approach based on most probable number dilution (MPN) series was established, targeting a wide variety of aerobic and anaerobic physiological groups. Since low biomass production was expected, in particular in the assays targeting lithotrophic bacteria, a recently developed very sensitive technique to quantify biomass formation during microbial growth by means of direct SybrGreen I staining (Martens-Habbena & Sass, 2006) was applied and combined with the chemical analysis of substrates.

Material and Methods

Sampling sites and sample retrieval

The Gotland and Fårö Deep are two adjacent basins in the Central Baltic Sea, located between Sweden and Latvia with water depths of approximately 240 and 180 m, respectively. Samples were taken from the Gotland Deep during cruises with the RV "Prof. Albrecht Penck" in July 2002 (57° 17.74'N, 20° 05.82'E) and August 2004 (57° 18.94'N, 20° 03.70'E) and from the adjacent Fårö Deep during a cruise with the RV "Alexander von Humboldt" in August 2003 (57° 59.47'N, 19° 53.58'E).

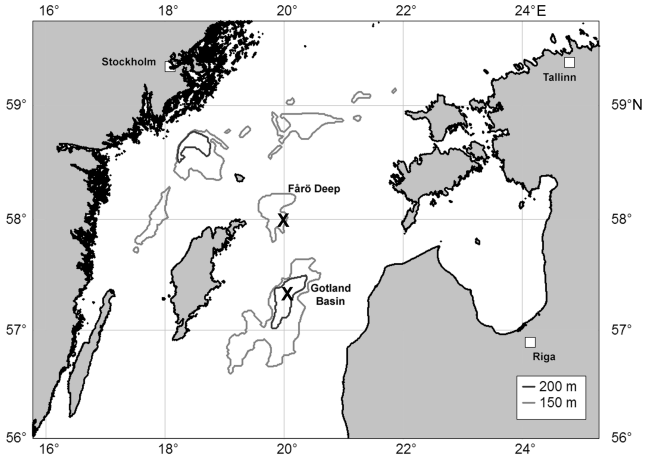

Fig. 1: Sampling locations at the Gotland Basin and the Fårö Deep in the Central Baltic Sea. Areas with water depth greater than 150 m and 200 m (grey and black lines) and exact sampling locations (crosses) are indicated. The map and depth lines were generated with datasets and PanMap software and available through the PANGEA Project at the AWI Bremerhafen (http://www.pangaea.de/Software/PanMap/).

Water samples were collected with GoFlow bottles attached to a rosette sampler (Hydro-Bios, Kiel, Germany) and processed immediately after retrieval. Data for temperature, oxygen concentration, conductivity, chlorophyll *a* fluorescence and pressure from the CTD probe (conductivity-temperature-depth; SBE 9Plus 6800M, Seabird Electronics, Bellevue, WA) were received and processed online.

Chemical determinations

Sulfide concentrations were determined after the methylene blue method of Cline (1969). Ammonium, nitrite, nitrate were analysed photometrically and accurate oxygen

concentrations by Winkler titration (Grasshoff *et al*., 1999). For the analysis of ammonium, nitrate, and nitrite in MPN series, samples (100 µl volume) had to be diluted 100 to 300-fold.

Determination of dissolved and particulate manganese

Water samples for the determination of dissolved and particulate manganese were collected from depths between 50 m and the sediment surface. Precisely 1000 ml of sample were filtered through a polycarbonate membrane filter (0.45µm pore size, Millipore HTTP04700, VWR, Darmstadt, Germany) mounted onto a polycarbonate filter holder (Type 16510, Sartorius, Göttingen, Germany). Each filter was subsequently rinsed with deionized water (electrical resistance > 18 MΩ) to remove residual salts, and was stored in sterile plastic petridishes in the dark until measuring particulate manganese.

For the determination of dissolved manganese 50 ml subsamples of the filtrate were collected, filled into polyethylene bottles (previously equilibrated with 2 % HNO_3 and rinsed with filtrate before filling), and acidified by addition of 1 ml redistilled HNO_3. Complete acid digestion of filters and analysis of manganese and iron was performed by inductively coupled plasma optical emission spectroscopy (ICP-OES) as described previously (Hinrichs *et al*., 2002).

Growth media

An artificial brackish water medium was used for preparing MPN series. This medium contained (in $g \cdot l^{-1}$): NaCl (5.6), $MgCl_2 \cdot 6H_2O$ (2.3), $CaCl_2 \cdot H_2O$ (0.34), KCl (0.15), Na_2SO_4 (0.91), KBr (0.023), H_3BO_3 (0.006), $SrCl_2 \cdot 6H_2O$ (0.009), NH_4Cl (0.021), KH_2PO_4 (0.0054), and NaF (0.0007). The medium was supplemented with $1 ml \cdot l^{-1}$ trace element solution SL10 (Widdel *et al*., 1983) and $0.2 ml \cdot l^{-1}$ of a selenite and tungstate solution (Widdel & Bak, 1992). After autoclaving, the anoxic medium was cooled under N_2/CO_2 (80/20, v/v) and per liter of cold medium 10 ml of a solution of ten vitamins (Balch *et al*., 1979) and $30 ml \cdot l^{-1}$ of a 1 M $NaHCO_3$ solution were added from sterile stocks. Finally, the medium was reduced by addition of Na_2S and acid $FeCl_2$ solutions to final concentrations of $1.2 mmol \cdot l^{-1}$ and $0.5 mmol \cdot l^{-1}$, respectively. The pH of the reduced medium was adjusted to 7.2 – 7.4 with sterile HCl or Na_2CO_3. The oxic medium differed from the anoxic one by being buffered with HEPES ($2.4 g \cdot l^{-1}$). The pH of the oxic medium was adjusted to 7.2 – 7.4 with NaOH before autoclaving and the medium was cooled under air and finally supplemented with vitamins and sodium bicarbonate (final concentration $0.2 g \cdot l^{-1}$).

Substrates used for MPN series

MPN series were prepared for heterotrophic and lithotrophic bacteria. For heterotrophic microorganisms a substrate mixture was used, containing a broad range of different carbon compounds (Süß *et al.*, 2004): the common 20 L-amino acids, the alcohols methanol, ethanol, *n*-propanol, and *n*-butanol, the short chain fatty acids formate, acetate, propionate, butyrate, valerate and caproate, and in addition glycerol, glucose, lactate, fumarate, malate, succinate (all compounds 0.1 $mmol^{-1}$ final concentration). Four different electron acceptors were offered for heterotrophic growth: oxygen (air), nitrate, manganese oxides and sulfate. For manganese and nitrate reducers the anoxic medium was used, but with sulfate replaced by manganese oxides (15 $mmol \cdot l^{-1}$ final concentration) or nitrate (6 $mmol \cdot l^{-1}$ final concentration). In 2004, a set of additional MPN series targeting fermenting microorganisms was prepared, lacking any of these electron acceptors.

MPN series for manganese-oxidising bacteria were prepared with oxic medium supplemented with $MnCl_2$ (7 $mmol \cdot l^{-1}$ final concentration) and those for nitrifying bacteria with NH_4Cl (6 $mmol \cdot l^{-1}$ final concentration) as electron donor, respectively. MPN series for anaerobic ammonium oxidisers were supplemented with 5 $mmol \cdot l^{-1}$ NH_4Cl and either with 15 $mmol \cdot l^{-1}$ manganese oxides or a combination of 5 $mmol \cdot l^{-1}$ $NaNO_3$ and 2 $mmol \cdot l^{-1}$ $NaNO_2$ as electron acceptors.

Preparation and incubation of MPN series

MPN series were inoculated onboard ship. All equipment needed for preparation of the MPN series was transferred into a polyethylene chamber (AtmosBag, 280 l, Aldrich, Milwaukee, Wisconsin, USA) that was flushed with nitrogen gas, evacuated and filled with N_2 again. The procedure was repeated up to five times to remove atmospheric oxygen.

For the preparation and incubation of the MPN series polypropylene 96-deep-well plates (Beckman, Fullerton, CA) were used. Each plate contained four different MPN series with three replicates and six tenfold dilutions each. Every well contained 900 µl medium. To the first dilution 100 µl sample were added and serially diluted into the subsequent wells. After inoculation the plates were covered with sterile lids (CAPMAT, Beckman, Fullerton, CA), sealing each well separately. In addition to the MPN series, on each plate four uninoculated dilution series were prepared as control. The inoculated and sealed MPN plates were placed into gas-tight plastic bags equipped with a gas generating and catalyst system for anoxic conditions (Anaerocult A mini, Merck, Darmstadt, Germany) and incubated for at least 12 weeks at 10°C in the dark.

Analysis of MPN series

The MPN plates were analysed for microbial growth by staining with SybrGreen I and fluorescence analysis (Martens-Habbena & Sass, 2006). Several MPN series were also checked by microscopy, mostly those for lithotrophic bacteria. In a microbiological cabinet the MPN plates were removed from the gas-tight plastic bags and the lid cut into pieces and carefully lifted using sterile tweezers. From each well 200 µl were transferred to a black microtiter plate and supplemented with 50 µl of SybrGreen I working solution. SybrGreen I working solution was freshly prepared each day at fivefold desired final assay concentration in sterile-filtered TE buffer concentrate (200 mM Tris-HCl, 50 mM Na_2EDTA, pH 8.0). All fluorescence measurements were carried out at 485 nm excitation and 520 nm emission on a fluorescence microplate reader (FLUOSTAR Optima, BMG Labtechnologies, Offenburg, Germany) with the following settings: three reading cycles with integration of 20 flashes, 0.5 sec delay between plate movement and reading, and 10 s shaking before each cycle and a detector gain of 1,300 arbitrary units. MPN counts were calculated using the software developed by Klee (1993).

Results

Stratification, temperature and salinity

During summer, the water column at the basins of the Central Baltic Sea generally exhibits three distinct water layers (I to III in Fig. 2) separated by thermal and haline stratification (Rheinheimer, 1995). This stratification pattern was confirmed during the sampling campaigns. The surface layer (layer I in Fig. 2) comprised most of the photic zone, as indicated by the depth profile of chlorophyll *a*, and extended to an approximate depth of 25 m. Temperatures in the surface layer ranged from 15 to 22°C during the sampling campaigns and rapidly dropped to values around 5°C in the intermediate layer beneath the thermocline (layer II in Fig. 2). Salinity did hardly change within the surface and intermediate layers with values around 7 practical salinity units (PSU), but gradually increased to approximately 10 PSU within the halocline between 75 and 90 m depth. Parallel to salinity, temperature also slightly increased. Within the stagnant bottom water (layer III in Fig. 2) a slight increase in salinity with depth was observed. While in the Gotland Deep in 2002 and in the Fårö Deep in 2003 maximum salinities were around 11.9 PSU, in the Gotland Deep in

2004 higher values of up to 13 PSU were recorded. These elevated values could be traced back to an influx of North Sea water in 2003 (Feistel *et al*., 2003).

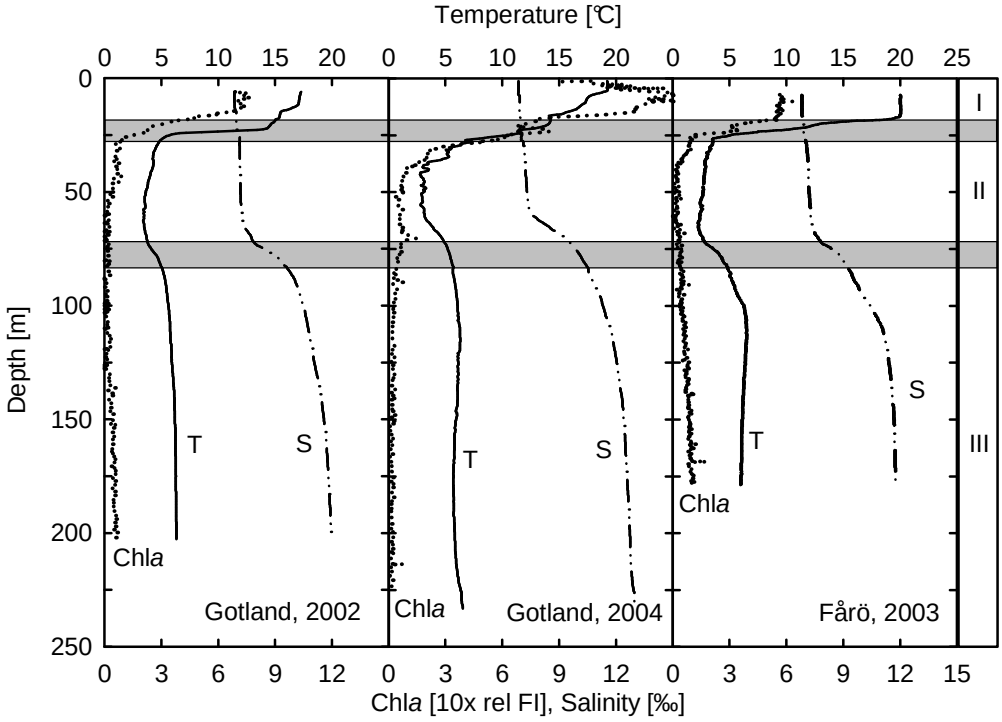

Fig. 2: Hydrographical structure of the Gotland Basin in 2002, and 2004, as well as the Fårö Deep, 2003. Given are the temperature (T), salinity (S), and Chlorophyll *a* fluorescence intensity (Chl*a*). Numbers on the right denote the three distinct water layers, separated by a thermocline and a halocline (shaded areas).

Gradients of oxygen and sulfide

In 2002 and 2004, oxygen concentrations along the surface and intermediate layers were in the range of 300 to 350 µM with little depth variation (Fig. 3). In 2003 oxygen concentrations were in a similar range in the intermediate layers but lower in the surface layers. All oxygen gradients, exhibited a strong decrease within the halocline. In the stagnant bottom water, however, differences between the single sampling campaigns were found. In 2002, along the upper 40 to 50 m low oxygen concentrations (less than 20 µmol·l^{-1}) were still present and anoxic conditions were reached only at a depth of 135 m. In contrast, in the Fårö Deep sampled in Summer 2003 anoxic conditions were present in most of the deep water and a sharp oxic-anoxic interface was found already at 105 m depth. In 2004, however, a very unusual oxygen profile was obtained. Oxygen had a distinct minimum at the lower boundary of the halocline. Beneath, concentrations increased again to values around 50 µM and

remained relatively constant down to a depth of approximately 200 m. However, a newly established oxic-anoxic interface was detected at 220 m depth.

Sulfide was generally detected just beneath the oxic-anoxic interface, except for 2002, when a slight overlap between the gradients of oxygen and sulfide was found. Sulfide concentrations generally increased with depth with a maximum of around 100 µmol·l^{-1} at 220 m depth in 2002.

Gradients of nitrate, nitrite and ammonium

Nitrate concentrations in the surface and intermediate layers were low, but strongly increased within the halocline. Elevated nitrate concentrations were generally found between the halocline and the oxic-anoxic interface. While in 2002 and 2003 maximum nitrate concentrations were about 6 µmol·l^{-1}, in 2004 up to 12 µmol·l^{-1} were detected. In the sulfidic layers no nitrate was found. In 2002, above the oxic-anoxic interface the nitrate profile appeared erratic with several local maxima and minima mirroring local variations in oxygen. These small-scale variations of oxic and anoxic conditions reflect the vertical sequence of individual water bodies separated due to tiny differences in salinity.

Ammonium concentrations were highest in the bottom waters above the sediment with concentrations of up to 30 µmol·l^{-1}, and with a steep decrease just beneath the oxic-anoxic interface. Between the halocline and the oxic-anoxic interface virtually no ammonium was detected. In the surface and intermediate layer, however, low concentrations of ammonium were present (up to 2 µmol·l^{-1}). Nitrite concentrations were generally very low. Local peaks with values reaching 0.25 to 0.41 µmol·l^{-1} were, however, observed at the upper and lower borders of the nitrate maximum zone (data not shown).

Gradients of dissolved and particulate manganese

Dissolved manganese showed a similar depth profile like ammonium with the highest concentrations in the bottom water, but with a steep drop just above the oxic-anoxic interface and a few meters above of that of ammonium. In 2002 and 2003, dissolved manganese profiles exhibited some small local maxima above the oxic-anoxic interface. In 2004, a small additional dissolved manganese maximum was detected parallel to the oxygen minimum at approximately 75 m depth. Elevated concentrations of particulate manganese were observed in a layer of 20 to 30 m above the oxic-anoxic interface exhibiting a double peak during all campaigns. Beneath the oxic-anoxic interface particulate manganese was consumed within a few meters.

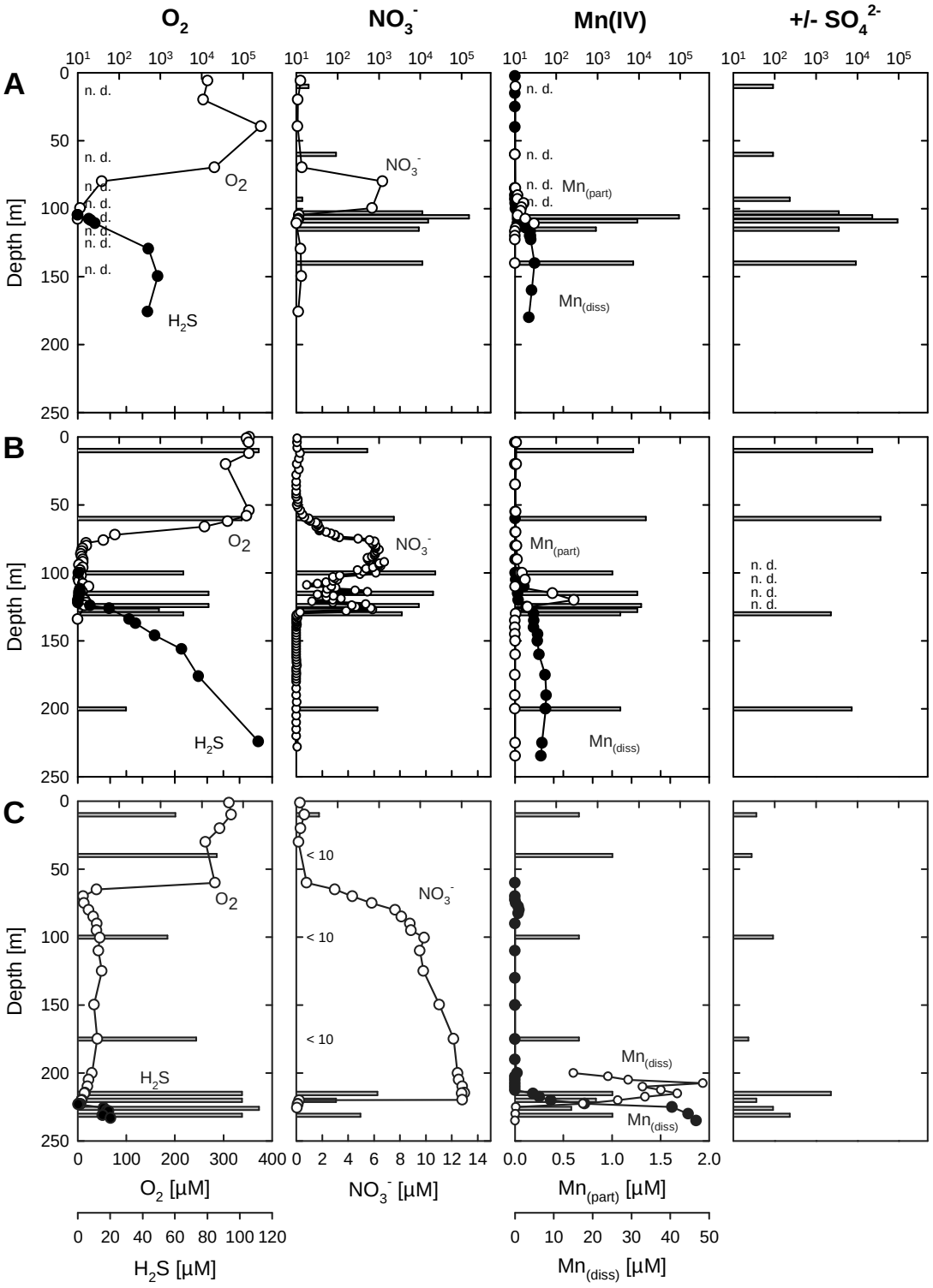

Fig. 3 Chemical profiles and viable counts of organotrophic microorganisms from the Fårö Deep (A), as well as the Gotland Basin, 2002 (B) and 2004 (C). Electron acceptors supplied together with the substrate mix are indicated on top.

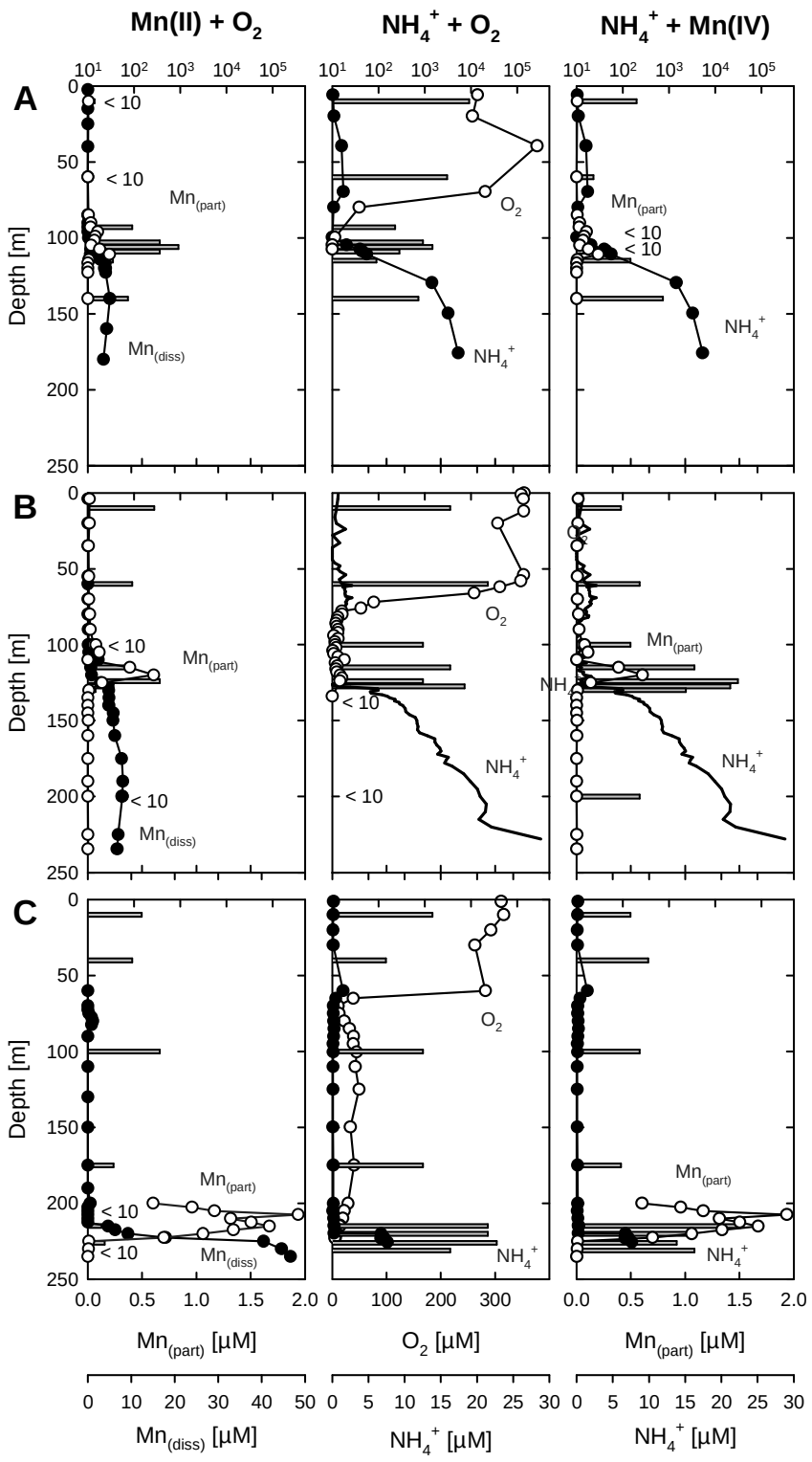

Fig. 4 Chemical profiles and viable counts in most probable number dilutions targeting lithotrophic microorganisms from the Fårö Deep (A), as well as the Gotland Basin, 2002 (B) and 2004 (C). The employed substrate combinations are given on top.

Viable counts of heterotrophic microorganisms

In August 2002, high numbers of aerobes were detected in the surface (240,000 cells ml^{-1}) and intermediate layers. Numbers in the bottom layer were at least one order of magnitude lower, but with a small local maximum at the oxic-anoxic interface. While no data are available for 2003, in summer 2004 a completely different situation was found, with the highest viable counts around the chemocline (up to 250.000 cells ml^{-1}) situated at approximately 220 m depth and with lower numbers in the oxygen-rich surface and intermediate layers.

Viable counts with nitrate as terminal electron acceptor were highest (up to 100,000 cells ml^{-1}) at the oxic-anoxic interface, concurrent with a strong decrease in nitrate. In the oxic surface and intermediate layers above, but also in the anoxic layers beneath the oxic-anoxic interface, MPN counts with nitrate were clearly lower. Similarly, highest viable counts with manganese oxides as terminal electron acceptor were observed with samples from the oxic-anoxic interface (up to 10^5 cells ml^{-1}). However, with exception of 2003, no pronounced decrease in MPN numbers towards the oxic surface or sulfidic deep layers was observed.

A similar situation was found for MPN series prepared with medium containing sulfate as terminal electron acceptor. In 2002 no clear trend in MPN counts with depth was found, while in 2003 and 2004 viable counts were highest around the oxic-anoxic interface (up to 10^5 cells ml^{-1}), like it was found for MPN series with nitrate and manganese oxide. MPN series for fermenting microorganisms were prepared only during sampling in summer 2004 (data not shown). The MPN counts obtained with this sulfate-free medium showed no difference in depth distribution or in numbers to the counts obtained with medium containing sulfate. Maximum counts were obtained around the chemocline. However, numbers obtained for layers above the chemocline (30-100 cells ml^{-1}) were just higher than the background numbers (20-40 cells ml^{-1}), determined in pure mineral media without additon of electron donors and electron acceptors (data not shown).

Chemolithotrophic microorganisms

MPN series targeting manganese-oxidising bacteria generally yielded low viable counts, rarely exceeding 100 cells ml^{-1} (Fig. 4). In 2002 and 2004, in the Gotland Deep, no correlation with the geochemical gradients was found. In 2003, however, a clear maximum was found within the upper part of the chemocline, and with 1000 cells ml^{-1} being higher than any other MPN count obtained with this medium combination.

Viable counts in MPN series prepared with oxic medium containing NH_4^+ as sole substrate were one to two orders of magnitude higher than those obtained with Mn^{2+}. Similar to the latter, for ammonium-amended medium only a weak correlation with the geochemical gradients was observed. Numbers were relatively high in the surface and intermediate layers (up to 23,000 cells ml^{-1} in 2002). In the chemocline, numbers were also elevated when directly compared to those in the adjacent layers, but were not exceeding those in the upper water layers. An exception was the situation in 2004, when the highest counts (up to 36,000 cells ml^{-1}) for aerobes with ammonium were obtained with samples from the chemocline at approximately 220 m depth.

The depth profile for MPN series targeting anaerobic ammonia-oxidising bacteria using manganese oxides as terminal electron acceptor roughly reflected the results obtained with oxic medium (Fig. 4). Maximum numbers of up to 30,000 cells per ml were obtained with samples from the upper part of the chemocline in 2002 and 2004, whereas in the lower part of the chemocline numbers were about one order of magnitude lower. Apart from the chemocline less than 500 cells were detected with this substrate combination. Samples from the Fårö Deep delivered in lower numbers (less than 700 cells per ml) and resulted in no significant correlation with the chemical gradients.

MPN series targeting anaerobic ammonium-oxidising bacteria with nitrite and nitrate as terminal electron acceptors were performed only in 2003 and 2004 (data not shown). Whereas, in 2003 no growth was obtained at all, in 2004 numbers at the surface and in 40 m depth (230 and 900 cells per ml, respectively) were significantly above the background obtained in substrate-free medium.

Analysis of MPN series by SybrGreen I staining and fluorescence measurement

In the present study MPN series were analysed for growth by staining with SybrGreen I and fluorescence measurement. This method gives a quantitative measure of the nucleic acid content that can be converted into cell numbers. The range of fluorescence and the corresponding cell numbers per ml for the different MPN series prepared in 2004 are depicted in Fig. 5. The results obtained reveal strong variability in cell yield achieved in the different wells of a single MPN. Highest total and average cell yields were obtained after oxic incubation with the medium for heterotrophic bacteria. Variations of fluorescence intensities between single positive wells corresponded to cell densities differing by approximately two orders of magnitude. Similarly high cell yields were achieved with manganese oxides as electron acceptors, although the average yield was clearly lower. With nitrate that offers an

only slightly lower energy yield than oxygen, cell densities of heterotrophic bacteria were generally over one order of magnitude lower than after oxic incubation. In the absence of any potential electron acceptors fewer cells were obtained, even compared to the MPN series with sulfate. Surprisingly high cell densities were achieved with media targeting lithotrophic bacteria. For these assays on average almost 10^7 cells per ml were estimated. However, assays with ammonium and manganese oxides even yielded up to 10^8 cells per ml.

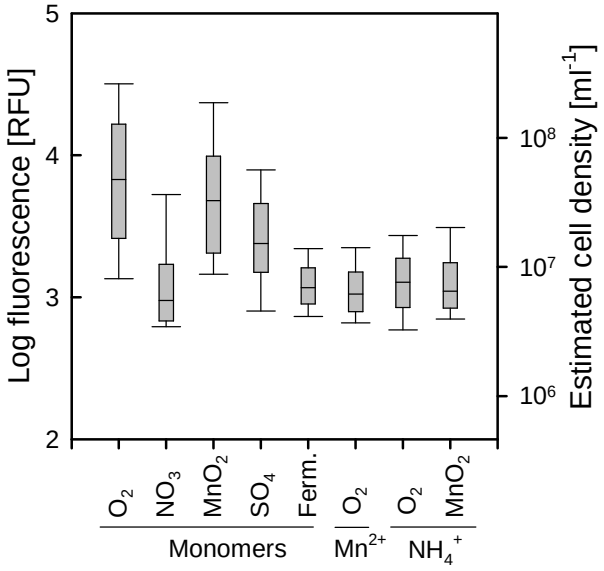

Fig. 5. Biomass formation in Most Probable Number Dilution Series according to fluorescence measurement of SybrGreen I stained subsamples. Given are the median, the 25 to 75 % (bars), as well as the 10 to 90 % confidence intervals (error bars) of enrichments with detectable growth from August 2004. The estimated cell density was calculated according to the calibration curve given by Martens-Habbena *et al.* (2006).

Chemical analysis of MPN series

Besides detection of growth by fluorescence analysis after staining with SybrGreen I, MPN series amended with ammonium, nitrite or nitrate were subjected to chemical analysis. Results obtained after analysing MPN series for heterotrophic nitrate reducers prepared in 2004 are presented in Fig. 6. Chemical analysis revealed that in all but two out of 22 wells that were proven positive after staining with SybrGreen I nitrate was almost completely removed. On the other hand, in several wells, particularly for 220 and 226 m depth, chemical analysis revealed nitrate reduction without detectable growth. In MPN series inoculated with sample from 215 m (above the oxic-anoxic interface) nitrate was mostly reduced to N_2, with exception of the lowest dilution in which nitrite was detected in reasonable amounts. Samples

from 231 m, in contrast, resulted mainly in incomplete reduction to nitrite. Nitrate ammonification played only a minor role, since less than 10 % of the nitrate was converted into ammonium (Fig. 6).

Different results were obtained for the MPN series targeting ammonium-oxidising bacteria. While nitrate and nitrite, when present, were clearly reduced. Ammonium concentrations showed, if even, only a weak decline (up to 50 µmol·l^{-1}), irrespective of the incubation conditions (data not shown). Determination of growth by fluorescence analysis upon SYBRGreen I staining was hampered by the presence of nitrite. In wells containing more than 500 µmol per liter a reduced fluorescence yield was obtained, depending on the nitrite concentration.

Discussion

In the present work the vertical distribution of heterotrophic and lithotrophic microbial communities were examined along the vertical gradients of two adjacent deep basins in the Central Baltic Sea. Under stably stratified conditions the presence of anaerobic communities was tightly correlated to the gradients of the respective electron acceptors, e.g. nitrate, or to sulfidic conditions in case of fermenting microorganisms. The lateral intrusion of oxic water into the bottom layer caused not only an oxidation of the ammonium and sulphide pools, but also a major disturbance of the in situ communities.

Hydrography and chemistry of the Gotland Basin and Fårö Deep

During sampling campaigns in 2002 and 2003 the Gotland Basin and the Fårö Deep exhibited thermo-haline stratification patterns typical for the Central Baltic Sea during late summer (Brettar & Rheinheimer, 1991; Rheinheimer, 1995; Feistel, 2003). The water column above the halocline was oxygen-saturated and almost depleted in nitrate and ammonium, whereas the layers between halocline and oxic-anoxic interface exhibited maxima in nitrate and particulate manganese. However, the two basins are separated by an embankment restricting water exchange to the upper part of the water column and hence had prevented the lateral intrusion that reached the Gotland Deep in 2003 from swashing over into the Fårö Deep. The two basins differed slightly in their temperature and salinity profiles along the halocline. This suggests that here the lateral intrusion had a slight effect on the Fårö Deep.

Such smaller intrusions of nearly iso-pycnic water appear to be relatively frequent, at least in the Gotland Basin, where they are thought to cause the erratic depth profiles of oxygen and nitrate that were observed at 80 to 130 m depth in 2002 (Fig. 3). The lateral intrusion of oxic water to the bottom layers of the Gotland Deep in spring 2003, in contrast, had a much more drastic effect. It was ensued by intense nitrification and manganese oxidation completely depleting the ammonium and dissolved manganese pools beneath the halocline. However, soon after this re-oxidation event a new chemocline established at the sediment surface and was slowly moving upwards. In Summer 2004, over one year after the intrusion, immediately beneath this new oxic-anoxic interface steep sulfide and ammonium gradients were detected, but maximum concentrations were still significantly lower than in 2002.

Impact of advective mixing and bottom water oxidation on microbial communities

Extent and vertical distribution of the different physiological groups analysed in the present work substantially varied between the single sampling campaigns. These variations, however, appear to have different reasons when regarding the upper and the bottom water layers.

During all sampling campaigns physico-chemical settings were similar above the halocline, while nonetheless viable counts varied considerably. Numbers of aerobes and nitrate reducers for example were apparently not related to changes in oxygen and nitrate concentrations and hence appear to depend on photosynthetic activity. It has been estimated that in the Central Baltic Sea about 45 % of the primary production is already remineralised above the halocline (Schneider *et al.*, 2002) and it has been shown that microbial communities rapidly respond to changes in photosynthetic activity, and hence exudation, but also to cessation of algal communities (Gocke, 1975; Rheinheimer, 1995). The excretion of nitrogen-poor exudates depends on nutrient supply and intensity of irradiance and is highest during spring and summer. Decaying phytoplankton blooms following the high productive periods, in turn, provide a more complex combination of different chemical compounds, like it was aimed with the substrate mixture for heterotrophs used in the present study. Therefore, it can be assumed that the changes in community composition found above the halocline reflect seasonal and meteorological effects.

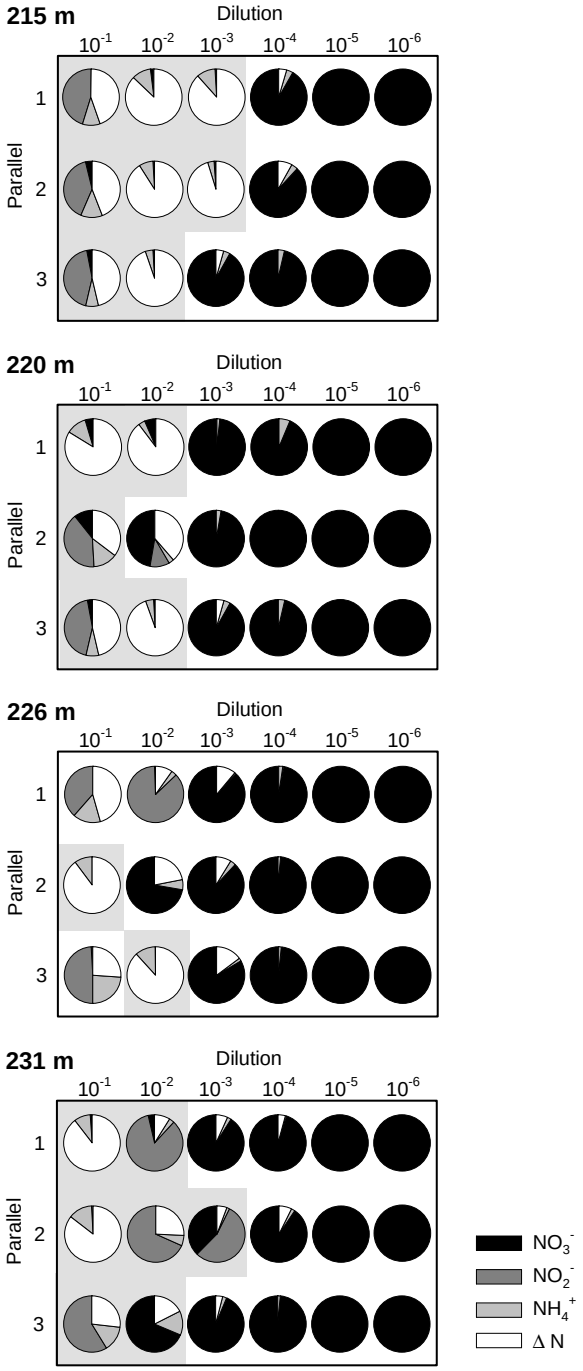

Fig. 6. Nitrate utilisation in MPN series targeting heterotrophic Nitrate-reducing bacteria from the chemocline of the Gotland Basin, 2004. The sampling depths and dilution steps are indicated above each panel. Dilutions with detectable growth as revealed by SybrGreen I staining are highlighted in gray.

Microbial communities beneath the halocline cannot benefit from algal exudates but nevertheless play an important role in the degradation of sedimenting organic material. Approximately 40 % of the primary production is remineralised here (Schneider *et al*., 2002). It is likely that the microbial communities in the bottom layers are more influenced by the chemical settings and the changes caused by the intrusion of oxic water. In 2004, a completely different picture emerged compared to the situation in the stably stratified basins sampled previously. The lack of nitrate-reducing bacteria and at the same time high numbers of aerobes in the MPN series indicates a strong decline of facultatively aerobic bacteria. One reason is that the deep water intrusion not only transported oxygen but also an allochthonous microbial community. On the other hand the relatively high oxygen concentrations beneath the halocline may favour different types of bacteria than the microaerophilic and anoxic conditions pervailing previously. It was reported that denitrification in the chemocline of the Central Baltic Sea is mainly driven by the oxidation of sulfide (Brettar & Rheinheimer, 1991). This lithotrophic metabolism helps saving organic material for assimilation and is advantageous under organic carbon-limited conditions. The dominance of lithoheterotrophic and mixotrophic nitrate reducers is supported by the analyses of the MPN series targeting nitrate reducers. Generally, very low growth yield was obtained (Fig. 5) and in all positive series FeS had completely vanished, whereas the negative wells still exhibited black FeS precipitates. After the oxic water intrusion sulfide in the bottom water was completely reoxidised leaving only organic material as potential electron donor *in situ*. If nitrate reducers can utilise organic material, it can be imagined that they are easily outcompeted by strict aerobes, explaining their apparent disappearance. At the newly established oxic-anoxic interface, however, where sulfide became available nitrate-reducing populations started to develop again, even in the presence of high numbers of aerobes.

Assessment of chemolithotrophic microorganisms

Previous investigations in the Black Sea and the Baltic Sea have shown, that microbial manganese oxidation at the oxic-anoxic interface significantly contributes to the overall electron balance (Tebo, 1991; Neretin *et al*., 2003). Several studies revealed aerobic and anaerobic ammonium oxidation in chemoclines of stratified water bodies (Enoksson, 1986; Kuypers *et al*., 2003). During the present study it was tried to correlate numbers of lithotrophic organisms to the respective gradients of their electron donors. However, true lithotrophic growth was rarely detected, although at least in a few cultures targeting

autotrophic manganese oxidisers the precipitation of manganese particles was found. Growth detection of lithotrophic bacteria is generally hampered by the low growth yield. Lithoautotrophic bacteria have to spend substantial amounts of energy for reversed electron transport to $NADP^+$ and this energy demand increases with increasing redox potential of the electron donor. On the other hand, a more positive redox potential of the electron donor reduces the ATP yield per molecule oxidised. For example two Mn^{2+} have to be oxidised to generate one ATP, while for each electron transferred from Mn^{2+} to $NAD(P)^+$ one to two ATP have to be spent. Hence, one can expect that autotrophic manganese oxidation would only support low growth if even at all. It is still matter of debate, whether manganese oxidation can be used for energy conservation (Tebo *et al.*, 2005). Nonetheless, it has been shown to be important *in situ* (Neretin *et al.*, 2003) and it has further been demonstrated that an *Erythrobacter* sp. strain can attain higher cell densities when growing under manganese-oxidising conditions (Francis, 2001). Therefore, it may be more likely that manganese is either utilized in a mixotrophic or in a chemolithoheterotrophic metabolism (Tebo & He, 1999; Tebo *et al.*, 2005).

In contrast, significant viable counts and cell densities were detected in dilution series supplemented with ammonium (Fig.4), but unexpectedly only a weak turnover of ammonium was detected. Viable counts inferred from these MPN series can thus not be considered as numbers of true lithotrophs. It has been shown that heterotrophic bacteria can outnumber enrichments of nitrifying bacteria (Aakra *et al.*, 1999), even in standard media. In the present work, however, a highly sensitive method for growth detection was used that was originally considered for lithotrophic and oligocarbophilic cultures (Martens-Habbena & Sass, 2006). But obviously this sensitive growth detection is prone to deliver false positive results, when not combined with chemical analysis of substrate turnover, like in the present work. If in the enrichment targeting lithotrophic bacteria organotrophic growth was obtained, there must have been organic carbon sources in the basal medium. To develop a more reliable growth assay for lithotrophic bacteria the potential organic carbon sources had to be identified. Besides the vitamin solution (sums up to 18 µmol C l^{-1}), chemical analysis revealed the presence of short chain volatile organic acids (formate, acetate, and propionate each up to10 µmol l^{-1}) in the basal medium. Assuming that 40 % of the carbon would be assimilated, and considering a carbon content of 15 fg per cell (Simon & Azam, 1989), the vitamin solution and volatile organic acids would support growth of up to 10^7 cells ml^{-1}, what is approximately the cell yield obtained in the lithotrophic MPN series in the present study.

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2.3 Carbon dioxide fixation below the chemocline of the Central Baltic Sea is not substantially supported by aerobic or nitrate-dependent sulfur oxidation

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Microbial Ecology (eingereicht)

Carbon dioxide fixation below the chemocline of the Central Baltic Sea is not substantially supported by aerobic or nitrate-dependent sulfur oxidation

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Abstract

Microbial carbon dioxide fixation is commonly found in pelagic oxic-anoxic interfaces. However, its exact location and extent, as well as microbial energy metabolisms supporting microbial CO_2 fixation are still a matter of debate. The present work provides a comprehensive study of nutrient, sulfur, and manganese chemistry in comparison to microbial CO_2 fixation around the oxic-anoxic interface of the Gotland Basin in the Central Baltic Sea. Typical opposite gradients of dissolved oxygen and sulfide approached the detection limits at 204 m water depth, accompanied by nitrate depletion at the same depth. In contrast, decreasing amounts of particulate manganese were detected down to 215 m water depth, stretching ten meters into the sulfidic zone. Whereas thiosulfate and sulfite were below the detection limit (< 50 nmol L^{-1}) throughout the entire interface, a peak of about 4 µmol L^{-1} zero-valent sulfur was detected around 210 m. CO_2 assimilation extended over 20 meters beneath the oxic-anoxic interface and a maximum of 0.7 to 1.1 µmol C L^{-1} d^{-1} was located at 215 m water depth, thus about ten to twelve meters below the chemocline. More than 70% of the measured CO_2 fixation could neither be attributed to aerobic, nor nitrate-dependent sulfur oxidation. Spiking experiments revealed that CO_2 fixation could only be enhanced by thiosulfate or polysulfides, whereas elevated sulfide concentrations, dithionite, as well as anthraquinone disulfonate inhibited CO_2 fixation. Chemical gradients, *in situ* CO_2 assimilation, and spiking experiments together suggested that bulk microbial dark CO_2 assimilation below the chemocline of the Gotland Basin was sulfur dependent and redoxsensitive. It is proposed that a microbial manganese oxide-dependent incomplete sulfide oxidation to thiosulfate or elemental sulfur accounts for a large fraction of the bulk CO_2 fixation and that sulfide is recycled by thiosulfate- and sulfur reducing microorganisms.

Introduction

Dark microbial carbon dioxide fixation has been observed at oxic-anoxic interfaces of several aquatic ecosystems. These include marine environments like Framvaren Fjord, Norway [43], Cariaco Trench, Caribbean Sea off Venezuela [74], brackish environments like the Black Sea [61], the Baltic Sea [14, 37], as well as freshwater systems like Lake Kinneret, Israel [24]. Although dark carbon dioxide fixation in oxic-anoxic interfaces can

make up a significant fraction of the overall inorganic carbon assimilation in these environments [e.g. 14, 65], knowledge about underlying microbial energy metabolisms is still scare.

Chemical gradients at pelagic oxic-anoxic interfaces in general suggest that several processes could support carbon dioxide fixation. These include, e.g., aerobic and anaerobic ammonia oxidation [5, 58, 60], disproportionation of sulfur compounds [2], aerobic methane oxidation [26], metal oxidation [21], and hydrogen oxidation [46]. Yet, none of these processes has been proven to be of quantitative importance. Aerobic and nitrate-dependent microbial sulfur oxidation, thus remain the most important reactions to explain the observed carbon dioxide fixation [e.g. 8, 9, 33]. This view is also supported by molecular analyses, which provided the presence and activity of sulfur-oxidizing genera of the *Gamma-* and *Epsilonproteobacteria* in pelagig oxic-anoxic interfaces of the Black Sea, as well as the Cariaco Trench [40, 42, 75]. Recently, it was also demonstrated that members of the *Epsilonproteobacteria*, closely related to the obligate chemolithoauthotrophic sulfur-oxidizing bacterium *Thiomicrospira denitrificans*, are present in the oxic-anoxic interfaces of the Gotland Basin and the Landsort Deep in the Baltic Sea and make up about 8% of the microbial community at the Gotland Basin redoxcline [36, 37].

However, considering the carbon and energy metabolism of known sulfur-oxidizing bacteria, the amount of sulfide supplied by a gradient from the sediment usually underscores the microbial assimilation of inorganic carbon at pelagic oxic-anoxic interfaces significantly [33, 65, 79]. Two mechanisms have been suggested to explain this discrepancy. Either the activity of sulfate reducing bacteria could lead to an additional sulfide supply for autotrophic sulfur oxidizing bacteria [33], or the activity of heterotrophic microorganisms itself could substantially contribute to the observed carbon fixation by inorganic carbon assimilation via anaplerotic reactions [14, 54, 79]. Some studies reported carbon dioxide fixation significantly below pelagic oxic-anoxic interfaces and it has therefore additionally been speculated, that novel pathways of microbial sulfur oxidation via reduction of particulate metal oxides such as manganese oxide and iron oxide could support microbial carbon dioxide fixation [33, 65, 79].

The present study attempted to gain insights into the vertical distribution, intensity, and characteristics of microbial carbon dioxide fixation in relation to chemical gradients observed at the oxic-anoxic interface of the Gotland Basin. Therefore, a comprehensive analysis of nutrients, sulfur-, and manganese species around the oxic-anoxic interface was

combined with the analysis of microbial carbon dioxide fixation *in situ* as well as in spiking experiments.

Methods

Study area and sampling. The Gotland Basin is a 248 m deep basin in the Central Baltic Sea (Fig. 1) and contains one of the largest coherent water masses of the Baltic Sea. Sampling was performed during cruise AL256 aboard R/V "Alkor" in May 2005 at the deepest part of the basin. Profiles of temperature, salinity, and oxygen were recorded with a Seabird SBE 911*plus* conductivity-temperature-depth profiler (Sea-Bird Electronics Inc., Washington, USA) mounted on a rosette water sampler. Water samples from discrete depths were collected with 5-Liter Free-Flow bottles (Hydrobios, Kiel, Germany) attached to the sampler. Subsamples were directly filled into calibrated 120 mL oxygen bottles with at least three flask volumes overflow. Special care was taken to avoid any oxygen contamination during filling procedure. For control purposes, the filling procedure was repeatedly done under a stream of argon to avoid any air contact of the water samples. No differences in chemical as well as activity measurements were observed between both procedures.

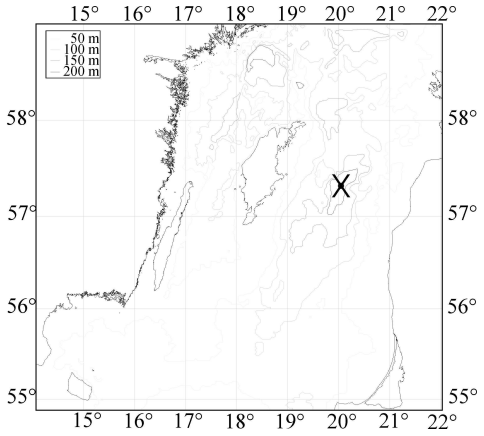

Fig. 1. Bathymetric map of the Central Baltic Sea. The sampling location at the central Gotland Basin (57° 19.2'N 19° 57'E) is indicated.

Chemical analyses. Analyses of inorganic nutrients, as well as oxygen and hydrogen sulfide were performed immediately after sampling by standard manual colorimetric methods [23]. Samples for total inorganic carbon concentration were taken, preserved with mercury chloride, and determined later by the coulometric SOMMA system [30].

Thiosulfate and sulfite concentrations were determined by HPLC analysis of monobromobimane-derivatized samples [16, 53, 78]. 500 µL water samples were taken from the Free-Flow bottles by means of a Hamilton syringe, filled into 1.5 mL reaction tubes and instantly derivatized by the addition of 100 µL monobromobimane reagent (see below). Samples were maintained for 30 min at room temperature in the dark, and the reaction was stopped by acidification with 50 µL of methanesulfonic acid (324 mmol L^{-1}). Samples were subsequently stored frozen at –80°C until HPLC analysis within three weeks. The stability of thiosulfate-bimane complex was reported to be somewhat sensitive to long-term storage [78]. Samples spiked with known amounts of thiosulfate were therefore derivatized and analyzed in parallel and did not exhibit significant decomposition of the thiosulfate-bimane complex within this storage period. Monobromobimane reagent was freshly prepared each day by mixing equal volumes of HEPES buffer (500 mmol L^{-1} HEPES, 50 mmol L^{-1} Na-EDTA, pH 8) and monobromobimane (Calbiochem, Carlsbad, CA; 45 mmol L^{-1} in acetonitrile). Standards of thiosulfate and sulfite were prepared in serum bottles, flushed with nitrogen, and stored at 4°C.

Analysis was carried out with a gradient HPLC System (Sykam, Fürstenfeldbruck, Germany) equipped with a low-pressure gradient controller (S8111), autosampler (S5200), and column oven (S4011). Separation was performed on a reversed-phased column (Adsorbospere OPA HR 5µ, 150x4.6 mm, Alltech, Unterhaching, Germany) thermostated to 25°C at a flow rate of 1.0 mL min^{-1}. Eluent A was 0.25% (v/v) acetic acid (pH 3.5) and eluent B was 100% HPLC gradient grade methanol (Carl Roth, Karlsruhe, Germany). The gradient employed was as follows: 0-0.5 min 10% B, 7 min 12% B, 15 min 30% B, 19 min 30% B, 23 min 50% B, 30 min 100% B, 36 min 100% B, 37 min 10% B, 44 min 10% B. Fluorescence emission was recorded on a Linear Instruments LC 305 fluorescence detector (excitation 380 nm, emission 480 nm). Sulfite and thiosulfate eluted after 3.3 and 5.9 min, respectively. Reproducibility of standards (1 µmol L^{-1}) was better than 5% and at an injection volume of 100 µL the detection limit was 50 nmol L^{-1}.

Zero-valent sulfur was determined in parallel samples as described by Zopfi et al. [78, 79] with the following modifications: One mL water sample was immediately fixed with 100 µL 2% (w/v) $ZnCl_2$ and stored at –80°C. Sulfur was extracted with 0.1 volume

chloroform for 2 hours and 70 µL of the chloroform extract was resuspended in 900 µL methanol and analyzed by HPLC (see below). Standard solutions were prepared in methanol and diluted in methanol:chloroform (90:10) prior to analysis. HPLC separation was carried out on the HPLC system and column described above with an isocratic eluent of 100% HPLC gradient grade methanol (Carl Roth, Karlsruhe, Germany). Absorption at 265 nm was recorded on a Linear Instruments UVIS 204 detector. Injection volume totalled 100 µL and cyclo-S_8 sulfur eluted after 3.1 min. The detection limit was 300 nmol L^{-1}, and reproducibility of 5 µmol L^{-1} standards was better than 5%.

Dissolved and particulate manganese analysis was performed according to Hinrichs *et al*. [27]. Exactly one liter of water sample was filtered through polycarbonate membranes (0.45 µm poresize, Millipore, Göttingen, Germany) mounted onto a polycarbonate filter holder (Type 16510, Sartorius, Göttingen, Germany) as soon as possible after sampling. Each filter was subsequently rinsed with 18 mΩ water and stored in sterile plastic Petri dishes in the dark until analysis. For the determination of dissolved manganese 50 mL subsamples of the filtrate were collected, filled into polyethylene bottles (equilibrated with 2% nitric acid and rinsed with sample filtrate), and acidified by addition of 1 mL redistilled nitric acid. Complete acid digestion of filters was done in closed PTFE autoclaves at 180°C in a mixture of nitric acid, perchloric acid and hydrofluoric acid. Extracts were resuspended in 25 mL of 2% nitric acid. Analysis of manganese was performed by inductively coupled plasma-optical emission spectroscopy (Perkin Elmer Optima 3000XL). Manganese was calibrated between 0.36 and 36 µmol L^{-1}. The detection limits for dissolved and particulate manganese were 0.36 µmol L^{-1} and 0.01 µmol L^{-1}, respectively.

In situ carbon dioxide dark fixation. Over a period of 10 days, multiple profiles throughout the redoxcline (180 to 220 m water depth) were taken for the determination of CO_2 dark fixation rates according to the method of Steemann Nielsen [34, 63]. The anoxic sodium [^{14}C]-bicarbonate stock solution (250 µCi mL^{-1}) was prepared as follows. Deionized water (18mΩ) was filled into serum vials and carefully flushed with argon to remove any oxygen. Weighted sodium [^{14}C]-bicarbonate crystals (SA 53.0 mCi $mmoL^{-1}$, Hartmann Analytic GmbH, Braunschweig) were added to the serum vials under argon atmosphere in an anaerobic chamber and dissolved.

Incubations were performed in 120 mL oxygen bottles, which were carefully filled as described above. 40 to 60 µL of an anoxic [^{14}C]-bicarbonate stock solution were added

with a gas-tight Hamilton syringe. Samples were incubated at *in situ* temperature (± 1°C) in the dark. Incubation was stopped by filtration. Prior to filtration of the samples, 50 µL sub-samples were drawn from all incubations, placed into scintillation vials containing 50 µL ethanolamine to quantify the amount of added [^{14}C]-bicarbonate. Samples were then filtered onto polycarbonate membrane filters (0.2 µm pore size, Whatman, via VWR International, Weiterstadt, Germany), exposed to HCl fumes for 30 min, mixed with scintillation cocktail (UltimaGold XR, Packard, USA), and counted on board in a Triathler liquid scintillation counter (Hidex Oy, Turku, Finland) and in the laboratory in a TriCarb 2560 TR/X liquid scintillation counter (Packard, USA). Carbon dioxide fixation of control samples immediately fixed with 1 mL formalin (37%) before adding [^{14}C]-bicarbonate was 0.0043 µmol C L^{-1} d^{-1} (standard deviation 0.0029 µmol C L^{-1} d^{-1}). All incubations were started within 15 min upon sample collection and lasted not longer than 24 hours.

Substrate spiking experiments. According to the results of *in situ* rate measurements water samples were taken from above, within, and below the zone of maximal *in situ* carbon dioxide fixation (204, 214, and 226 m water depth, respectively). Carbon dioxide fixation was analyzed as described above and parallel samples were immediately spiked with working solutions (see below) of the following potential electron donors and acceptors: oxygen, sodium nitrate, manganese oxide, iron oxide, AQDS (anthraquinone disulfonate), sodium thiosulfate, polysulfide, sodium sulfide, and sodium dithionite. Working solutions (2.4 mmol L^{-1}) of sodium nitrate, manganese oxide, iron oxide, AQDS, and sodium thiosulfate were prepared by dilution of concentrated sterile stocks (1 mol L^{-1}) into sterile filtered deionized water, filled into butyl rubber-stoppered serum vials, and flushed with argon for at least 15 minutes. Stock solution of polysulfide was made by short heating of 50 mL sodium sulfide solution (1 mol L^{-1}) to 90°C, after addition of 0.11 moles of elemental sulfur under nitrogen atmosphere. Working solutions of polysulfide and sodium sulfide were flushed with argon prior to the addition of concentrated stock solutions. Sodium dithionite is a strong reducing agent and rapidly decomposes in aqueous solution. The working solution was therefore prepared from sodium dithionite crystals directly on board. Oxygen solution was made from sterile-filtered air saturated water. All working solutions were prepared within 4 hours prior to use. One mL of substrate working solutions was injected in the lower part of 120 mL oxygen flasks (final concentration 20 µmol L^{-1}, oxygen approximately 5 µmol L^{-1}) by means of disposable syringes after filling

with sample water and before starting the incubation by addition of [^{14}C]-bicarbonate. All incubations were made in duplicate and analysis of samples was performed as described above.

Sulfide spiking experiments were done to estimate the relation between sulfide concentrations and carbon dioxide fixation. Increasing amounts of sodium sulfide working solution (see above) were added to water samples from the depth of maximum carbon dioxide fixation (216 m water depth) resulting in final sulfide concentrations of 10 to 110 µmol L^{-1}. Sulfide concentrations were confirmed by colorimetric analysis of control samples (see above). Incubations were started and analyzed as described above.

Results

Hydrography. Over a period of nine days CTD casts were taken. Depth profiles of temperature, salinity, and oxygen (sensor data) showed a typical stratification of the water column of the central Gotland Basin (Fig. 2A). A thermocline at around 40 m water depths and a permanent halocline at 75 m separated the water column into three layers: the upper warmer water, the cold winter water, and the long term stagnant deep water. The comparison of temperature and salinity profiles revealed small anomalies below the halocline, indicating recent lateral water intrusions between 80 and 142 m (Fig. 3). Below this depth, only one anomaly due to a major seawater inflow in 2003 was detected.

Chemistry. At the water surface super saturated oxygen concentrations of about 450 µmol L^{-1} were accompanied by nitrate concentrations below the detection limit. Below the halocline, nitrate concentrations increased steadily to 6 µmol L^{-1}, whereas the oxygen concentrations dropped rapidly to 50 µmol L^{-1} (Fig. 2B). Between 100 to 175 m oxygen concentrations decreased slowly and nitrate increased to more than 9 µmol L^{-1}. The pelagic chemocline was found at a depth of about 202 to 204 m. At these depths, the measured oxygen was below 5 µmol L^{-1} and sulfide was below 0.3 µmol L^{-1}. From all depth profiles between 185 m and the chemocline, mean oxygen and nitrate gradients of 0.26 and 0.13 µmol L^{-1} m^{-1}, respectively, were calculated. Below this interface, nitrate was completely depleted and sulfide increased steadily by about 1.1 µmol L^{-1} m^{-1} to approximately 20 µmol L^{-1} at 226 m, accompanied by an increase of ammonium to about 8 µmol L^{-1}.

However, ammonium started to increase 4 meters above a significant increase of sulfide (Fig. 4 A and C). During the sampling campaign, the depth of the chemocline, i. e., the zone of oxygen < 5 µmol L^{-1} and no detectable sulfide, did not change significantly. However, the sulfide concentrations near the sediment surface increased from about 25 to about 50 µmol L^{-1} during the last sampling days (data not shown).

The analysis of thiosulfate and sulfite revealed concentrations below the detection limit of 50 nmol L^{-1} between 180 and 221 m. Zero-valent sulfur, i. e., the sum of cyclo-S_8 sulfur plus sulfane sulfur of polysulfides, was detected at concentrations between 0.8 and 4.2 µmol L^{-1}, with the maximum located at 210 m (Fig. 4A). This was in accordance with microscopic observations of filtered water samples, which revealed the highest density of sulfur globules at this depth.

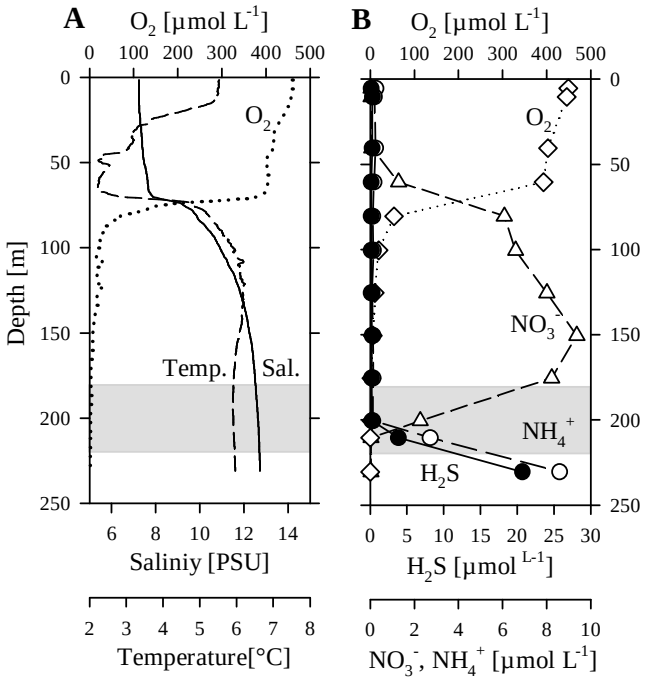

Fig. 2. Depth profiles of temperature, salinity, and oxygen (sensor) (A), oxygen (titrated), nitrate, ammonium and sulfide (B) of the water column at central Gotland Basin (May, 4th 2005). The shaded area denotes the section of the water column shown in figure 4.

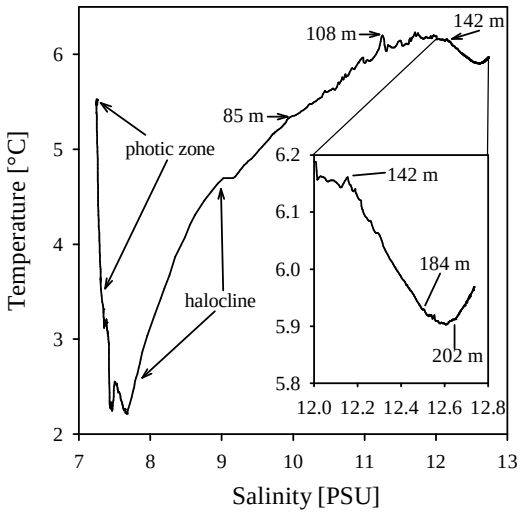

Fig. 3. Temperature - salinity diagram of the whole water column and the part below 133 m water depth (insert) of the central Gotland Basin (May, 4th 2005). Arrows and numbers indicate water depths throughout the depth profile.

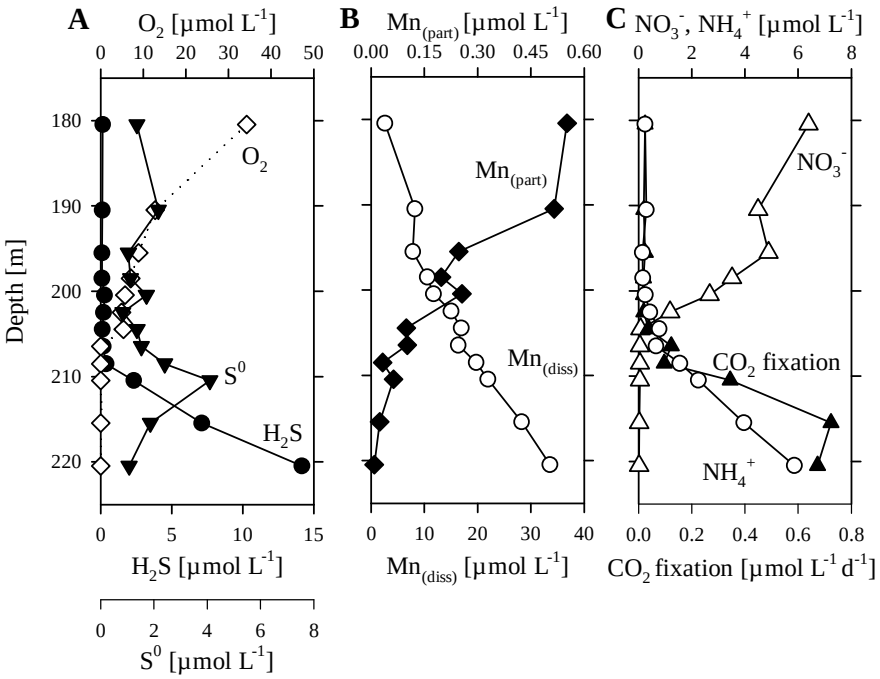

Fig. 4. High-resolution profiles of oxygen, zero-valent sulfur, and sulfide (A), particulate and dissolved manganese (B), as well as nitrate, ammonium and dark carbon dioxide fixation (C) across the redoxcline from 180 to 220 m water depth (May, 4th 2005).

Dissolved and particulate manganese were analyzed and detected between 180 and 230 m. Dissolved manganese increased from 2.5 µmol L^{-1} at 180 m to 32 µmol L^{-1} at 220 m (Fig. 4B). Above the sediment surface, at 230 m the concentration was 42 µmol L^{-1} (data not shown). Particulate manganese decreased from 0.5 µmol L^{-1} above 190 m to about 0.2 µmol L^{-1} at the oxic-anoxic interface. Particulate manganese was also detected below the oxic-anoxic interface. From 208 m down to 220 m, the concentrations decreased from 0.1 to 0.01 µmol L^{-1}.

In situ carbon dioxide dark fixation. From 180 m down to 225 m (Fig. 4C) fixation of carbon dioxide was detectable. Above 205 m however, the fixation rate remained below 0.1 µmol C L^{-1} d^{-1}. Beginning at 205 m, it increased steadily to maximum values between 0.7 – 1.1 µmol C L^{-1} d^{-1} at 215 m water depth. The maximum carbon dioxide fixation was about 10 m below the oxic-anoxic interface within the anoxic environment. Below 215 m a slight decrease was observed down to 220 m followed by an almost complete decline below 225 m.

Three profiles of carbon dioxide fixation were compared to the respective sulfide concentrations (Fig. 5). This comparison shows, that already the presence of small concentrations of sulfide significantly stimulates carbon dioxide fixation in comparison to the situation just above the oxic-anoxic interface.

The peak of CO_2 fixation was always found in a water depth of around 215 m with hydrogen sulfide concentrations between 5 and 10 µmol L^{-1}. Carbon dioxide fixation decreased with further increasing hydrogen sulfide concentrations below 215 m but remained significantly higher than above the chemocline until hydrogen sulfide concentrations exceeded 20 µmol L^{-1} (Fig. 4C and 5). The integrated carbon dioxide fixation in the anoxic layer was about 9.3 mmol C m^{-2} d^{-1}.

Effects of substrate spiking on in situ carbon dioxide dark fixation. Three distinct water layers, (1) the layer of just elevated CO_2 fixation below the chemocline, (2) the layer of maximum CO_2 fixation, and (3) the layer with declining fixation rates, were chosen to test the effect of substrates which may potentially fuel or inhibit autotrophic carbon dioxide fixation.

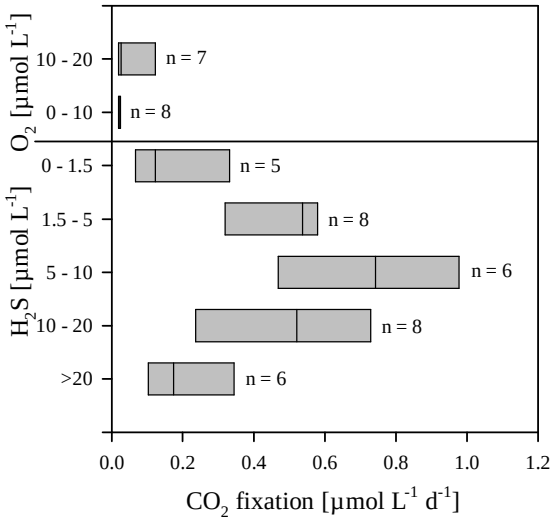

Fig. 5. Box plot of grouped *in situ* dark carbon dioxide fixation rates versus *in situ* concentration ranges of oxygen and sulfide. The number (n) of measurements included in each box is given.

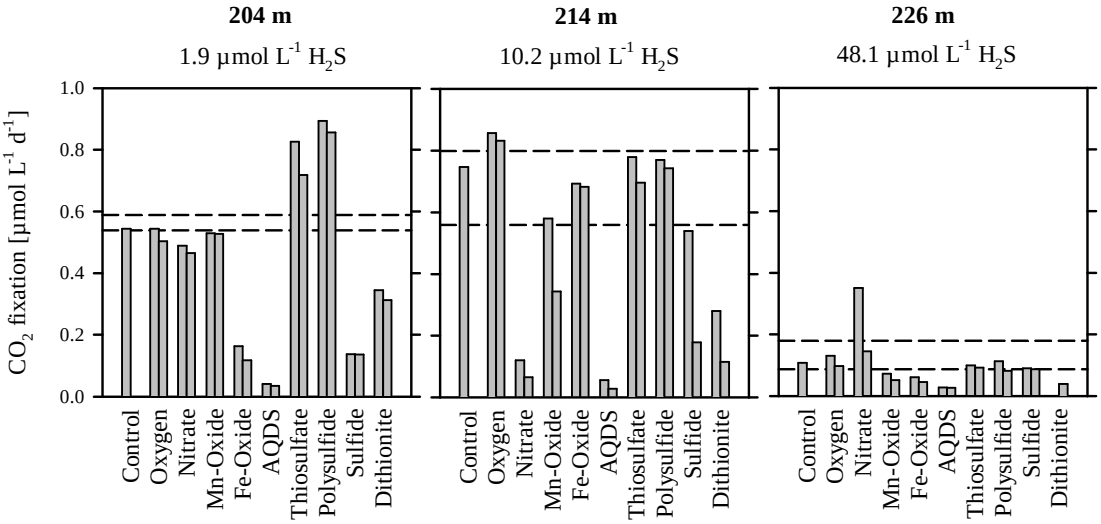

Fig. 6. Effect of single potential electron donors, acceptors, and reducing agents on the dark carbon dioxide fixation rate above (204 m), within (214 m) and below the fixation maximum (226 m depth). Given are the data of two replicate spikings and one control, spiked with nitrogen flushed water. Two parallel determinations of the *in situ* CO_2 fixation are indicated by dashed lines. *In situ* sulfide concentrations are given above each graph.

In water samples from near the oxic-anoxic interface (204 m) with a sulfide concentration of about 2 µmol L^{-1} and *in situ* carbon dioxide fixation of 0.54 and 0.59 µmol C L^{-1} d^{-1}, stimulation of the fixation rate by 35-50% was observed by the addition of thiosulfate and polysulfide, respectively (Fig. 6). Whereas the addition of oxygen, nitrate, or manganese oxide had neither stimulating nor inhibiting effect, iron oxide, AQDS, sulfide, and dithionite had a strong inhibitory effect on the carbon dioxide fixation. The addition of dithionite reduced the fixation rate by 50% to 0.33 µmol C L^{-1} d^{-1}, and iron oxide, sulfide, and hydrogen reduced the rate by 75 to 80% to approximately 0.15 µmol C L^{-1} d^{-1}. AQDS showed the strongest inhibitory effect. Its addition caused a decrease of the rate to less than 0.05 µmol C L^{-1} d^{-1} in all three depths (Fig. 6).

In water samples from the fixation maximum (214 m) or below (226 m), only slight or no stimulation of the *in situ* rate was observed during these experiments. In samples taken from the layer of highest carbon dioxide fixation the *in situ* fixation rates were 0.56 and 0.8 µmol C L^{-1} d^{-1}. The addition of oxygen, thiosulfate, polysulfide, and iron oxide had neither stimulating, nor inhibiting effect to samples from this depth. On the other hand, nitrate, and manganese oxide (one parallel), as well as sulfide (one parallel) and dithionite, significantly reduced the fixation rate (from 30 to more than 80% reduction of the *in situ* rate) (Fig. 6).

Water samples from 226 m with 48 µmol L^{-1} sulfide and an *in situ* fixation of 0.09 and 0.18 µmol C L^{-1} d^{-1} carbon dioxide fixation were not significantly stimulated by any of the added electron donating or electron accepting compounds. The addition of nitrate led to an increase of fixation by 0.2 µmol C L^{-1} d^{-1} in one incubation. The addition of manganese oxide, iron oxide, AQDS, and dithionite caused a reduction of the *in situ* carbon dioxide fixation rate (Fig. 6).

Effect of sulfide on in situ carbon dioxide fixation. As described above, the *in situ* carbon dioxide fixation decreased, when sulfide concentrations exceeded 25 µmol L^{-1}. This phenomenon was further addressed by spiking several parallel samples from the depth of fixation maximum with increasing amounts of sodium sulfide (Fig. 7). At *in situ* sulfide concentrations of 11 µmol L^{-1}, the fixation was between 0.57 and 0.8 µmol C L^{-1} d^{-1}. The addition of 10 µmol L^{-1} sulfide had neither increasing, nor decreasing effect on the carbon dioxide fixation. However, the fixation rate already decreased in samples, which received additional 20 µmol L^{-1}, and declined to values below 0.2 µmol C L^{-1} d^{-1} at added sulfide concentrations of more than 30 µmol L^{-1} (Fig. 7).

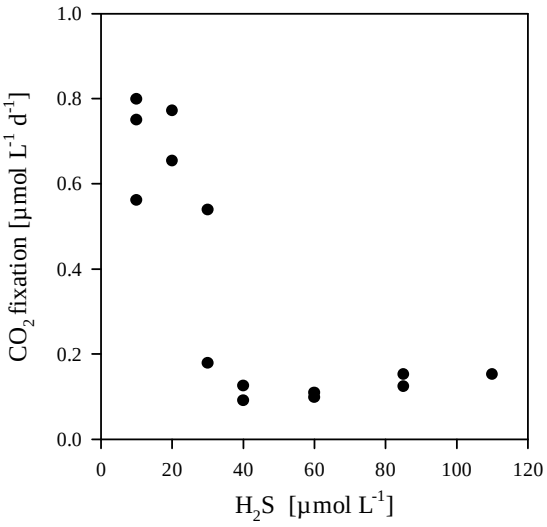

Fig. 7. Effect of sulfide on the dark carbon dioxide fixation in samples from the fixation maximum. Given are the CO_2 fixation rates in relation to the final sulfide concentrations (11 µmol L^{-1} H_2S *in situ* concentration plus spiking).

Discussion

Hydrographic and chemical characteristics of the Gotland Basin. The hydrographical structure of the central Gotland Basin is characterized by a thermo-haline stratification [17, 49]. Below the permanent halocline between 60 and 80 m water and nutrient exchange occur only by lateral mixing or eddy diffusion [49]. Following a stagnant period of ten years, a large inflow of oxygenated seawater into the deep basin and a complete oxidation of the sulfidic bottom water were recorded during spring 2003 [17]. Since summer 2003, stagnant conditions have been observed and sulfidic conditions began to reestablish in the bottom water (Fig. 2 and 3). The oxic-anoxic interface moved from the sediment surface upwards to 225 m in August 2004 [37]. During the present study in May 2005, the chemocline was located between 202 to 204 m. The rise of the chemocline by about 30 m per year demonstrates the electron acceptor limitation, i. e., oxygen limitation, of the stagnant water body below the halocline.

Due to the proximity of the chemocline to the sediment surface, we expected steep gradients of sulfide and ammonium. However, assuming a mixing coefficient of about 0.053 cm^2 s^{-1} [59], the ammonium, and hydrogen sulfide fluxes were about 0.17 and 0.52 mmol $m^{-2}d^{-1}$, respectively. These fluxes were in the same range as in the years 1999 to

2001, when the chemocline was located between 120 and 140 m water depth [49]. Sulfide oxidation was additionally indicated by the presence of zero-valent sulfur throughout the redoxcline even in the absence of oxygen and nitrate (Fig. 4A). However, thiosulfate and sulfite were not detectable. Similar settings, even though with lower concentrations of zero-valent sulfur have been observed at the Black Sea and other anoxic basins [33, 41, 79]. Zopfi et al. [79] reported that concentrations of sulfur intermediates increased during enhanced mixing of oxygenated and sulfidic water, and afterwards decreased remarkably within days. In turn, the low concentrations of thiosulfate and sulfite observed during the present study, may be explained by a stable setting of the oxic-anoxic interface (Fig. 3), where sulfur transformations are preferentially mediated by microbial activity and lead to a rapid depletion of these sulfur compounds readily accessible by a broad range of autotrophic and heterotrophic microorganisms.

Dissolved and particulate manganese around the chemocline. During the renewal of the bottom water and associated oxygenation of sulfidic water layers, like in January 2003 [17], the entire manganese pool is oxidized and large amounts of particulate manganese oxides accumulate at the sediment surface [25, 28]. The concentration maxima of dissolved and particulate manganese around the chemocline were about two times higher than in 2002, when the chemocline was located at about 130 m water depth. Usually, similar concentrations of manganese oxides of more than 1 µmol L^{-1} have only been observed in much smaller or shallower water bodies [e. g. 67, 76, 79]. In the Black Sea, similar manganese oxide concentrations have been reported at near shelf stations [57, 66] whereas lower concentrations were recorded at off shore stations [38, 51]. Thus, the potential for manganese cycling across the chemocline in this study is among the largest known for pelagic systems. The data presented here, additionally show that detectable amounts of particulate manganese are present up to ten meters within the sulfidic zone. To our knowledge, this has not been reported from any pelagic redoxcline before. Usually, particulate manganese disappeared immediately at the oxic-anoxic interface [e. g. 49, 79]. Although, this discrepancy remains puzzling, a possible explanation could be that during collection of particulate manganese by membrane filtration, sulfide present in the samples is filtered through the manganese particles on top of the filter surface and speed up manganese dissolution during the filtration procedure. Detection of particulate manganese within sulfidic water may thus rely on filter size, filtration volume, as well as the amount of particulate manganese present in those water samples. A comparatively large pool of

particulate manganese associated with low sulfide concentrations could have allowed its detection during the present study below the chemocline.

Carbon dioxide dark fixation below the chemocline. In the present study, carbon dioxide fixation was analyzed at high resolution along the oxygen and sulfide gradients. Our data suggest that in spring 2005, only the CO_2 fixation directly at the oxic-anoxic interface could be related to the activity of aerobic or denitrifying bacteria. Oxygen and nitrate concentrations dropped below the detection limit at 204 m water depth (Fig. 4A and C). The fluxes of both electron acceptors were about 0.33 and 0.13 mmol m^{-2} d^{-1}, respectively. Thus, the oxygen flux provided about 65% and the nitrate flux about 35% of the electron uptake capacity. The corresponding CO_2 fixation was about 0.03 mmol C m^{-3} d^{-1}. In this layer, significant carbon dioxide assimilation could be expected due to the activity of aerobic ammonia-oxidizing bacteria. Given that nitrifying bacteria oxidize about 8.3 moles of ammonia per mole CO_2 assimilated into biomass [4], the calculated ammonia flux of 0.17 mmol m^{-2} d^{-1} could account for carbon dioxide assimilation of 0.01 mmol C m^{-3} d^{-1} within a two meters layer of active ammonia-oxidizing bacteria. Thus, one third of the detected inorganic carbon assimilation in this layer could be due to the activity of ammonia-oxidizing bacteria, whereas the remaining carbon dioxide assimilation may be due to aerobic as well as nitrate-dependent sulfur-oxidizing bacteria.

The detected maximum CO_2 fixation rates between 0.7 of 1.1 µmol C L^{-1} d^{-1} were in the same range as reported from other pelagic oxic-anoxic interfaces [33, 43, 65, 79] (Table 1). Also, the fixation maximum was located significantly below the oxic-anoxic interface and was more than twentyfold higher than directly at the interface. In contrast, Gocke [22] and Detmer *et al.* [14] reported a distinct peak of about 4.5 µmol C L^{-1} d^{-1} near the oxic-anoxic interface of the Gotland Basin and a broader secondary maximum below this layer of about 2 µmol C L^{-1} d^{-1}. At that time, the interface was located at 130 m, and higher maximum values of carbon dioxide fixation were related to higher areal fixation rates as well as higher sulfide fluxes. The maximum carbon dioxide fixation at the Black Sea redoxcline seems in general to be slightly lower, between 0.2 and 0.5 µmol C L^{-1} d^{-1} [33, 62] (Table 1). Significantly higher fixation rates between 5.8 and 11.2 µmol C L^{-1} d^{-1}, as well as up to 86 µmol C L^{-1} d^{-1} were reported from Framvaren Fjord and Mariager Fjord, respectively, which showed a much steeper gradient of hydrogen sulfide [43, 79] (Table 1).

In the present study, the zone of maximum carbon dioxide assimilation in the Central Baltic Sea was consistently found about ten meters below the oxic-anoxic interface and, therefore, clearly under anoxic conditions (Fig. 4 and 5). This was also shown for other ecosystems [33, 43, 65, 74, 79]. A comparison of reported sulfide fluxes and carbon dioxide fixation rates revealed a mean ratio of about 20 µmol CO_2 fixation per µmol H_2S diffusion. This value exceeded reported ratios for microbial non-photosynthetic carbon dioxide fixation by a factor of 100 [33, 43, 62], suggesting that either the calculated sulfide flux is strongly underestimated or the primary sulfide flux would not substantially support microbial chemoautotrophy (see below). However, studies reporting significantly higher sulfide fluxes also reported higher CO_2 fixation rates [43, 79]. Thus, even in those environments the sulfide flux is far from providing enough electron donors for the detected chemoautotrophic CO_2 fixation.

Stimulation and inhibition of in situ CO_2 dark fixation. Spiking experiments with single electron donors or acceptors were conducted with water samples from above, within, and below the maximum carbon dioxide fixation to find out, if chemolithoautotrophic microorganisms may be limited by either electron donating (above) or electron accepting compounds (below the fixation maximum).

The only significant stimulation observed during our experiments was due to the addition of the potential electron donating compounds thiosulfate and polysulfide above the fixation maximum, pointing towards a possible electron donor limitation in this layer. Interestingly, sulfide and dithionite additions caused a significant decrease of CO_2 fixation in all three depths. None of the electron donating compounds stimulated CO_2 fixation at the fixation maximum as well as ten meters below the maximum (Fig. 6). These results indicate that sulfide concentrations higher than 30 µmol L^{-1} itself, significant changes of the redox potential, or a combination of both may strongly affect microbial CO_2 fixation below the chemocline.

Surprisingly, none of the tested electron accepting compounds significantly stimulated carbon dioxide fixation (Fig. 6). A possible explanation could be that above the fixation maximum, electron acceptors may not limit growth of chemolithoautotrophic microorganisms and therefore cause no stimulation of CO_2 fixation. Within the fixation maximum no stimulation of autotrophic activity was expected, as electron donating and accepting compounds should be available in balanced amounts. The addition of single electron donating or -accepting compounds could thus not lead to increased carbon dioxide

assimilation. The inhibitory effect of nitrate addition at the fixation maximum seems conspicuous and could only be attributed to facultative microorganisms, which might exhibit a lag phase while switching from one electron acceptor to another. This effect, however also supports the hypothesis, that nitrate is not the dominating electron acceptor for autotrophic microorganisms at the carbon dioxide fixation maximum *in situ*.

Below the fixation maximum, it was expected that autotrophic microorganisms could be limited by the availability of electron accepting compounds, but surprisingly, none of the tested electron acceptors caused increased carbon dioxide assimilation. The absence of oxygen and nitrate in this layer may explain why these electron acceptors cause no stimulating effect, whereas such stimulation has previously been observed in long-term enrichment experiments [37], where significant microbial growth is possible. In contrast, particulate manganese was detected down to the fixation maximum. An addition of manganese oxide particles could therefore cause an increase of autotrophic activity below the fixation maximum. Yet, this effect was not observed during the present study. On the one hand, this may be explained by the chemical structure of the synthetic manganese oxide particles employed. On the other hand, in samples from the oxic-anoxic interface of the Gotland Basin analyzed by fluorescence microscopy, we found natural manganese rich particles to be highly colonized by microorganisms (data not shown). The addition of sterile manganese oxide particles may not provide a significant amount of available electron acceptor, and thus stimulate carbon dioxide assimilation, unless bacteria have successfully colonized these particles.

The *in situ* CO_2 fixation maximum at the Central Baltic Sea was restricted to sulfide concentrations below 25 $\mu mol\ L^{-1}$ (Fig. 7). A similar restriction of carbon dioxide assimilation was found at the Black Sea redoxcline [33] and surprisingly also at the Mariager Fjord, where significantly steeper gradients and an about sixtyfold higher sulfide flux has been determined [79]. The results of our sulfide spiking experiments (Fig. 7) demonstrate that carbon dioxide fixation at its maximum rate at 215 m water depth was also strongly susceptible to sulfide concentrations higher than 30 $\mu mol\ L^{-1}$, indicating that increasing sulfide concentrations may be responsible for decreasing carbon dioxide fixation rates in deeper water layers below 220 m. This is also supported by the inhibitory effect of dithionite addition observed in our spiking experiments (Fig. 6). Dithionite acts as a reducing agent and significantly lowers the redox potential and additionally facilitates chemical dissolution of manganese oxides.

The detected susceptibility to high sulfide concentrations may not contradict the importance of sulfide oxidation for chemolithotrophic microbial CO_2 fixation. Jørgensen *et al*. [33] determined a surprisingly reasonable molar ratio of ^{35}S-sulfide oxidation to carbon dioxide fixation below the Black Sea chemocline. In our carbon dioxide assimilation experiments, all control experiments poisoned by formaldehyde showed negligible CO_2 fixation, contradicting chemical effects. In our spiking experiments, we additionally included anthraquinone disulfonate (AQDS). AQDS contains quinone-moieties and serves as an electron shuttle, e. g. by mediating the electron transfer between bacteria and metal oxide surfaces [3, 11, 50, 55]. It therefore serves as an alternative electron acceptor for a variety of bacteria [e. g. 3, 12, 13, 50]. AQDS is also immediately reduced chemically in the presence of sulfide. It thus removes free sulfide rapidly and provides an electron short circuit between sulfide and metal oxides. In our experiments, AQDS caused the strongest reduction of carbon dioxide fixation above, within, and below the fixation maximum (Fig. 6), supporting the hypothesis that sulfide and metal species may be involved in the metabolism of chemolithoautotrophs *in situ*.

Taken together, our spiking experiments suggest that carbon dioxide fixation of chemolithoautotrophic microorganisms below the oxic-anoxic interface of the Gotland Basin was related to a redox-sensitive sulfur oxidation. Stimulation was observed only by addition of reduced sulfur compounds, which do not significantly alter the apparent redox potential. Intentional lowering of the redox potential by sulfide or dithionite addition, as well as chemical electron shuttling from sulfide to metal surfaces induced by AQDS addition strongly impaired carbon dioxide fixation.

Chemical versus biological oxidation of sulfide in the presence of manganese oxide. Several studies have provided strong evidence that chemical oxidation of free sulfide, as well as iron sulfide, and pyrite by manganese oxide is fast, if not even spontaneous [1, 6, 10, 56, 68, 69, 77]. Consequently, a direct microbial coupling of sulfide oxidation and manganese reduction has not been considered to compete chemical sulfide oxidation. Moreover, chemical sulfide oxidation in sediments has been shown to be fast enough to support microbial sulfur disproportionation [6, 19, 69].

Yao and Millero [77] established the kinetic behavior of chemical sulfide oxidation ($-d[H_2S]_T/dt$) by manganese oxide in seawater as to be first order with respect to total sulfide ($[H_2S]_T$) and manganese oxide concentrations ($[MnO_2]$):

$$-d[H_2S]_T / dt = k * [H_2S]_T * [MnO_2] \quad (1)$$

The rate constant *k* was determined to decrease with temperature from 436 M^{-1} min^{-1} at 25°C to 285 M^{-1} min^{-1} at 5°C, respectively [77]. Following this equation, sulfide oxidation rates of 7.2 µmol cm^{-3} d^{-1} have been estimated for manganese-rich marine sediments [68]. During the studies mentioned above, the high rates of chemical manganese-dependent sulfide oxidation were observed at sulfide and manganese oxide concentrations in the micromolar or even millimolar range, typical for manganese-rich marine sediments [68]. However, throughout the oxic-anoxic interface of the Gotland Basin, sulfide concentrations of up to 10 µmol L^{-1} and manganese oxide concentrations of 0.1 down to 0.02 µmol L^{-1} at 6°C would result in a chemical manganese-dependent sulfide oxidation between 0.05 and 0.07 µmol L^{-1} d^{-1}. The chemical manganese oxide-dependent sulfide oxidation would thus account for a daily oxidation of less than one percent of the present sulfide pool. This demonstrates that in contrast to marine sediments microorganisms could potentially compete with chemical sulfide oxidation at pelagic oxic-anoxic interfaces.

Complete and incomplete microbial sulfide oxidation. Anaerobic oxidation of sulfide has been proven to act via reduction of nitrate and several microorganisms have been described turning out this reaction [35, 71, 72]. However, the data presented here suggest, that nitrate is not the predominant electron acceptor for autotrophic microorganisms at the fixation maximum in the Gotland Basin. It has been described, that in the absence of oxygen and nitrate, microorganisms catalyze the autotrophic disproportionation of sulfur species [2]. And a connection of the marine sulfur and metal cycles has been found to act via microbial sulfur disproportionation reactions [69]. In marine sediments, the disproportionation of sulfur species becomes energetically favorable, if sulfide is efficiently trapped as FeS or rapidly oxidized chemically [68]. However, under *in situ* conditions at the fixation maximum the chemical oxidation of sulfide by manganese oxide is far too slow (see calculations above) to supply sufficient sulfur intermediates for the energetically unfavorable sulfur disproportionation by autotrophic microorganisms (reactions 14 to 16, Table 2) to account for the detected *in situ* carbon dioxide fixation.

Table 1: Comparison of dark carbon dioxide assimilation and sulfide gradients reported from five marine pelagic redoxclines. Hydrogen sulfide gradients were estimated or taken from the respective literature, because flux calculations were not always reported.

Site	**Maximal rate CO_2-fixation [µmol C L^{-1} d^{-1}]**	**Total assimilation CO_2-fixation [mmol C m^{-2} d^{-1}]**	**Integrated depth [m]**	**H_2S flux [mmol S m^{-2} d^{-1}]**	**H_2S gradient [µmol L^{-1} m^{-1}]**	**Reference**
Black Sea	0.19	2.0	14	0.2 [1)]	0.68	[33]
	0.32	5.3	25	n.r. [6)]	0.51	[33]
Baltic Sea	4.5 [2)]	36 [2)]	23 [2)]	n.r.	2.0 [2)]	[22]
	4.6 [3)]	34 [3)]	23 [3)]	n.r.	3.6 [3)]	[14]
	1.1	9.3	21	0.52	1.1	This study
Cariaco Basin	0.33	14.2	136	n.r.	n.r	[74]
	0.4 - 2.5	26 - 157	80 - 100	0.71 - 1.35	0.08 - 0.18	[65]
Mariager Fjord	86	10.4	0.3	6.11	> 65 [4)]	[79]
Framvaren Fjord	5.8 / 11.2	n.r.	n.r.	n.r.	> 50 [5)]	[43]

[1)] From Brewer & Spencer (1974) cited in [33]
[2)] Estimated from Gocke [22, figure 2].
[3)] Estimated from Detmer et al. [14, figure 2].
[4)] Estimated from Zopfi [79, figure 6].
[5)] From Millero [44].
[6)] N.r., not reported.

Table 2: Potential anaerobic sulfur oxidation reactions involving nitrate and manganese oxide, as well as disproportionation reactions of sulfur and thiosulfate. For each reaction the Gibbs standard free energy change (pH 7; 25°C) is given.

	Reaction			ΔG_0' [kJ mol^{-1}][1]
(2)	$5\ S_2O_3^{2-} + 8\ NO_3^- + H_2O$	=>	$10\ SO_4^{2-} + 4\ N_2 + 2\ H^+$	- 383.1
(3)	$5\ S^0 + 6\ NO_3^- + 2\ H_2O$	=>	$5\ SO_4^{2-} + 3\ N_2 + 4\ H^+$	- 548.0
(4)	$10\ S^0 + 4\ NO_3^- + 3\ H_2O$	=>	$5\ S_2O_3^{2-} + 2\ N_2 + 6\ H^+$	- 164.9
(5)	$5\ HS^- + 8\ NO_3^- + 3\ H^+$	=>	$5\ SO_4^{2-} + 4\ N_2 + 4\ H_2O$	- 744.3
(6)	$10\ HS^- + 8\ NO_3^- + 8\ H^+$	=>	$5\ S_2O_3^{2-} + 4\ N_2 + 9\ H_2O$	- 361.3
(7)	$5\ HS^- + 2\ NO_3^- + 7\ H^+$	=>	$5\ S^0 + 1\ N_2 + 6\ H_2O$	- 196.4
(8)	$S_2O_3^{2-} + 4\ MnO_2 + 6\ H^+$	=>	$2\ SO_4^{2-} + 4\ Mn^{2+} + 3\ H_2O$	- 274.0
(9)	$S^0 + 3\ MnO_2 + 4\ H^+$	=>	$SO_4^{2-} + 3\ Mn^{2+} + 2\ H_2O$	- 384.3
(10)	$2\ S^0 + 2\ MnO_2 + 2\ H^+$	=>	$S_2O_3^{2-} + 2\ Mn^{2+} + H_2O$	- 110.4
(11)	$HS^- + 4\ MnO_2 + 7\ H^+$	=>	$SO_4^{2-} + 4\ Mn^{2+} + 4\ H_2O$	- 526.1
(12)	$2\ HS^- + 4\ MnO_2 + 8\ H^+$	=>	$S_2O_3^{2-} + 4\ Mn^{2+} + 5\ H_2O$	- 252.2
(13)	$HS^- + MnO_2 + 3\ H^+$	=>	$S^0 + Mn^{2+} + 2\ H_2O$	- 141.7
(14)	$S_2O_3^{2-} + H_2O$	=>	$SO_4^{2-} + HS^- + H^+$	- 10.9
(15)	$4\ S^0 + 4\ H_2O$	=>	$SO_4^{2-} + 3\ HS^- + 5\ H^+$	+ 10.3
(16)	$4\ S^0 + 3\ H_2O$	=>	$S_2O_3^{2-} + 2\ HS^- + 4\ H^+$	+ 15.7

[1] Refers to the Gibbs free energy change of one mole S atoms oxidized or reduced. Thermodynamic constants from Thauer *et al.* [70], Stumm and Morgan [64], Lide [39].

Jørgensen [32] argued that metal oxides, such as manganese oxide or iron oxide could serve as the terminal electron acceptors for sulfur oxidizing bacteria under such conditions. This view is supported by the detection of particulate manganese down to the CO_2 fixation maximum at the central Gotland Basin (Fig. 4B), by the observed susceptibility to high sulfide concentrations and lowering of the redox potential (Fig. 7), as well as by the dense colonization of manganese-rich particles. However, to date there is neither a description of a pure culture turning out one of these reactions, nor a detailed analysis of the microbial assemblage associated to manganese-rich particles *in situ*.

Chemostate experiments with sulfur oxidizing bacteria revealed that *Thiobacillus denitrificans* oxidizes about 2.5 moles of sulfide (~1530 kJ under chemostate conditions) to sulfate (reaction 5, Table 2) to assimilate one mole of carbon dioxide under denitrifying

conditions [72]. An incomplete oxidation of sulfide to thiosulfate under similar conditions would yield approximately half of the energy (reaction 6, Table 2), and thus five moles of sulfide had to be oxidized to thiosulfate per mole carbon dioxide fixed. In comparison, a potential manganese-dependent incomplete oxidation of sulfide to thiosulfate would yield only 33% of the energy (reaction 12, Table 2) and thus the oxidation of about 7.5 moles of sulfide was required to allow the fixation of one mole carbon dioxide. This estimation fits surprisingly well to the experimentally determined ratio of seven to nine moles of sulfide oxidized per mole carbon dioxide fixed and the fact that up to 80% of sulfide was incompletely oxidized to thiosulfate below the chemocline of the Black Sea [32, see above].

Sulfide recycling by heterotrophic bacteria. It seems likely that chemoautotrophic sulfur bacteria are predominantly responsible for the detected dark carbon dioxide assimilation [see above, 32, 65], but the consumption of sulfide by sulfide-oxidizing bacteria exceeds the sulfide flux from the sediment more than 100-fold [32]. It has therefore been argued, that sulfate-reducing bacteria could recycle sulfide via sulfate reduction. However, the sulfate reduction rates determined at the Black Sea, as well as at the Gotland Basin chemocline were very low and could not supply substantial amounts of sulfide [32, L. Neretin, pers. communication].

The calculations above, as well as the ^{35}S-sulfide oxidation experiments by Jørgensen [32] suggest that sulfide-oxidizing bacteria may oxidize sulfide to thiosulfate (reaction 12, Table 2) or elemental sulfur (reaction 13, Table 2), instead of sulfate. Yet, thiosulfate and elemental sulfur can readily serve as an electron acceptor for a broad variety of heterotrophic microorganisms [18, 20, 45, 48, 52, 73]. Such organisms have repeatedly been isolated from oxic-anoxic interface of the Gotland Basin, the Black Sea, and other oxic-anoxic interfaces [e. g. 7, 47, 48, 73]. Additionally, molecular surveys have shown that thiosulfate- and sulfur reducing bacteria are among the abundant members of the microbial communities below the chemoclines of anoxic basins [37, 42, 75]. It is thus likely, that heterotrophic sulfur-reducing bacteria, instead of sulfate-reducing bacteria, are responsible for the recycling of sulfide and that sulfate reduction rates significantly underestimate the potential of sulfide recycling by heterotrophic bacteria in such environments (Fig. 8).

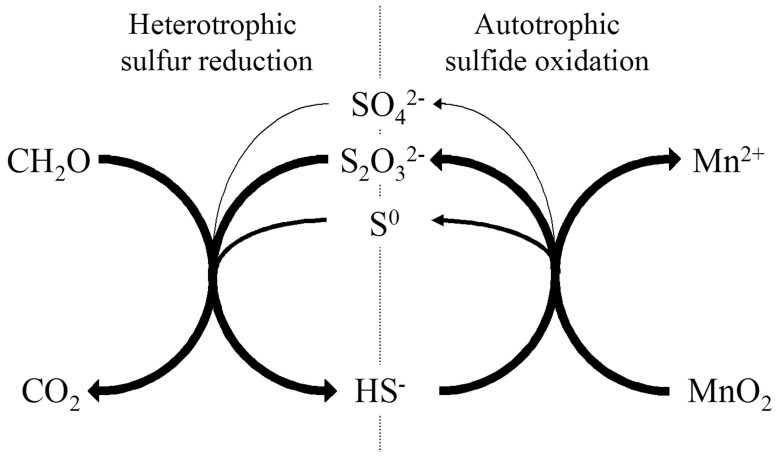

Fig. 8. Proposed scheme of the interaction between incomplete hydrogen sulfide oxidation coupled to manganese reduction and heterotrophic sulfur reduction at the dark CO_2 fixation maximum below the redoxcline of the central Gotland Basin.

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2.4 Organotrophic and lithotrophic microbial communities along chemical gradients at the chemocline of the Central Baltic Sea

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(in Bearbeitung)

Organotrophic and lithotrophic microbial communities along chemical gradients at the chemocline of the Central Baltic Sea

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Keywords

cultivation, bacteria, heterotrophy, lithotrophy, denitrification, manganese, oxic-anoxic interface, chemocline

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Abstract

The vertical distribution of organotrophic and lithotrophic microbial communities throughout the water column of the Gotland Basin was investigated by an integrated cultivation approach based on miniaturised Most Probable Number (MPN) dilution series. Sensitive quantification of microbial growth was combined with chemical and molecular analysis of selected enrichments. High viable counts of aerobic organotrophic bacterioplankton were predominantly obtained from the photic zone and from around the chemocline at 204 m depth (>1.5 x 10^6 cells ml^{-1}). At the chemocline up to 10^5 cells ml^{-1} of manganese-reducing or fermenting bacteria were stimulated to grow, while MPN counts of nitrate reducers reached 10^4 ml^{-1}. Numbers of sulphate-reducing bacteria were just above the detection limit. Aerobic sulphur oxidisers were detected throughout the water column with maximum numbers of about 7.5 x 10^5 cells per ml at 100 m depth and around the chemocline. Nitrate-dependent sulphur oxidisers were detected in similar numbers but appeared to be more restricted to the chemocline, with maximum numbers in the zone of maximum dark carbon dioxide fixation at 215 m water depth. Thiosulphate disproportionating bacteria were detected only in very low numbers in the anoxic layers at 215 and 220 m depth, while nitrifying enrichments were obtained exclusively from 40 m depth. Molecular analysis of enrichments from above (195 m) and below the oxic-anoxic interface (215 m) revealed the presence of eleven separate phylotypes affiliated with the *Alpha-* and *Gamma-*, and *Epsilonproteobacteria*, of which only two were found in enrichments from both layers. The results of the present study indicate that microbial communities in the water column of the Gotland Basin are highly structured, even on a small vertical scale throughout the chemocline. The heterotrophic microbial community at the chemocline apparently harbours a significant fraction of facultatively lithotrophic sulphur-oxidising microorganisms.

Introduction

Aquatic microbial communities harbour a large diversity of microorganisms that is now routinely investigated based on 16S rRNA gene sequence analysis (e.g. Rappé & Giovannoni, 2003). Direct insights into the metabolic diversity of environmental microbial assemblages are however still scarce. Only in a few cases it has yet been possible to obtain clues about microbial metabolisms *in situ* by means of molecular approaches (Allen & Banfield, 2005; Béjà *et al.*, 2000; Sharkey *et al.*, 2004). Hence, insights into metabolic properties of

phylotypes abundant in the environment still often rely on inferring phenotypic properties from those cultured close relatives, if this is possible at all. Therefore, the cultivation and unravelling of the metabolism of novel microorganisms still remains an important task in microbial ecology (Zengler *et al.*, 2002). Isolation of microorganisms is usually laborious and rarely gains *in situ* abundant phylotypes (Amann *et al.*, 1995). Recent advances, however, have been made by the application of sensitive techniques for the detection of growth in combination with providing close to *in situ* growth conditions, what has helped to stimulate growth of several so far uncultured types and to bring a larger diversity into culture (Connon & Giovannoni, 2002; Kaeberlein *et al.*, 2002; Rappé *et al.*, 2002). On the other hand, it has been shown that the systematic variation of cultivation conditions can also increase the cultivation success (Köpke *et al.*, 2005; Stevenson *et al.*, 2004).

During the present study, an integrated cultivation approach has been used to gain insights into the metabolic diversity of organotrophic and lithotrophic microbial communities along the water column of the Gotland Basin. The Central Baltic Sea represents a physically and chemically highly structured environment (Aitsam *et al.*, 1984; Feistel *et al.*, 2003). A thermocline developing usually from April to October separates the surface layer that contains the photic zone with its active phytoplankton assemblage from permanently cold intermediate layer characterised by significantly low microbial abundance and activity. A stagnant deep-water body extends from a permanent halocline found at 60 to 100 m depth down to the sediment (Rheinheimer *et al.*, 1989; Schneider *et al.*, 2002). These deep layers are typically characterised by low microbial activities that are significantly increased only after oxygen is depleted and the sediment releases sulphide and other electron donors fuelling lithotrophic microbial communities at the oxic-anoxic interface (Rheinheimer *et al.*, 1989). It was shown that microbial chemosynthesis at such pelagic chemoclines can provide a significant carbon source for the microbial food web (Taylor *et al.*, 2001). As a consequence, the composition of microbial assemblages along the chemical gradients differs remarkably (Madrid, 2000; Vetriani *et al.*, 2003). Knowledge on the vertical structure of the water column and the respective chemical gradients can help to identify horizons of specific metabolic activities (Jørgensen *et al.*, 1991; Rheinheimer *et al.*, 1989) and hence should provide guidance for developing target-oriented cultivation assays.

In a previous study on the Gotland Deep, cultivation-based methods targeting different physiological types likes aerobes, nitrate or manganese reducers were applied to samples taken with high spatial resolution from the different parts of the chemocline. Heterotrophic populations exhibited a vertical sequence with the subpopulations utilising different electron

acceptors reflecting the respective chemical gradients. Lateral intrusions of oxygen-rich water, however, lead to an almost complete disappearance of this facultatively anaerobic community (Martens-Habbena *et al.*, submitted). Chemolithoautotrophic bacteria were not detected, although there is ample indication for microbially mediated oxidation of manganese, sulphide and ammonium (Bauer, 2003; Brettar & Rheinheimer, 1991; Neretin *et al.*, 2003). As a consequence, in the present study, we have accordingly modified the set of substrate combinations, targeting aerobic and anaerobic organotrophic as well as lithotrophic bacteria. However, lithoheterotrophic bacteria might also play an important role *in situ* and it can be expected that they yield only low cell densities in the respective media but on the other hand would be detectable by their activities. Therefore, microbial growth in the MPN series was quantified with high sensitivity (Martens-Habbena & Sass, 2006) and most assays were also analysed for substrate turnover and finally subjected to molecular analysis for identification of the enriched microorganisms.

Material and Methods

Study area and sampling. Water samples were collected from the Gotland Basin (Baltic Sea) during cruise AL256 with the RV "Alkor" in Mai 2005. Samples were collected using rosette samplers equipped with online sensors for conductivity, density, temperature, and turbidity (Sea-Bird Electronics Inc., Washington, USA) and twelve 5 Liter Goflow bottles (Hydrobios, Kiel, Germany). Water samples for chemical analyses and cultivation experiments were taken directly upon sample retrieval. Samples for cultivation procedures were taken from the sampling bottles and directly filled into autoclaved glass tubes with at least five volumes overflow and sealed without air access.

Chemical analyses *in situ*. Determination of dissolved oxygen, sulphide, nitrate, nitrite, and ammonia was carried out manually as described (Grasshoff *et al.*, 1999). Dissolved and particulate manganese were determined as follows. One litre of water sample was filtered through nucleopore filters (0.45 µm poresize, Millipore, Göttingen, Germany). The filtrate was acidified by addition of 0.02 volumes redistilled HNO_3 and stored in screw-capped polyethylene bottles, preconditioned with redistilled HNO_3 for the determination of dissolved manganese. Analysis was carried out in the laboratory by means of ICP-OES as described in detail elsewhere (Hinrichs *et al.*, 2002). The filters containing particulate manganese were rinsed with 18 MΩ water and stored in sterile plastic Petri dishes until further treatment in the

laboratory. The filters were completely digested in a mixture of HNO_3, $HClO_4$, and HF in closed PTFE autoclaves and manganese was analysed by means of ICP-OES.

Carbon dioxide assimilation. Dark CO_2 fixation rates were determined according to the method of Steemann Nielsen (Steemann Nielsen, 1952). Incubations were performed in 120 ml oxygen bottles, carefully filled with several volumes of sample overflow. Anoxic [^{14}C]-bicarbonate (40 to 60 µl, 250 µCi ml^{-1}) was added from a stock solution prepared with ^{14}C labelled sodium bicarbonate (specific activity 53.0 mCi mM^{-1}, Hartmann Analytic, Braunschweig, Germany). Samples were incubated not longer than 24 hours at 6 ± 1°C in the dark. The exact amount of [^{14}C]-bicarbonate added was determined by mixing of 50 µl sub-samples with 50 µl ethanolamine in scintillation vials. Samples were fixed by filtration onto polycarbonate membrane filters (0.2 µm pore size, VWR International, Weiterstadt, Germany), exposed to HCl fumes for 30 min, mixed with scintillation cocktail (UltimaGold XR, Packard, USA), and counted after return to the laboratory in a TriCarb 2560 TR/X liquid scintillation counter (Packard, USA). Activity of negative controls immediately fixed with 1 ml formaldehyde (37% *v/v*) before adding [^{14}C]-bicarbonate was 0.0043 µM C d^{-1} (standard deviation 0.0029 µmol C l^{-1} d^{-1}).

Growth media for MPN dilution series. Oxic and anoxic sulphate-free artificial brackish water medium was designed according to the salinity present in the deep water of the Gotland Basin (Bruns *et al.*, 2002). The medium contained the following salts (in g l^{-1}) NaCl (5.6), $MgCl_2 \cdot 6H_2O$ (2.3), $CaCl_2 \cdot H_2O$ (0.34), KCl (0.15), KBr (0.023), H_3BO_3 (0.006), $SrCl_2 \cdot 6H_2O$ (0.009), NH_4Cl (0.021), KH_2PO_4 (0.0054), and NaF (0.0007). After autoclaving the mineral salts solution, the oxic and anoxic media were cooled under air or N_2/CO_2 (80/20, v/v), respectively and were aseptically supplemented with 1 ml · l^{-1} trace element solution SL10, 0.2 ml · l^{-1} of a selenite and tungstate solution, 10 ml of a ten vitamin solution, and 30 ml · l^{-1} of a 1 M $NaHCO_3$ (Köpke *et al.*, 2005). The anoxic medium was reduced by addition of Na_2S and acid $FeCl_2$ solutions to final concentrations of 1 mmol l^{-1} and 0.6 mmol l^{-1}, respectively. Finally, the pH of the media was adjusted to 7.2 – 7.4 with sterile HCl or Na_2CO_3. Oxic medium that received organic substrates, was buffered with HEPES (2.4 g · l^{-1}) instead of $NaHCO_3$ and the pH was adjusted to 7.2 – 7.4 prior to autoclaving by addition of NaOH.

Substrate combinations for MPN series. The basal oxic and anoxic mineral media were supplemented with substrates from concentrated sterile stock solutions. For organotrophic microorganisms a monomer substrate mix (Süß *et al.*, 2004) was combined with (final concentration in mM) oxygen (air), sodium nitrate (5), manganese oxide (20), or

sodium sulphate (10). For dilution series targeting fermenting organisms no electron acceptor was added. For dilution series targeting lithotrophic organism the monomer mix was replaced by thiosulfate (5), ammonium chloride (oxic incubations: 5, anoxic incubations: 2), or manganese chloride (5). One set of dilution series was prepared without addition of an electron donor, and combined with the given electron acceptors.

Preparation of MPN dilution series. MPN dilution series were set up with samples from eight water depths (10 m, 40 m, 100 m, 195 m, 202 m, 208 m, 215 m, and 220 m) in 96-deep-well plates (Beckman, Fullerton, CA) with 900 µl growth medium and 100 µl inoculum per well as described previously (Köpke *et al.*, 2005; Süß *et al.*, 2004). A parallel dilution series without inoculum served as contamination control. After inoculation the plates were sealed with sterile lids (CAPMAT, Beckman, Fullerton, CA), placed into gas-tight plastic bags equipped with a gas generating and catalyst system for anoxic conditions (Anaerocult A mini, Merck, Darmstadt, Germany). All cultivation experiments were prepared under nitrogen atmosphere in an anaerobic cabinet (AtmosBag, 280 l, Aldrich, Milwaukee, Wisconsin, USA). Plates intended for oxic incubation were removed from the anaerobic cabinet and subsequently placed into plasic bags on top of wet paper towels to prevent water loss due to evaporation. All MPN series were incubated for 16 weeks at 10ºC in the dark.

Analysis of microbial growth. Growth of microorganisms was quantified by means of SybrGreen I staining of 200-µl sub-samples (Martens-Habbena & Sass, 2006). Deep-well plates were transferred into an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA), removed from the plastic bags and opened. 200 µl of sub-sample were removed aseptically from each well and transferred into black, non-treated 96-well microplates (Nunc F 96, VWR International, Weiterstadt, Germany). Samples were stained by the addition of 50 µl of a freshly prepared 1:2000 dilution of SybrGreen I dye (Sigma-Aldrich, Schnelldorf, Germany) in 0.2 µm sterile-filtered buffer (200 mM Tris, 50 mM Na_2EDTA, pH 7.8). Incubation was performed overnight and fluorescence intensity was subsequently analysed on a fluorescence microplate reader as described previously (Martens-Habbena & Sass, 2006). The varying media composition caused a background fluorescence between 50 and 150 RFU. Growth was scored positive only when fluorescence was exceeding 200 RFU, corresponding to about 1.7×10^6 *E. coli* cells ml^{-1}. The performance of the fluorescent staining procedure was routinely checked by combined light- and fluorescence microscopy directly following fluorescence analysis.

Chemical analyses of MPN dilution series. Selected samples were subjected to analysis of substrate utilisation and product formation. For this, 50-µl sub-samples were taken

from the respective MPN series and diluted as necessary with anoxic water in HPLC autosampler vials. Analysis of nitrite, nitrate, sulphite, sulphate, and thiosulphate was carried out by ion chromatography on a HPLC System (Sykam, Fürstenfeldbruck, Germany) equipped with an anion exchange column (LCA22), autosampler (S5200), column oven (S4110), regeneration unit (S2210/S6330) and conductivity detector (S3110). Anoxic eluent was prepared by carefully flushing a solution of 22 mM sodium hydroxide and 15 % (v/v) ethanol with helium for 15 min. Subsequently, 0.74 g l^{-1} sodium carbonate were added under a stream of helium to avoid air contact. Separation was performed at 70°C and at a flow rate of 1.0 mL min^{-1}. Chromatograms were recorded and analysed using the ChromStar 6.3 software package (SCPA, Weyhe, Germany).

Colorimetric analysis of ammonium and nitrite were done as described elsewhere (Grasshoff *et al.*, 1999).

Isolation of nucleic acids, PCR, DGGE, and DNA sequence analysis. A subset of MPN dilution series inoculated with water sample from 195 m water depth and all dilution series with inoculum from 215 m water depth was selected for the extraction of nucleic acids. Due to the small sample size, it was necessary to pool 100-µl aliquots out of the three parallels of the two highest positive dilutions. Genomic DNA was extracted from the pooled samples using the UltraClean™ Soil DNA Kit (Mobio, Carlsbad, CA, USA). Direct PCR amplification of a 550 bp fragment for subsequent DGGA analysis using the primers GC-341f (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C-CC TAC GGG AGG CAG CAG-3') and 907r (5'-CCGTCAATTCCTTTGAGTTT-3') was not successful. Therefore, the almost entire 16SrRNA gene was PCR-amplified using the primers 8f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3'). The 50-µl PCR mixtures contained 5 µl of 10-fold ThermoPol PCR buffer (New England Bioloabs, Ipswich, MA, USA), 200 µM of each dNTP, 200 nM of each primer (MWG Biotech, Ebersberg, Germany), and 1 unit of Taq DNA polymerase (New England Biolabs). PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany) and subsequently re-amplified using the primers GC341f and 907r.

PCR products were separated by DGGE using the IngenyPHOR U System (Ingeny, Leiden, The Netherlands) as described by Wilms *et al.* (2006). Bands were excised with a sterile scalpel, frozen, crushed, rinsed with water to remove excess of urea and formamide, and DNA was eluted in 50 µl Tris buffer (10 mM Tris-HCl, pH 8) at 4°C overnight. Subsequent re-amplification and sequence determination were done as described elsewhere (Köpke *et al.*, 2005).

A specific PCR protocol targeting a 16S rRNA gene sequence type belonging to the *Epsilonproteobacteria* (Höfle *et al.*, 2005) was employed to screen the MPN dilution series for this uncultured but in situ abundant phylotype. The PCR mixtures were composed as described above and combined with the primers 8f and OST1r (5'-CTT AGC GTC AGT TAT GTT CCA GG-3', Höfle *et al*, 2005). PCR reactions were performed for 35 cycles with an annealing temperature of 66°C. Products of this specific PCR were purified and directly used for cycle sequencing as described above.

Phylogenetic analysis. Partial 16S rRNA gene sequences retrieved from 53 DGGE bands and specific PCR reactions were compared to those in GenBank using the BLAST program (Altschul *et al.*, 1997). Sequences were grouped into operational taxonomic units (OTU) after alignment to their closest database matches using the arb program package (Ludwig *et al.*, 2004).

DNA sequences obtained during this study are available from GenBank under accession no. to .

Results

Chemical gradients. During the present study in May 2005, super-saturated oxygen concentrations of more than 400 µM at the surface layers, were accompanied by very low concentrations of nitrate and ammonia (Fig.1). Beneath the halocline, dissolved oxygen rapidly decreased to values below 50 µM, whereas nitrate increased with depth to about 9 µM at 150 m. Beneath nitrate declined until both oxygen and nitrate approached the detection limit at a water depth of 204 meters. From 205 meters downwards, sulphide was detectable and steadily increased to about 40 µM at the sediment surface. Similarly, ammonium concentrations started to increase at the oxic-anoxic interface to 8.5 µM at 220 meters (Fig. 1).

Dissolved and particulate manganese was analysed in the layers adjacent to the oxic-anoxic interface. Gradients of dissolved and particulate manganese showed a large overlap and extended some tens of meters into the oxic or anoxic layers. Dissolved manganese increased from 2.5 µM at 180 m depth to values around 40 µM at the sediment surface. Particulate manganese, in turn, decreased rapidly from around 0.5 µM detected at 180 m to

less than 0.2 µM at the oxic-anoxic interface. Beneath the chemocline, however, particulate manganese was still detected but rapidly decreased to below 0.01 µM at 220 meters (Fig. 1).

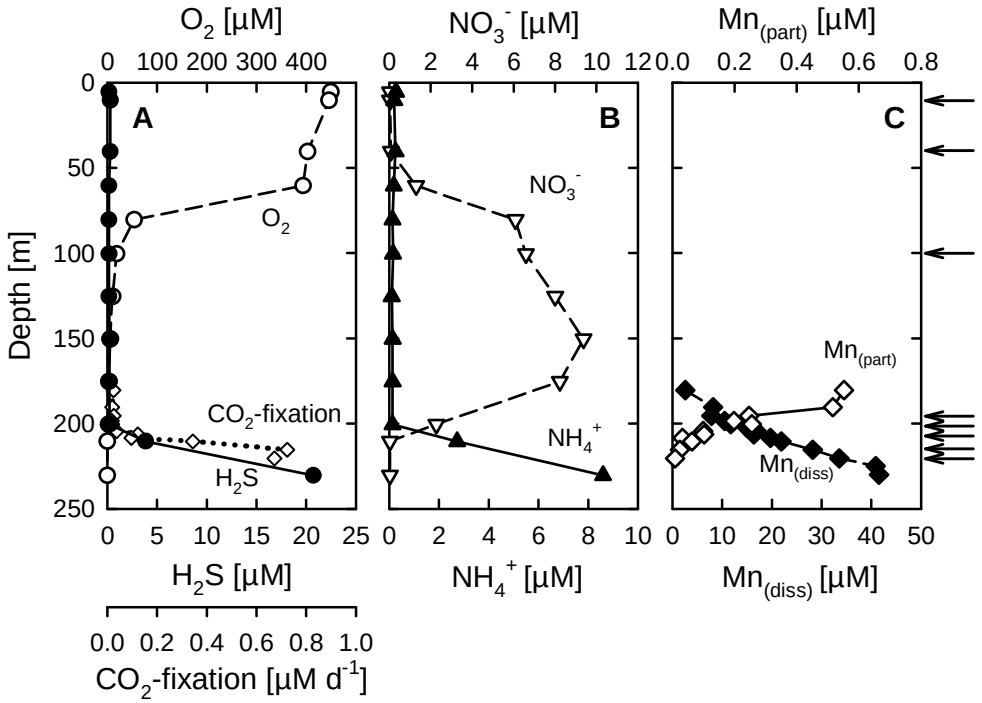

Fig.1. Chemical structure and dark carbon dioxide assimilation at the Gotland Basin in Mai 2005. Given are depth profiles of dissolved oxygen and sulphide, as well as microbial dark carbon dioxide assimilation (A), nitrate and ammonia (B), as well as dissolved [$Mn_{(diss)}$] and particulate manganese [$Mn_{(part)}$] (C). Arrows indicate sampling depth for preparation of Most Probable Number dilution series (see Fig. 2).

Carbon dioxide assimilation. Microbial dark carbon dioxide assimilation was analysed to assess the distribution of active chemoautotrophic bacteria. Above the chemocline, in the presence of dissolved oxygen, carbon dioxide assimilation activity was generally around 0.02 µM d^{-1} and slightly increased to about 0.04 µM d^{-1} at the oxic-anoxic interface. Beneath the interface, a further increase in carbon dioxide assimilation rates was found, reaching maximum values around 0.7 µM d^{-1} at 215 to 220 m depth. Below 220 m the rates decreased again to values around 0.3 µM d^{-1} (not shown).

MPN counts of organotrophic microorganisms. Highest viable counts of organotrophic microorganisms were obtained after oxic incubation and using the monomer mix as electron donor and carbon source. More than 10^6 cells ml^{-1} were detected in the surface layer, as well as above (195 m) and beneath the oxic-anoxic interface (215 m, 220 m). MPN counts obtained with samples from 40, 100, and 208 m were about one order of magnitude lower and did not significantly differ from those without electron donor (Fig.2).

Significantly lower counts were observed in the different anoxic incubations. Highest MPN counts with nitrate reached 10^4 cells ml^{-1} at 195 m and 215 m depth. At the other depth the viable counts reached only 1000 cells ml^{-1} or even less. Generally the counts were lower than those in the controls dilution series without monomer mix but with nitrate.

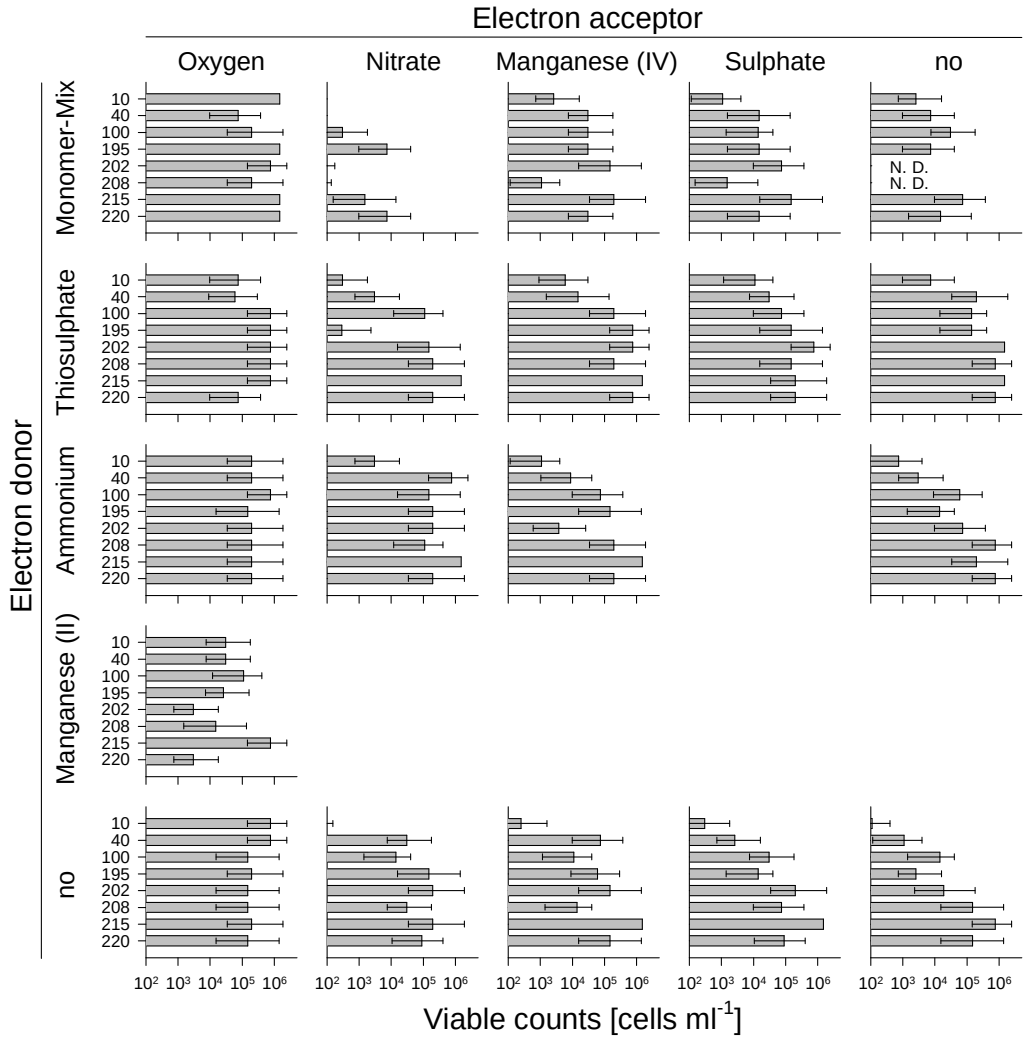

Fig. 2: Viable counts in MPN dilution series with given substrate combinations throughout the water column of the Gotland Basin in May 2005 as revealed by SybrGreen I staining. Error bars represent lower and upper 95% confidence intervals. Bars without error bars are given in case that all six dilutions were positive.

Viable counts obtained with monomer medium supplemented with manganese oxide, with sulphate, or even without electron acceptor were in a similar range and clearly exceeded those with nitrate. While about 10^3 cells ml^{-1} were detected within the photic zone, numbers increased by one order of magnitude in the intermediate layers. Highest numbers (around 10^5

cells ml^{-1}) were found around the oxic-anoxic interface and at the CO_2 fixation maximum at 215 m.

The utilisation of nitrate could be confirmed by ion-chromatography for positive wells that exhibited growth (data not shown). Decrease of sulphate as an indication of actively sulphate reducing bacteria was found only in series inoculated with samples from 215 and 220 m water depth and only in the lowest (10^1) dilution steps. Therefore, the main metabolism in these series must have been fermentation. Attempts to detect dissolved manganese by colorimetric analysis were not successful, probably due to the precipitation of manganese carbonates in the carbonate-buffered medium. But nevertheless, the microbial reduction of manganese became evident from decolourisation and from the formation of whitish precipitates that was absent in the controls.

MPN counts of sulphur-oxidising microorganisms. Dilution series targeting sulphur-oxidising bacteria were supplied with thiosulphate as electron donor. In oxic dilution series about 750.000 cells ml^{-1} were detected in samples from the layers between 100 and 215 m depth. In the layers above and below, MPN did not significantly differ from the background obtained with the control series without electron donor. Anoxic series generally yielded low numbers (10^2 to 10^4 cells ml^{-1}) above the halocline. Beneath, viable counts increased and reached maximum numbers at the oxic-anoxic interface exceeding those obtained for aerobes. In nitrate and manganese oxide-amended MPN series, highest counts (>10^6 cells ml^{-1}) were obtained in the anoxic layers at 215 m water depth. Surprisingly, in series supplemented with sulphate or without electron acceptor, maximum counts were in a similar range (around 10^6 cells ml^{-1}), but these counts were obtained with obtained in samples from 202 m depth, above the oxic-anoxic interface.

With exception of a few samples, from above the halocline and from 215 m depth, viable counts were significantly higher than in dilution series without electron donor. However, analysis of thiosulphate consumption in MPN series inoculated with samples from 215 m depth revealed that under oxic conditions thiosulphate was completely oxidised in most cases (Fig. 3), except for the highest positive dilutions. Generally, only part of the thiosulphate was oxidised to sulphate.

From the analysis of the anoxic series, a slightly different picture emerged. Thiosulphate consumption increased from the lower (less 1.5 mM, Fig. 3) to the higher dilution steps (up to 3 mM), but was in all cases lower than in the oxic MPN series. Like for aerobic incubations, only part of the thiosulphate was oxidised to sulphate. However, thiosulphate reduction or disproportionation can be excluded at least for the higher dilutions

as indicated by the absence of FeS precipitates, including the reducing agent in the medium that apparently was oxidised, too. Nonetheless, the viable counts determined according to the Sybr Green I staining matched those obtained by analysis of thiosulphate turnover.

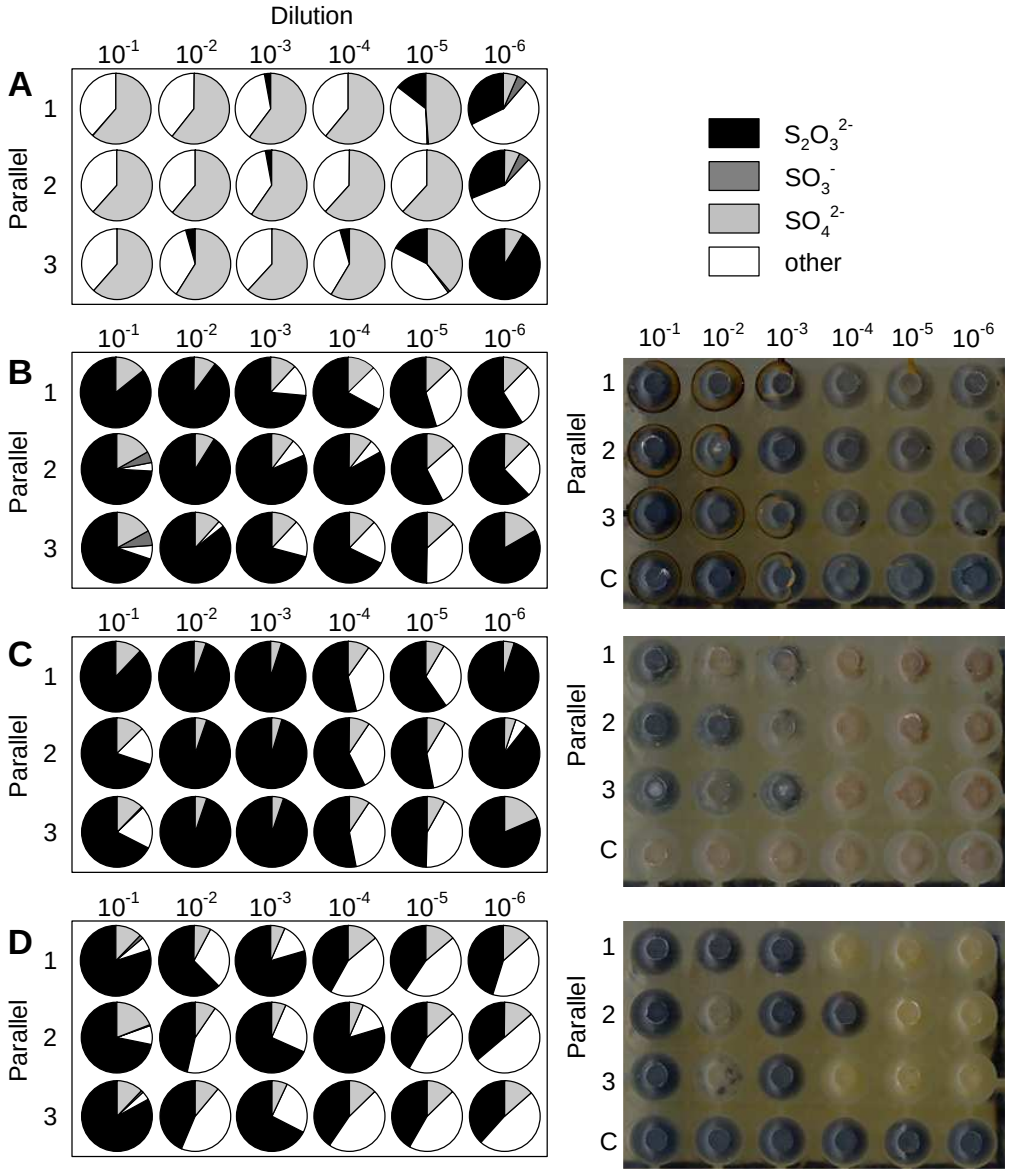

Fig. 3. Left panel: Thiosulfate utilisation in MPN dilution series with A) oxygen, B) nitrate, C) manganese oxide, and D) without electron acceptor, inoculated with water sample from 215 m water depth. Right panel: Images of the dilution series, illustrating the presence or absence of ferrous sulphide precipitations. The total amount of sulphur atoms present in the incubations was 10.5 mM (5 mM thiosulphate, 0.5 mM ferrous sulphide). For construction of the pie charts 1 mole thiosulphate was converted to two moles sulphur atoms.

MPN counts of ammonium and manganese-oxidising microorganisms. Except for 100 m depth, viable counts in dilution series targeting aerobic ammonium oxidisers did not

differ from those in the control dilution series (Fig. 2). Chemical analysis, however, revealed nitrite formation (up to 50 µM) in the 10^{-2} and 10^{-3} dilutions of the 40 m-series indicating the presence of nitrifying bacteria (data not shown), but not in any other samples.

Anoxic dilution series supplemented with ammonium and nitrate exhibited viable counts of 7.5 x 10^5 at 40 m and about 1.4 x 10^6 cells ml^{-1} at 215 m water depth. For the other depths numbers were in a similar range as in the control dilution series. Chemical analysis revealed nitrate consumption, but only a weak turnover of ammonium. Similarly, cell counts obtained in incubations with ammonium as potential electron donor and with manganese oxide did neither differ significantly from the control incubations (Fig. 2), nor exhibited ammonium turnover (data not shown).

Viable counts in dilution series supplemented with Mn(II) under oxic conditions, were generally even lower than the background detected in control dilutions. A surprising exception was the series inoculated with water from 215 m depth yielding about 750.000 cells ml^{-1}. However, oxidation of manganese could not be verified by microscopical investigation and was not analysed further.

Growth in medium without substrate additions. It was previously recognized that by means of SybrGreen I staining, growth could be detected even with the substrate-free basal medium alone (Martens-Habbena & Sass, 2006). To estimate the effect of this unspecific growth, in particular on assays targeting lithotrophic microorganisms, control dilutions were prepared by omitting the electron donor, the electron acceptor or both. In all these controls, either under oxic or anoxic conditions, surprisingly high viable counts were obtained (Fig. 2). In several assays up to 10^6 cells ml^{-1} were found, for example under oxic conditions with samples from above the halocline or under anoxic conditions with samples from 215 m depth, even without added electron acceptor nor donor.

Biomass formation in Most Probable Number Dilution Series. All MPN dilution series were analysed fluometrically after staining with SybrGreen I, what allowed not only a sensitive detection despite low growth yields but also offered the opportunity to quantify the biomass formed. Fig. 4 depicts an estimate of the biomass produced in the 158 different dilution series. As expected, highest cell yields were obtained in dilution series supplemented with monomer substrate mix. Median cell densities varied from 1.3 x 10^7 in series with manganese oxide (40 m depth) to 6 x 10^7 cells per ml in oxic dilution series inoculated with surface water. Surprisingly, cell yields in the anoxic MPN series with monomer mix often equalled or exceeded that in the oxic assays, for example for the depths beneath the oxic-anoxic interface. Quantification of biomass in assays with nitrate was strongly hampered by a

strong interference of nitrite (>500 µM), that was formed in several of the wells, with Sybr Green I fluorescence.

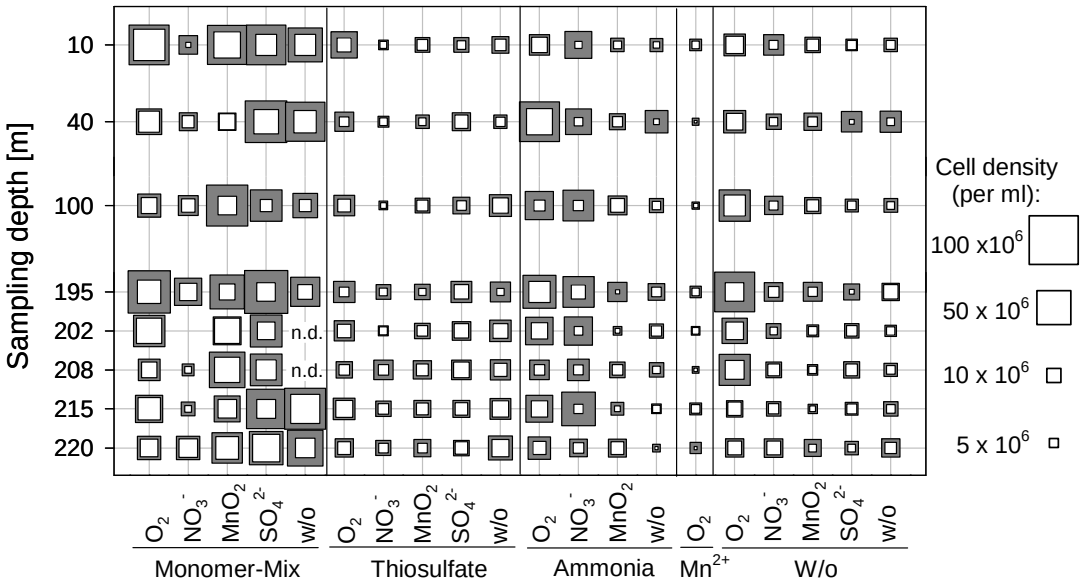

Fig. 4 Variability of biomass formed in MPN series as revealed by SybrGreen I staining. Given are the 25 to 75 % intervals of cell densities, white and grey squares, respectively, formed in wells with detectable growth in each of the 158 dilution series shown in Fig. 2. Substrate combinations and water depth of sample origin are indicated. Fluorescence data were converted to cell densities (legend) according to a calibration curve obtained with *E. coli* cells.

In the dilution series without electron donor addition less variation in biomass formation was found among the single series supplemented with the different electron acceptors but also from the different depths. (Fig. 4). After oxic incubation between 10 and 24 x 10^6 cells per ml were obtained, whereas anoxic incubation resulted in 3 to 13 x 10^6 cells per ml, irrespective of the electron acceptor provided. This means that generally biomass formation in anoxic MPN series supplemented with monomer mix was significantly higher than in the electron donor-free control. For aerobes, in turn, a more ambiguous result was obtained. After oxic incubation the monomer mix apparently supported a higher average cell yield in series inoculated with samples from the surface and from around the oxic-anoxic interface, except for 208 m. Samples from this depth and from the intermediate layers above and just below the halocline (40 and 100 m), however, resulted in average cell numbers generally not exceeding those in the electron donor-free control (Fig. 4). Interestingly, in the lowest one to two dilutions only low cell numbers were observed in all series, even those yielding higher

averages. These series, inoculated with samples from 10, 195, 202, 215, and 220 m depth, tended to exhibit highest cell yields in the highest positive dilutions (Fig.5).

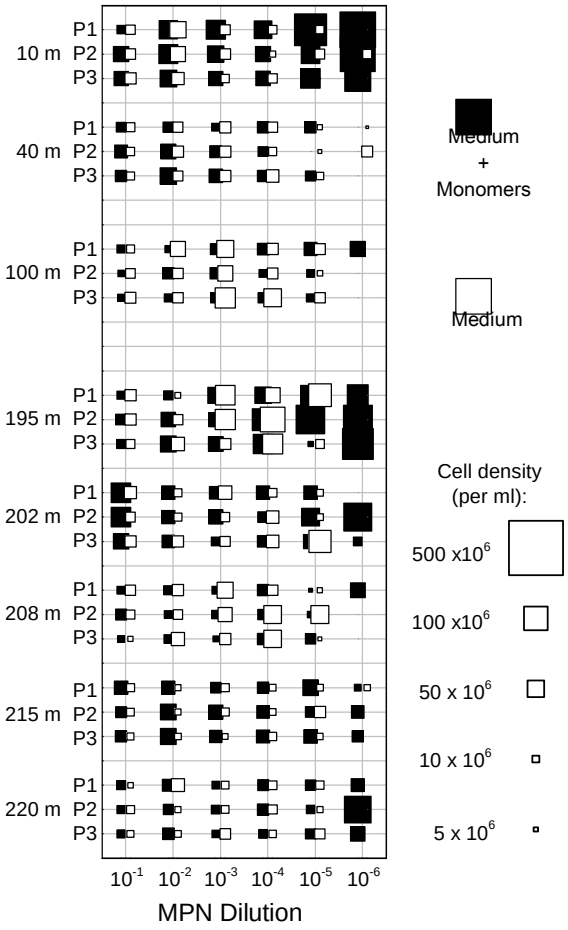

Fig. 5. Comparison of biomass formation in oxic MPN dilution series with monomer substrate mix (black squares) and without substrate (open squares) from given water depth. Fluorescence data were converted to cell densities using a calibration curve obtained with *E. coli* cells.

Cell densities in dilution series with thiosulphate were lower and showed less variation than those obtained with monomer media. In oxic dilution series median cell numbers varied between 8 and 18 x 10^6 cells ml^{-1}, and were only slightly higher than in the anoxic MPN series. However, cell numbers obtained in MPN series supplemented with thiosulphate did not significantly exceed those obtained in electron donor-free controls.

In contrast, MPN series with ammonium as potential electron donor and oxygen or nitrate as electron acceptor yielded clearly higher cell numbers than the respective controls.

Interestingly, highest numbers (5.1 x 10^7 cells ml^{-1}) were obtained with samples from 40 m depth after oxic incubation, the only wells in which conversion of ammonium to nitrite was observed. Cell numbers in the other oxic series numbers were at least 50 % lower.

Dilution series supplemented with ammonium and nitrate exhibited more variation among the single wells. Cell numbers clearly higher than in the electron donor-free control were only found in the low dilutions (10^{-1} to 10^{-3}), in contrast to those in the high dilutions, in the assays with manganese oxide or without electron acceptor. MPN series targeting manganese-oxidising bacteria yielded the lowest cell yield and were even lower than in the electron donor and electron acceptor-free control.

Molecular analysis of MPN enrichments. Molecular analysis focussed on dilution series prepared with samples from 195 m and 215 m depth. The first sample originated from above the chemocline, a layer characterised by a steep gradient of nitrate and trace amounts of oxygen, whereas the latter sample was from the zone of highest *in situ* dark carbon dioxide assimilation (Fig. 1). In addition, the dilution series showing nitrification activity was also analysed.

Out of 28 dilution series analysed, sequences of 16S rRNA gene fragments were obtained from all 53 excised DGGE bands. Together with one sequence obtained by a specific PCR protocol all sequences could be affiliated to eleven operational taxonomic units (OTU) according to their closest cultured relatives (Table 1). Four OTUs belonged to the *Alphaproteobacteria*, five to the *Gammaproteobacteria*, and one OTU from 40 m water depth belonged to the *Betaproteobacteria*. From the 195 m samples three OTUs were affiliated to the *Gammaproteobacteria*, one OTU was affiliated to the *Alphaproteobacteria*, and one to the *Epsilonproteobacteria*. Three OTUs affiliated to the *Gammaproteobacteria* and four related to *Alphaproteobacteria* were detected in the dilution series from 215 m.

Generally, not more than three different genera were detected within a single dilution series. Most notably, this was the case in the oxic control dilution series from 195 m water depth. In all other dilution series analysed one or two different OTUs were found. Only two OTUs, *Thalassospira lucentensis* and *Pseudomonas* sp. were retrieved from both depths (Table 1). Whereas, *Thalassospira*-related sequences were found in four different substrate combinations, *Pseudomonas*-related sequences were detected in all substrate combinations.

Table 1: Phylogenetic affiliation of enrichments in highest positive dilutions of MPN series from 195 and 215 m sampling depth.

Sampling Depth (m)	OTU[1]	DGGE Band No.	Group[2]	Sequence Similarity (%)	Closest relative	Substrate combination																				
						Monomer mix					Thiosulphate					Ammonium				Mn(II)	no					
						Oxygen	Nitrate	Mn(IV)	Sulphate	no	Oxygen	Nitrate	Mn(IV)	Sulphate	no	Oxygen	Nitrate	Mn(IV)	no	Oxygen	Oxygen	Nitrate	Mn(IV)	Sulphate	no
40[3]	1	25	*Alpha*	99.0	*Tistrella mobilis*[T]											+									
	5	26	*Beta*	96.6	*Limnobacter thiooxydans*[T]											+									
195[3]	2	1, 9, 14, 43	*Alpha*	97.1-99.4	*Thalassospira lucentensis*[T]	+		+			+										+				
	6	5, 30, 44	*Gamma*	90.8-97.8	*Marinobacter excellens*[T]		+										+				+				
	7	7	*Gamma*	96.8	*Thalassolituus oleivorans*[T]		+																		
	8	3, 15, 32, 45-48	*Gamma*	92.6-100	*Pseudomonas* sp. SN1[4]	+					+						+				+				
	11	-[5]	*Epsilon*	100	uncult. G138eps1[4]																	+			
215	1	19	*Alpha*	99.1	*Tistrella mobilis*[T]							+													
	2	27, 49, 50	*Alpha*	96.2-98.2	*Thalassospira lucentensis*[T]											+					+				
	3	4	*Alpha*	97.3	*Sulfitobacter dubius*[T]	+																			
	4	17	*Alpha*	99.4	*Citreicella thiooxydans*[T]						+														
	8	10, 12, 18, 20, 22-24, 29, 35-38, 40, 42, 51, 52, 53	*Gamma*	95.6-100	*Pseudomonas* sp. SN1[4]		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	9	39	*Gamma*	97.0	*Shewanella baltica*[T]														+						
	10	21	*Gamma*	100	*Halomonas aquamarina*[T]								+												

[1] Operational taxonomic units.

[2] Refers to the subgroup of the Proteobacteria.

3 Enrichments yielding low viable counts were not analysed.

[4] Assignment to a type strain not unambiguously possible.

[5] Detected by specific PCR, not by DGGE.

Sequences related to *Marinobacter* were detected in three different substrate combinations, *Tistrella*-related sequences in two, while all other OTUs were retrieved from a single substrate combination. Interestingly, all sequences detected in the MPN series, except from the *Thalassolituus oleivorans*-related one, are known to gain energy from oxidation of sulphur compounds. However none of them belong to genera typically representing autotrophic sulphur oxidisers.

In a previous study, however, a so-far uncultured *Epsilonproteobacterium* was shown to dominate the denitrifying community at the Gotland Deep chemocline (Labrenz *et al.*, 2005). Since a specific PCR protocol for this organism is available, MPN series supplied with nitrate as electron acceptor were checked. While it was not found in the DGGE approach applying general bacterial primers, the organism was detected by specific PCR in the MPN series supplemented with nitrate but without an electron donor.

Due to low viable counts detected in some dilution series from 195 m these enrichments were not considered to harbour microbes abundant in the environment. These enrichments were thus not included in the molecular analysis.

Discussion

Similar to the Black Sea, the water column of the Central Baltic Sea possesses three major compartments. During summer, the warm surface layer that is characterised by intense photosynthesis is separated by a thermocline from a cold and DOC-poor intermediate layer that extends down to the permanent halocline and the stagnant bottom waters (Aitsam *et al.*, 1984; Feistel *et al.*, 2003). Between these layers exchange of organic carbon and nutrients is, except for mixing between the upper two compartments in spring and autumn, restricted to sedimentation. Accordingly, the activity as well as plate counts of heterotrophic bacterioplankton change considerably along the water column (Bruns *et al.*, 2002; Rheinheimer *et al.*, 1989). The aim of the present study was to gain more detailed insights into the metabolic diversity within the different compartments of the water column of the Gotland Basin with special attention paid to the chemocline.

Physiologic types of microbes in the photic zone and intermediate layers. During late spring season, the heterotrophic bacterioplankton thrives on polysaccharide- and amino

acid-rich exudates released by an active *Achnanthes* and *Skeletonema*-dominated diatom bloom (Granum *et al.*, 2002; Rheinheimer, 1995). Therefore, the high MPN counts obtained for the surface layers might be explained by the substrate combination, e.g. glucose and amino acids, used in the present study that partly reflects the most important substrates in situ. However, the availability and in particular the composition of the algal and cyanobacterial exudates varies considerably between spring and the nitrogen-limited conditions during summer (Granum *et al.*, 2002), and likely affects the cultivation success (Bruns *et al.*, 2002, Martens-Habbena et al., submitted). Anaerobic growth, as well as thiosulphate oxidation may not play an important role for microorganisms at the water surface.

Analysis of glucose turnover rates and potential exoenzyme activities revealed that the cold intermediate layers harbour less active microbial communities (Rheinheimer *et al.*, 1989), that also exhibits a lower cultivation success (Bruns *et al.*, 2002). Decreased viable counts as well low cell yields (Figs. 4 and 5) might indicate that these communities rely on different sets of substrates than in the photic zone. This is easy to imagine since the sedimenting organic matter consists of rather complex polymeric substrates and is depleted in the easily degradable compounds (Rheinheimer *et al.*, 1989). However, microbes might then grow at least on some of the substrate compounds and should be able to use some of the others for assimilation, e.g. amino acids. The comparatively high background growth in the basal oxic medium without monomer substrates indicates that HEPES buffer (10 mM) and the mixture of vitamins (equal to approximately 20 µM of organic carbon) might at least in part serve as a potential substrate.

Aerobic and anaerobic organotrophs at the chemocline. The microbial community at the chemocline differs from that in the layers above for two main reasons: First, the sulphide-oxygen interface stimulates growth of autotrophic sulphur oxidisers (e.g. Jannasch *et al.*, 1991; Sorokin, 1972; Zopfi *et al.*, 2001), which provide a significant contribution of organic material for the heterotrophic microbial community (Jørgensen *et al.*, 1991; Taylor *et al.*, 2001). Secondly, oxygen depletion compels the acquisition of alternative electron acceptors by heterotrophic, but also lithotrophic microorganisms (Brettar & Rheinheimer, 1991; Brettar & Rheinheimer, 1992; Rheinheimer *et al.*, 1989).

Increasing counts of organotrophic microorganisms at the chemocline are generally in accordance with the increased glucose turnover and exoenzyme activities as well as carbon availability at the oxic-anoxic interface (Rheinheimer *et al.*, 1989; Taylor *et al.*, 2001). The presence of large numbers of aerobes points towards a substantial fraction of facultative aerobes what is in line with previous investigations that demonstrated reduced but still

considerable viable counts of aerobes also in the anoxic water layers (Bruns *et al.*, 2002; Martens-Habbena *et al.*, submitted; Rheinheimer *et al.*, 1989).

The local maxima of aerobic and anaerobic heterotrophs at the upper part of the chemocline (195 to 202 m depth) could be caused by the vertical sequence of electron acceptors available across the chemocline.

Heterotrophic nitrate-reducers are overwhelmingly outnumbered by lithotrophic ones. This finding is consistent with previous reports (Martens-Habbena *et al.*, submitted) and fits well to findings that denitrification at chemocline of the Gotland Basin is mainly driven by the oxidation of sulfide (Brettar & Rheinheimer, 1991; Brettar & Rheinheimer, 1992). If organotrophic nitrate reduction does not play an important role in situ, manganese oxides might serve as alternative electron acceptors for organic matter degradation. This view is supported by the steep gradients of particulate and dissolved manganese and the relatively high MPN counts obtained with this electron acceptor. In fact, it was shown that at pelagic chemoclines an intense manganese cycle can occur (Neretin *et al.*, 2003) and a number of manganese-reducing bacteria were isolated from these habitats (e. g. Nealson & Myers, 1992; Nealson & Scott, 2003).

Surprisingly, most physiological groups including heterotrophic aerobes, MPN numbers showed a second local minimum at the CO_2 fixation maximum at 215 to 220 m depth. Additional organic carbon substrates may become available to the heterotrophic microbial community through autotrophic microorganisms (Taylor *et al.*, 2001). However, due to the lack of electron acceptor in these depths, the maximum of aerobes can hardly be explained. Faint amounts of manganese oxides (Fig. 1) and intermediate oxidation products of sulphide, e.g. elemental sulphur or thiosulphate might become available to the heterotrophic microbial community in situ. Therefore, it seems likely that growth of aerobes in samples from these depths might be due to facultative microorganisms.

Molecular analysis of the MPN series identified members of the genera *Thalassospira*, *Halomonas* and *Pseudomonas* in dilution series with manganese oxides as electron acceptors; organisms that at first sight do not belong to the ‘typical’ genera of manganese-reducing bacteria. However, manganese reduction took place in the MPN series and it has been reported that a *Pseudomonas* strain was able to reductively dissolve manganese oxide (Hernandez *et al.*, 2004). On the other hand tests for manganese reduction are included only in a very few species descriptions, indicating that the knowledge of how wide this capacity is distributed among the different phylogenetic groups is still very limited.

The viable counts of fermenting organisms are in a similar range like those of other anaerobes. Assuming that nitrate is effectively depeleted by sulphur-oxidising bacteria (Brettar & Rheinheimer, 1992), and manganese oxides are only available to particle-associated bacteria, then fermentative metabolisms may become important for free-living microbes beneath the oxic-anoxic interface. However, fermenters generally rely on the activity of terminal oxidisers that consume their metabolic end products, for example sulfate-reducing bacteria. The low number of sulphate reducers detected at the chemocline of the Baltic Sea is in good agreement with the very low rates of sulphate reduction at the chemoclines of the Gotland Basin (L. Neretin, pers. communication) but also of the Black Sea (Albert *et al.*, 1995; Jørgensen *et al.*, 1991). On the other hand, the MPN results might in fact underestimate the actual numbers *in situ*. It has been shown that the chemocline of Mariager Fjord harbours significant numbers of SRB (Teske *et al.*, 1996) and just recently in a molecular investigation 16S rRNA genes closely related to the genus *Desulfobacula* were detected in the chemocline of the Gotland Basin (Labrenz *et al.*, submitted). These SRB, however, can be expected to utilize only a restricted numbers of the substrates offered with the monomer mix, e.g. the fatty acids that were present at 0.1 mM each, and might therefore escaped recognition due to the low amount of sulfate reduced. But when SRB are present in significant numbers they apparently do not reduce sulphate. It seems therefore likely that they may also reduce partly oxidised sulphur compounds provided by the sulphur-oxidising bacteria.

Lithotrophic microorganisms at the chemocline. The surprisingly high viable counts of aerobic sulphur oxidising bacteria exceed previous reports by at least two orders of magnitude (Jørgensen *et al.*, 1991; Sorokin *et al.*, 1995). This is most likely due to the sensitive detection method applied during the present study.

Pseudomonas-, *Citreicella*-, and *Thalassospira*-related *Alpha*- and *Gamma-proteobacteria* dominated the oxic MPN enrichments from 195 m and 215 m. Although sulphur oxidation is known at least for *Pseudomonas* and *Citreicella* spp., none of these genera was shown to grow autotrophically (López-López *et al.*, 2002; Sorokin, 2003; Sorokin *et al.*, 2005) suggesting an chemolithoheterotrophic metabolism.

The confined occurrence of nitrate-dependent sulphur oxidisers at the oxic-anoxic interface fits well to the observation that active denitrification takes place only within a 10 m-interval around the chemocline (Brettar & Rheinheimer, 1991). Interestingly, the highest viable counts of nitrate-dependent sulphur oxidisers were found within the zone of highest carbon dioxide assimilation, while the bacteria identified in the MPN enrichments (closely

related to *Pseudomonas* sp. and *Tistrella* sp.) can be considered to lack the capacity for autotrophic growth. Moreover, nitrate was not detected at this depth, suggesting that the enrichments only grow facultatively with nitrate. In fact, previous studies suggested that a putative autotrophic sulphur-oxidising phylotype, related to *Sulfurimonas denitrificans* (Takai *et al.* 2006), is abundant in the chemocline of the Gotland Basin and involved in sulphur oxidation (Höfle *et al.*, 2005; Labrenz *et al.*, 2005). Obviously, the 'electron donor-free' assay supplemented with nitrate, which contained the potential electron donors ferrous iron and sulphide from the reducing agent, selected best for this lithoautotrophic type. It is known that thiosulphate oxidation can be performed by a huge diversity of bacteria and it is easy to imagine that these putatively autotrophic bacteria are outcompeted by lithoheterotrophic bacteria in the thiosulfate-rich medium.

Cultivation-based assessment of microbial diversity. The goal of the present study was to gain insights into the microbial diversity at the water column of the Central Baltic Sea by cultivation-based means. The combination of cultivation assays with molecular identification of enrichments has previously been successfully applied to isolate novel phylotypes from marine bacterioplankton and soil (Connon & Giovannoni, 2002; Rappé *et al.*, 2002; Stevenson *et al.*, 2004). Whereas, the use of agar plates in principle allows the enrichment of various different microbes on a single plate, liquid enrichments are usually dominated only by a very few if not a single phylotype. This limitation has been overcome by application of dilution techniques, which enable the establishment of hundreds of parallel enrichments (Bruns *et al.*, 2003; Connon & Giovannoni, 2002), that require considerable efforts checking and identifying positive cultures (e.g. Bruns *et al.*, 2003; Connon & Giovannoni, 2002; Rappé *et al.*, 2002). Additionally, such cultivation assays have so far been restricted to the cultivation of aerobic microorganisms. During the present study a miniaturized MPN dilution series approach suitable for aerobic and anaerobic microorganisms (Köpke *et al.*, 2005; Süß *et al.*, 2004) was combined with a new highly sensitive method for the quantification of biomass (Martens-Habbena & Sass, 2006). Subsequently, only a subset of enrichments potentially harbouring environmentally abundant microorganisms was subjected to chemical and molecular analysis. Focussing on two depths was in fact a successful strategy. Two out of eleven phylotypes enriched, were previously detected by molecular fingerprints at the chemocline of the Gotland Basin (Labrenz *et al.*, submitted), those related to the metabolically versatile genus *Pseudomonas* group detected in almost all enrichments from 215 m, and a so far uncultured *Epsilonproteobacterium*, related to *Sulfurimonas denitrificans* found in a single enrichment.

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3. Diskussion und Ausblick

Die vorliegende Arbeit beschäftigte sich mit mikrobiellen Gemeinschaften an der Chemokline anoxischer Becken in der zentralen Ostsee. Ziel der Arbeit war es, mit Hilfe chemisch-analytischer und mikrobiologischer Methoden einen Einblick in die chemische Zonierung der Wassersäule und der oxisch-anoxischen Übergangszone sowie der Verteilung mikrobieller Energiestoffwechseltypen entlang der chemischen Gradienten zu gewinnen.

3.1 Die chemische Zonierung der Chemokline und die Rolle des Mangans

Die Untersuchung der chemischen Gradienten über einen Zeitraum von mehreren Jahren ergab ein umfassendes Bild. Von besonderem Interesse war die vollständige Oxidation des stagnierenden sulfidischen Tiefenwassers im Gotland Becken und die nachfolgende Neubildung eines anoxischen Wasserkörpers. Solche Ereignisse wurden bis Mitte der 1970er Jahre regelmäßig im Abstand weniger Jahre beobachtet und sind Folge großer Salzwassereinströme aus der Nordsee (Matthäus & Franck, 1992). Kleinere Einströme wurden in der südlichen Ostsee regelmäßig registriert, jedoch sind in den vergangenen dreißig Jahren nur zwei Einströme so massiv gewesen, dass sie die tiefen Becken in der zentralen Ostsee erreichten (1993 und 2003, Feistel *et al.*, 2003). Durch die Verdrängung von Wassermassen kommt es bei solchen Ereignissen zum Eintrag von Sauerstoff in die tiefen Becken.

Die Analysen zeigten, dass zu Beginn der vorliegenden Untersuchungen die chemischen Gradienten eine typische Langzeitstagnation anzeigten, wie sie auch in früheren Studien gefunden wurde (Brettar & Rheinheimer, 1991; Neretin *et al.*, 2003; Rheinheimer *et al.*, 1989). Während die Gradienten von Sauerstoff, Nitrat, Nitrit, Ammonium und Sulfid seit langem detailliert analysiert werden, gab es bisher nur sehr wenige Analysen von gelöstem und partikulärem Mangan (Neretin *et al.*, 2003; Pohl & Hennings, 1999). Aus den Untersuchungen von Neretin *et al.* und der vorliegenden Arbeit kann geschlossen werden, dass im Gotland Becken und im Fårö Tief langzeitstagnierende Bedingungen mit partikulären Mangankonzentrationen oberhalb der Chemokline von 0.3 bis 1 µM verbunden sind. Die Konzentrationen von gelöstem Mangan an der Sedimentoberfläche betragen dabei bis zu 15 µM (Kap. 2.2; Neretin *et al.*, 2003). Die im Laufe der vorliegenden Untersuchung beobachtete

Oxidation des Tiefenwassers hatte jedoch wesentlichen Einfluss auf die Konzentrationsverhältnisse. An der neugebildeten Chemokline in 220 m Wassertiefe wurde im Jahr 2004 bis zu 2 µM an partikulärem Mangan und bis zu 45 µM gelöstem Mangan nahe der Sedimentoberfläche detektiert. Im Laufe der Oxidation der Wassersäule muss daher ein lateraler Eintrag oder die Sedimentation manganreicher Partikel zur Akkumulation von Mangan nahe der Sedimentoberfläche geführt haben. Dieses Ereignis hatte nachhaltigen Einfluss auf die chemische Zonierung an der Chemokline. Die hochaufgelöste Analyse von gelöstem und partikulärem Mangan an der Chemokline im Mai 2005 zeigte, dass unter diesen Bedingungen partikuläres Mangan in messbaren Mengen in sulfidisches Wasser hinein sedimentiert und bis zu 10 m innerhalb des schwach sulfidischen Wassers unterhalb der Chemokline nachweisbar war. Im Gegensatz zu marinen Sedimenten, in denen hohe Manganoxid-Konzentrationen eine schnelle chemische Oxidation von Sulfid bewirken (Thamdrup, 2000), ist unter den Bedingungen im Freiwasser die chemische Oxidation um Größenordnungen langsamer und trägt nicht wesentlich zur Sulfidoxidation bei. Unter diesen Bedingungen könnten daher Mikroorganismen die Sulfidoxidation an die Reduktion von Manganoxiden koppeln (Kap. 2.3).

Ein weiterer wichtiger strukturierender Effekt der biologischen oder chemischen manganoxidabhängigen Sulfidoxidation ist die auftretende Trennung von Sulfid- und Sauerstoffgradienten. So konnte in der vorliegenden Arbeit ebenfalls gezeigt werden, dass die Gradienten von Sauerstoff und Nitrat nicht mit dem Sulfidgradienten überlappen und daher, im Gegensatz zu stagnierenden Bedingungen, eine mikrobielle Oxidation von Sulfid mit Sauerstoff oder Nitrat unter hohen Manganoxid-Konzentrationen oberhalb der Chemokline unwahrscheinlich erscheint (Kap. 2.3). Diese Ergebnisse sind konsistent mit abnehmenden Denitrifikationsraten, die im selben Untersuchungszeitraum an der Chemokline bestimmt wurden (Hannig *et al.*, 2006 eingereicht). Darüber hinaus wurden von Hannig *et al.* nach der Neubildung der Chemokline im Jahr 2003 und 2004 anaerobe Ammoniumoxidation nachgewiesen und mittels FISH die Gegenwart von Planktomyzeten der Gruppe Candidatus *Scalindula* sp. gezeigt. Da Sulfid- und Nitratgradienten nicht mehr überlappen, wohl aber die Gradienten von Ammonium und Nitrat, könnte das Verhalten von partikulärem Manganoxid eine Nische für Anammox-Bakterien in der Konkurrenz mit sulfidoxidierenden Bakterien um den Elektronenakzeptor Nitrat schaffen. Mangan hat nicht nur einen Einfluss auf die mikrobiellen Prozesse in manganreichen marinen Sedimenten, sondern offensichtlich auch einen wesentlichen Einfluss auf die Prozesse an der Chemokline anoxischer Becken.

Die Besonderheiten des Mangankreislaufs an der Chemokline lassen jedoch nach wie vor wichtige Fragen offen. So ist unklar, wie Partikelsedimentation und Aufwärtsdiffusion von gelöstem Mangan bilanziert werden können. Die Partikelsedimentation verläuft deutlich schneller als durch Eddy-Diffusion gesteuerter Fluss von gelöstem Mangan (Neretin *et al.*, 2003). Das Manganoxid oberhalb der Chemokline müsste daher deutlich schneller abnehmen als während der vorliegenden Arbeit beobachtet. Die Reduktion von Manganoxiden durch autotrophe und heterotrophe Mikroorganismen ist seit langem bekannt. Entsprechende Mikroorganismen wurden unter anderem aus der Chemokline des Gotland Beckens und anderer mariner anoxischer Becken isoliert.

Jüngste Untersuchungen zur mikrobiellen Manganoxidation zeigen jedoch, dass Pseudomonaden, die in der vorliegenden Untersuchung auch an der Chemokline des Gotland Beckens in großer Zahl nachgewiesen wurden, Mn^{2+} zunächst zu löslichen Mn(III)-Komplexen oxidieren (Parker *et al.*, 2004; Webb *et al.*, 2005). Erst in einem zweiten Schritt disproportioniert das Mn(III) aus diesen Komplexen entweder chemisch zu partikulärem Manganoxid und Mn^{2+}, oder es wird in einem zweiten enzymatischen Schritt vollständig zu partikulärem Manganoxid oxidiert (Parker *et al.*, 2004; Webb *et al.*, 2005). Darüber hinaus gibt es erste Hinweise auf die Gegenwart von gelösten Mn(III)-Komplexen auch an der Chemokline des Schwarzen Meeres (B. Tebo, pers. Mitteilung). Die Bedeutung der mikrobiellen Manganoxidation für die beteiligten Organismen ist bis heute nicht aufgeklärt (Tebo *et al.*, 2005). Der Nachweis von Mn(III)-Verbindungen sowohl in Reinkulturexperimenten als auch unter natürlichen Bedingungen lässt jedoch vermuten, dass der Kreislauf von Manganoxidation und -reduktion nicht zwangsläufig partikuläre Verbindungen einschließen muss. Es konnte bereits gezeigt werden, dass gelöste Mn(III)-Pyrophosphat-Komplexe von *S. oneidensis*, einem typischen metallreduzierenden Bakterium, als Elektronenakzeptoren verwendet werden können (Kostka *et al.*, 1995). Gelöste oxidierte Manganverbindungen könnten auch weniger spezialisierten Mikroorganismen als Elektronenakzeptor zur Verfügung stehen und Manganreduktion einer größeren phylogenetischen Diversität nicht partikelassoziierter Mikroorganismen erlauben.

3.2 Verbesserte Strategien zur Detektion und Kultivierung von Mikroorganismen

Die Kultivierung von Mikroorganismen ist bis heute eine wichtige Voraussetzung für eine detaillierte mikrobiologische Untersuchung (Harold, 2005; Leadbetter, 2003; Tyson & Banfield, 2005). In den vergangenen Jahren hat insbesondere die Kombination von neuen Kultivierungsstrategien mit der molekularbiologischen Analyse von Anreicherungen zur wesentlichen Verbesserung des Kultivierungserfolges beigetragen. Dennoch konnte bis heute ein großer Anteil der phylogenetischen Diversität nicht nachweislich unter Laborbedingungen kultiviert werden (Leadbetter, 2003). Es besteht daher nach wie vor großer Bedarf an methodischen Weiterentwicklungen zur Kultivierung von Mikroorganismen.

Während der vorliegenden Untersuchung wurden kultivierungsbasierte Verfahren zur Analyse mikrobieller Gemeinschaften an oxisch-anoxischen Grenzschichten verwendet. Mittels spezifischer Substratkombinationen in MPN-Verdünnungsreihen wurden einzelne physiologische Gruppen selektiv angereichert, deren Lebendzellzahl bestimmt und anhand von 16S rRNA-Gensequenzen identifiziert. Bisherige Methoden zur derartigen Analyse einer großen Zahl von Anreicherungen basieren auf einer fluoreszenzmikroskopischen Detektion von Wachstum und anschließender Bestimmung von 16S rRNA-Gensequenzen der isolierten Phylotypen (z. B. Connon & Giovannoni, 2002).

Im Rahmen der vorliegenden Arbeit wurde ein neues Verfahren zur Analyse des Wachstums in den verschiedenen Anreicherungen entwickelt. Gleich einer fluoreszenzmikroskopischen Detektion, basiert dieses Verfahren ebenfalls auf der Anfärbung von Zellen durch nukleinsäurebindende Fluoreszenzfarbstoffe. Ausgehend von der Hypothese, dass einzelne fluoreszierende Zellen nicht nur im Mikroskop, sondern auch durch die Messung der gesamten abgestrahlten Fluoreszenz ausreichend genau detektierbar sein müssten, wurden dazu verschiedene Farbstoffe und Versuchsbedingungen getestet. Die Ergebnisse dieser Untersuchungen (Kap. 2.1) zeigten, dass insbesondere die standardmäßig zur Fluoreszenzmikroskopie eingesetzten Fluoreszenzfarbstoffe, DAPI und Acridine Orange, zu diesem Zweck ungeeignet sind. Jedoch wurden mit neueren Farbstoffen, wie SybrGreen I und II sowie PicoGreen, wesentlich bessere Ergebnisse erzielt. Dieses Verfahren erlaubte es, die mikroskopische Untersuchung von Anreicherungen vollständig durch die Fluoreszenzmessung zu ersetzen. Auf diese Weise wurde es möglich, eine große Anzahl von parallelen MPN-Verdünnungsreihen mit verschiedenen Substratkombinationen anzulegen und insgesamt über 10.000 Einzelproben sehr sensitiv auf Wachstum zu untersuchen. Einzelne

MPN-Reihen konnten so einfach für chemische und molekularbiologische Untersuchungen ausgewählt und ebenfalls analysiert werden.

In jüngerer Vergangenheit wurde wiederholt gezeigt, dass der Einsatz geringer Substratkonzentrationen und die sensitive Detektion von Wachstum wesentlich zur Verbesserung des Kultivierungserfolges beitragen (Connon & Giovannoni, 2002; Köpke *et al.*, 2005; Rappé *et al.*, 2002; Süß *et al.*, 2004). Während der vorliegenden Untersuchungen wurden daher Kultivierungsmedien für organotrophe Mikroorganismen verwendet, die viele verschiedene Substrate in geringer Konzentration enthielten. Darüber hinaus wurden Medien für lithotrophe Mikroorganismen und Medien ohne zusätzliche Substrate verwendet. Die Ergebnisse zeigten, dass unter diesen Bedingungen reproduzierbar hohe Kultivierungserfolge erzielt und abundante Phylotypen angereichert werden können.

Mit Hilfe der einfachen und sensitiven Detektion von Wachstum können künftige Studien wesentlich detaillierter Kultivierungsbedingungen testen und optimieren. Darüber hinaus wird es nicht mehr notwendig sein, Substratgemische zu verwenden, sondern es kann beispielsweise in parallelen Ansätzen gezielt der Kultivierungserfolg mit einzelnen Kohlenstoffsubstraten untersucht werden. Derartige Kultivierungsstudien könnten nicht nur dazu dienen, neue Phylotypen zu kultivieren, sondern auch einen detaillierteren Einblick in die potentiellen Substratspektren natürlicher mikrobieller Gemeinschaften geben.

3.3 Mikrobielle Gemeinschaften an der Chemokline des Gotland Beckens

Ein wichtiges Ziel der vorliegenden Untersuchung war es, einen Einblick in die Strukturierung der mikrobiellen Gemeinschaften an der Chemokline der tiefen Becken in der zentralen Ostsee zu gewinnen. Die Analyse verschiedener Energiestoffwechseltypen in natürlichen Gemeinschaften stellt bis heute eine große Herausforderung dar. Bis auf wenige Ausnahmen ist es nicht möglich, eine molekularbiologische Detektion mit einem direkten Nachweis der Verwendung bestimmter Elektronenakzeptoren zu koppeln. In der vorliegenden Arbeit wurden daher MPN-Verdünnungsreihen verwendet, um einen Einblick in die Verteilung von Mikroorganismen entlang der chemischen Gradienten zu gewinnen. Anhand der Ergebnisse ist es zwar ebenfalls nicht möglich, die aktive Verwertung der verschiedenen Elektronenakzeptoren durch verschiedene Mikroorganismen *in situ* nachzuweisen. Dennoch lassen sich aus den Ergebnissen wesentliche Schlussfolgerungen über die Verteilung der mikrobiellen Gemeinschaften an der Chemokline ziehen.

Während langzeitstagnierender Bedingungen, wie sie zu Beginn der vorliegenden Arbeit im Gotland Becken vorgefunden wurden, scheint ein recht ausgeglichenes Verhältnis von aeroben und fakultativ oder strikt anaeroben Mikroorganismen vorzuliegen. Im Anschluss an die Reoxidation des Beckens und der Neubildung der Chemokline änderte sich dieses Verhältnis dramatisch. Erhöhten Lebendzellzahlen von aeroben standen deutlich reduzierte Zahlen von anaeroben Mikroorganismen gegenüber. Der laterale Eintrag von sauerstoffreichem Wasser in das Becken scheint zu einem deutlichen Eintrag von aeroben Mikroorganismen in das Tiefenwasser geführt zu haben. Darüber hinaus blieben die Gemeinschaften strikt oder fakultativ anaerober Mikroorganismen im oxidierten Wasser nicht erhalten. Unter diesen Bedingungen ist der Abbau organischen Materials vermutlich vorwiegend an oxische Verhältnisse gebunden, bis sich eine neue anaerobe Gemeinschaft an und unterhalb der Chemokline etabliert hat. Diese Ergebnisse bestätigen die schon erwähnten geringen Denitrifikationsraten und zeigen zudem, dass der gleiche Trend auch für Manganreduzierer und Fermentierer gelten könnte. Obwohl mit Hilfe molekularbiologischer Methoden unter stagnierenden Bedingungen an der Chemokline des Fårö Tiefs auch typische Sulfatreduzierer der Gattung *Desulfobacula* detektiert wurden (Labrenz *et al*., eingereicht), zeigen die kaum detektierbaren Sulfatreduktionsraten im Gotland Becken (L. Neretin, pers. Mitteilung) und dem Schwarzen Meer, sowie die geringen Lebendzellzahlen (Kap. 2.4), dass mikrobielle Sulfatreduktion an der Chemokline großer anoxischer Becken vermutlich kaum von Bedeutung ist (Jørgensen *et al.*, 1991). Ein wichtiger Aspekt, den es noch zu untersuchen gilt, ist die Verwendung von Schwefel, Thiosulfat und Sulfit als Elektronenakzeptoren. Die hohen Raten von Sulfidoxidation und CO_2-Fixierung durch lithotrophe Mikroorganismen lassen vermuten, dass intermediäre Schwefelverbindungen wichtige Elektronenakzeptoren für heterotrophe Mikroorganismen im Freiwasser darstellen.

Lithotrophe Mikroorganismen spielen an der Chemokline eine wichtige Rolle. Viele Untersuchungen deuten darauf hin, dass insbesondere chemolithoautotrophe Schwefeloxidierer einen signifikanten Anteil der mikrobiellen Gemeinschaft ausmachen. Darüber hinaus sind die Raten der CO_2-Fixierung an der Chemokline solcher Becken nur durch hohe Sulfidoxidationsaktivität zu erklären (Jørgensen *et al.*, 1991). Passend dazu wurden auch an der Chemokline des Gotland Beckens 5 bis 12 % der Gesamtzellzahl einem unkultivierten Phylotyp der Epsilonproteobakterien zugeordnet (Labrenz *et al.*, 2005), der den lithoautotrophen Schwefeloxidierer, *Sulfurimonas denitrificans*, als nächsten kultivierten Verwandten aufweist. Während der vorliegenden Untersuchung konnte dieser Phylotyp ebenfalls in MPN-Reihen mit eisensulfidhaltigem Medium und Nitrat als Elektronenakzeptor

angereichert werden. Jedoch stammte diese Anreicherung nicht aus der Zone der maximalen CO_2-Fixierung, sondern der sauerstoff- und nitrathaltigen Zone oberhalb der Chemokline (Kap. 2.4). Es erscheint fraglich, ob dieser Organismus auch für die maximalen Raten der CO_2-Fixierung unterhalb der Chemokline verantwortlich sein könnte. Die chemischen Messungen und Stimulationsexperimente legten nahe, dass die CO_2-Fixierung an der Chemokline von Mikroorganismen durch eine Kopplung von Sulfidoxidation und Manganreduktion geleistet wird. Dieser Prozess konnte bisher jedoch nicht durch entsprechende eindeutige Kulturversuche nachgewiesen werden. Es ist daher nach wie vor unklar, ob dieser abundante Phylotyp neben Sauerstoff und Nitrat eventuell auch gelöste oder partikuläre Manganverbindungen als Elektronenakzeptor nutzen kann.

Die Ergebnisse der vorliegenden Arbeit zeigen auch, dass nicht nur lithoautotrophe Schwefeloxidierer von Bedeutung sein könnten, sondern ebenfalls ein großer Anteil der heterotrophen Bakteriengemeinschaft Energie aus der aeroben und anaeroben Oxidation von Schwefelverbindungen gewinnen kann. Schwefeloxidation könnte für heterotrophe Organismen eine wichtige Rolle spielen, um weniger von der Verfügbarkeit organischer Substrate abhängig zu sein und letztere nur als Kohlenstoffquelle verwenden zu müssen. Dieses könnte insbesondere von Vorteil sein, wenn die verfügbaren organischen Kohlenstoffverbindungen schwer abbaubar sind. Tatsächlich wurden überraschend viele 16S rRNA-Gensequenzen von typischen Aromaten- und Kohlenwasserstoffverwertern, wie *Acinetobacter, Pseudomonas, Thalassolituus, Marinobacter, Thauera* und *Desulfobacula* direkt in der Wassersäule der zentralen Ostsee oder in Anreicherungen detektiert (Kap. 2.4; Labrenz et al., 2005; Labrenz *et al*., 2006 eingereicht). Bisher unveröffentlichte Ergebnisse der vorliegenden Untersuchung zeigten, dass mit Huminsäure-Präparationen als Kohlenstoffsubstrat ebenfalls hohe Lebendzellzahlen erzielt werden können. Die Wege und Mechanismen des Abbaus komplexer organischer Substanz sind bis heute nicht ausreichend verstanden (z. B. Amon *et al.*, 2001) und es sind noch wesentlich detailliertere Studien nötig, um die Mechanismen der Konkurrenz und Einnischung der Mikroorganismen an oxisch-anoxischen Grenzschichten im Detail zu verstehen.

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Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig verfasst und nur die angegebenen Hilfsmittel verwendet habe.

Oldenburg, im Juni 2006 Willm Martens-Habbena